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Oral administration of Hsp65-producing *Lactococcus lactis* attenuates allergic asthma in a murine model

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Keywords

airway hyperresponsiveness, allergic asthma, heat shock protein, lactic acid bacteria, *Lactococcus lactis*, mucosal immunotherapy.

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Abstract

Aims: Allergic asthma is a chronic inflammatory lung disease characterized by a Th2-type immune response pattern. The development of nonspecific immunotherapy is one of the primary goals for the control of this disease.

Methods and Results: In this study, we evaluated the therapeutic effects of *Lactococcus lactis*-producing mycobacterial heat shock protein 65 (LLHsp65) in an ovalbumin (OVA)-induced allergic asthma model. OVA-challenged BALB/c mice were orally administrated with LLHsp65 for 10 consecutive days. The results demonstrate that LLHsp65 attenuates critical features of allergic inflammation, like airway hyperresponsiveness and mucus production. Likewise, the treatment decreases the pulmonary eosinophilia and the serum level of OVA-specific IgE. In addition to deviating immune responses towards Th1-cytokine profile, increase regulatory T cells, and cytokine levels, such as IL-6 and IL-10.

Conclusions: Our results reveal that the mucosal immunotherapy of LLHsp65 significantly reduces the overall burden of airway allergic inflammation, suggesting a promising therapeutic strategy for allergic asthma treatment.

Significance and Impact of the Study: This research reveals new perspectives on nonspecific immunotherapy based on the delivery of recombinant proteins by lactic acid bacteria to treat of allergic disorders.

Introduction

Asthma is a global public health problem that affects approximately 235 million people and is responsible for 300 000 deaths a year (WHO 2017). Allergic asthma is a chronic inflammatory lung disease characterized by air-flow obstruction, hyperresponsiveness, and bronchial remodelling (Bousquet *et al.* 2000; Lambrecht and Hammad 2014).

These pathophysiological changes are a result of an exacerbated type 2 immune response (Th2), marked by IL-4, IL-5, and IL-13 cytokine production. These cytokines are responsible for the recruitment of inflammatory cells, such as basophils and eosinophils; allergen-specific

IgE production, mucus overproduction, and smooth muscle contraction (Lambrecht and Hammad 2014). Even though symptomatic treatments can contain allergic disorders, there is still an urgent need for an effective treatment that alters the natural course of allergic asthma (Bahceciler 2012; Gamazo *et al.* 2014).

The allergen-free immunotherapy can potently induce regulatory T cells (T regs) and Type 1 immunity (Th1) cells that counterbalance the effects of pro-allergic Th2 responses, thereby altering the course of the disease (Ye *et al.* 2011; Jutel *et al.* 2016). Heat shock proteins (Hsp) have been reported as potential immune modulators, which can elicit specific protective immunity (Srivastava 2002; Quintana and Cohen 2011; Binder 2014). Our

group has established that allergen-free immunotherapy with mycobacterial Hsp65 can polarize the immune response to a Th1-cytokine profile and induce T reg cells and IL-10 production. As such, it is responsible for preventing airway inflammation and hyperresponsiveness (Rha *et al.* 2002; Fonseca *et al.* 2012, 2015; Shin *et al.* 2012).

Some studies indicate that the mucosal delivery of therapeutic proteins can improve its ability to induce both mucosal and systemic immunity (Neurath *et al.* 2002; Ye *et al.* 2011; Bahceciler 2012). Lactic acid bacteria, such as *Lactococcus lactis*, are excellent candidates for the development of a mucosal delivery vector due to their safety certified as 'generally recognized as safe' (GRAS) by the US Food and Drug Administration. Besides, it is a well-established genetic tool for heterologous protein production (Bermúdez-Humarán 2009; Bermúdez-Humarán *et al.* 2011).

Accordingly, the oral administration of *L. lactis* expressing therapeutic proteins exhibits excellent immunomodulatory effects capable of preventing immune disorders (Wu *et al.* 2006; Marinho *et al.* 2010; Ai *et al.* 2014). However, most studies focus on prophylactic treatment, and insufficient data demonstrate that mucosal immunotherapy is useful as an allergic asthma treatment.

Therefore, this study aims to evaluate whether the oral administration of Hsp65-producing *L. lactis* (LLHsp65) can alleviate established airway inflammation in the OVA-induced asthma model.

Materials and methods

Animals

Female BALB/c mice, 6- to 8-week old, were acquired from the Animal Facility of the Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo (Brazil). These mice were housed in temperature-controlled rooms (22–25°C) under barrier conditions and provided with sterile food and water. Experiments were conducted following the Ethics Committee of the School of Medicine of Ribeirão Preto (protocol 062/2017).

Bacterial strain, plasmids and growth conditions

A recombinant *L. lactis* NCDO2118 strain that secretes *M. leprae* Hsp65 (LLHsp65) was used, as described elsewhere 2012; Gomes-Santos *et al.* 2017). The vector pXylT: SEC:hsp65, using a xylose-inducible expression system, directed the expression of the recombinant Hsp65 to the extracellular medium. The wild-type *L. lactis* NCDO2118 (LL-CT) strain was used as a control group.

The *L. lactis* NCDO2118 strains were grown in the M17 medium (DifcoTM, BD), supplemented with 0.5% glucose or 2% xylose at 30°C. Plasmids were selected by chloramphenicol (10 µg ml⁻¹).

Conditions of xylose induction

First, a single colony of recombinant *L. lactis* NCDO2118 harbouring the pXylT: SEC: hsp65 (LLHSP65) was incubated overnight at 30°C in 10 ml of M17 medium with 0.5% glucose and chloramphenicol (Cm, 10 µg ml⁻¹). On the second day, the culture was diluted in a ratio of 1 : 10 in a fresh M17 medium supplemented with 2% xylose and Cm (10 µg ml⁻¹), incubated for 24 h at 30°C to induce the expression of Hsp65. On the third day, the culture was centrifuged for 7 min at 13 000 g and 4°C. The pellet was resuspended in sterile phosphate-buffered saline (PBS) containing 20% glycerol and stored as aliquots of 5 × 10¹⁰ CFU per ml in an ultra-freezer (–80°C). The counting and viability of these aliquots were periodically checked through the plating dilutions in M17 agar medium containing 0.5% glucose and 10 µg ml⁻¹ chloramphenicol, as previously described (de Azevedo *et al.* 2012).

Analysis of Hsp65 expression

For western blot analysis, protein extracts were prepared from two 1 ml aliquots of each sample (LLHSP65 and LL-CT), as described in the former section. First, the samples were centrifuged for 7 min at 13 000 g, 4°C. Pellet was resuspended in TES-lysozyme buffer (0.01 mol l⁻¹ Tris-HCl, 0.01 mol l⁻¹ EDTA, 0.3 mol l⁻¹ NaCl, 10 mg ml⁻¹ lysozyme, 0.01 mol l⁻¹ DTT, Complete), incubated for 1 h at 37°C, and added 20% sodium dodecyl sulphate (SDS; J. T. Baker). The resuspension was vortexed and added to a fivefold concentrate (5x) sample buffer (0.2 mol l⁻¹ Tris-HCl, 40% glycerol, 10% SDS, 0.1% bromophenol blue, 2-mercaptoethanol). Samples were heated at 100°C for 5 min to obtain the protein extract.

Western blot analysis was conducted according to Sambrook *et al.* (1989). Briefly, protein extract was separated in 10% SDS-PAGE and blotted onto a nitrocellulose membrane (Life Technologies, Carlsbad, CA, USA). Detected with polyclonal anti-Hsp65 antibody (1 : 500; Farmacore Biotecnologia Ltda, Ribeirão Preto, SP, Brazil) and peroxidase-labelled mouse IgG (1 : 1000; Molecular Probes, San Diego, CA, USA), followed by a peroxidase substrate 3,3'-diaminobenzidine (DAB; Peroxidase Substrate Kit SK-4100; Vector Laboratories, Burlingame, CA, USA).

Experimental asthma model and immunotherapy

The acute allergic airway inflammation was induced via sensitization and challenged with fraction V of the chicken egg albumin protein, also called ovalbumin-grade V (OVA; Sigma-Aldrich, St Louis, MO, USA). Figure 1 summarizes the experimental procedure. In brief, on days 0 and 7, mice groups ($n = 10$) were sensitized with 10 μg of OVA and 2.25 mg of aluminum hydroxide, $\text{Al}(\text{OH})_3$ (Fisher Scientific, Ottawa, QC, Canada) via intraperitoneally (IP) injections. On days 14, 22, and 23, mice were intranasally (IN) challenged with 10 μg of OVA. After the first challenge, mice were orally administered with 5×10^9 CFU of LL-CT or for 10 consecutive days. At the endpoint of assessment, airway inflammation parameters were assessed. The control group received only a saline solution, and the OVA group was treated with saline solution.

Cell profile of bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid (BALF) was collected, as previously described (Fonseca *et al.* 2011). Concisely, the BALF was obtained following two consecutive injections of cold PBS into the lungs. The total volume was centrifuged at 400 g for 10 min at 4°C. Supernatants were stored at -20°C and cells were counted in a Neubauer chamber; BALF cells were centrifuged onto the microscope slides and stained with panoptic (Clozap-Hemogram, Inc., Curitiba, PR, Brazil). Percentages of the eosinophils, lymphocytes, macrophages, and neutrophils were multiplied by the total cell number from each sample.

Cytokine levels

IFN- γ , IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17, and TNF- α were quantified in BALF supernatants

and lung homogenates using MILLIPLEX MAP Mouse Cytokine Magnetic Bead Assay (EMD Millipore, Bedford, MA, USA). The cytokines levels were quantified via a MAGPIX platform and calculated by MILLIPLEX Analyst software (EMD Millipore, Bedford, MA, USA).

The platform uses the mean fluorescence intensity of the samples, and the cytokine concentrations were calculated in pg ml^{-1} . The cytokine detection limits were as follows: 0.4 pg ml^{-1} for IL-4; 0.5 pg ml^{-1} for IL-17; 1.0 pg ml^{-1} for IL-5; 1.1 pg ml^{-1} for IFN- γ and IL-6; 2.0 pg ml^{-1} for IL-10; 2.3 pg ml^{-1} for TNF- α ; 4.8 pg ml^{-1} for IL-12; and 8.3 pg ml^{-1} for IL-13.

Serum levels of OVA-specific antibodies

Blood samples were collected by retro-orbital puncture, centrifuged at 2000 g for 10 min at 4°C. The absorbance of OVA-specific IgE, IgG1, and IgG2a was measured using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (BD Biosciences-PharMingen, San Diego, CA) using biotin-conjugated anti-mouse IgE (R35-118), IgG1 (A85-1), and IgG2a (R19-15).

Histological analysis

Sections of 5 mm of the left upper lobes of the lung were stained with haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). Perivascular and peribronchial inflammation and mucus production were measured using the following arbitrary score: 0 (absence of inflammation; no mucus production), 1 (occasional peribronchial inflammation; focal bronchial mucus), 2 (frequent peribronchial and perivascular inflammation plus eosinophilic infiltration; recurrent bronchial mucus) and 3 (diffuse

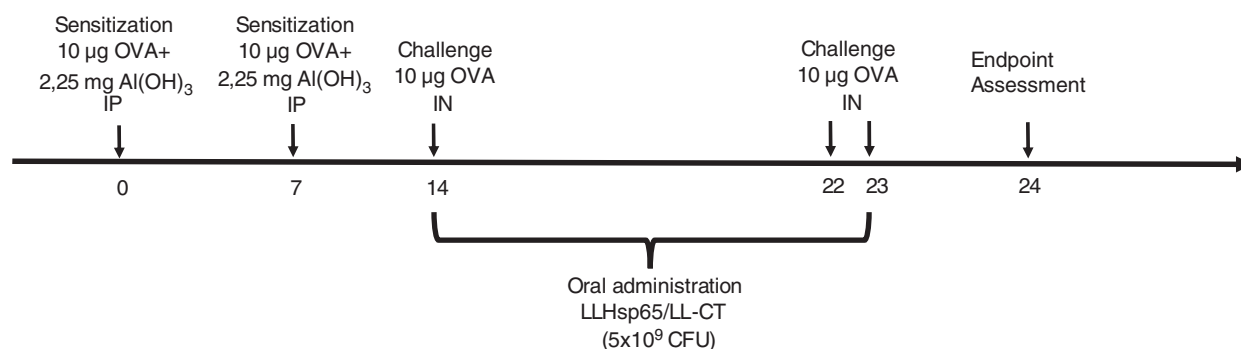


Figure 1 Experimental protocol for OVA-induced allergic asthma model and immunotherapy with LLHsp65. On days 0 and 7, BALB/c mice were sensitized with of OVA and alumen, via IP. On days 14, 22, and 23, mice were challenged with OVA, via IN. After the first challenge, mice were orally administered with LL-CT or LLhsp65 for 10 consecutive days. At the endpoint of assessment, airway inflammation parameters were assessed. The control group received only a saline solution, and The OVA group was treated with saline solution.

peribronchial and perivascular inflammation with the predominance of eosinophilic infiltrate; spread bronchial mucus). A pathologist analysed all the samples in a double-blind test, as described elsewhere (Fonseca *et al.* 2009).

Flow cytometry

At the endpoint assessment, a single-cell suspension was prepared from the middle-lower lobes of the lung. Mouse Treg cells were analysed for CD4⁺CD25⁺Foxp3⁺ expression using FITC-labelled anti-CD3, APC-labelled anti-CD4, PE-Cy7-labelled anti-CD25, PE-labelled anti-Foxp3 (BD Biosciences), and Mouse Foxp3 Buffer Set (BD Biosciences) according to the manufacturer's instructions. Samples were acquired on a FACSCanto II cytometer (BD Bioscience Pharmingen) with CellQuest software (BD Biosciences, Mountain View, CA) and analysed using the FlowJo software (TriStar, San Carlos, CA). At least 150 000 events were acquired for each sample. Determinations were performed in duplicate.

Measurement of airway hyperresponsiveness

Twenty-four hours after the last OVA challenge, airway hyperresponsiveness (AHR) was measured by pulmonary tissue resistance and elastance in response to increased concentration of methacholine challenges (MCh; Sigma-Aldrich, St Louis, MO, USA), as previously described (Borges *et al.* 2013). Briefly, mice were anaesthetized, tracheotomized, and mechanically ventilated using FlexiVent (Scireq, Montreal, QC, Canada). Data were obtained at baseline and after delivery of increasing concentrations of aerosolized MCh (6.25, 12.5, 25, and 100 mg ml⁻¹) for 10 s via an ultrasonic nebulizer (Hudson RCI, Teleflex Medical, Temecula, CA). The peak response of each parameter was determined using the coefficient of determination (COD) >0.9.

Statistical analysis

Experimental results were reported as mean $\pm$ standard error and analysed using the GraphPad Prism software version 6.0c. The results were compared using one-way

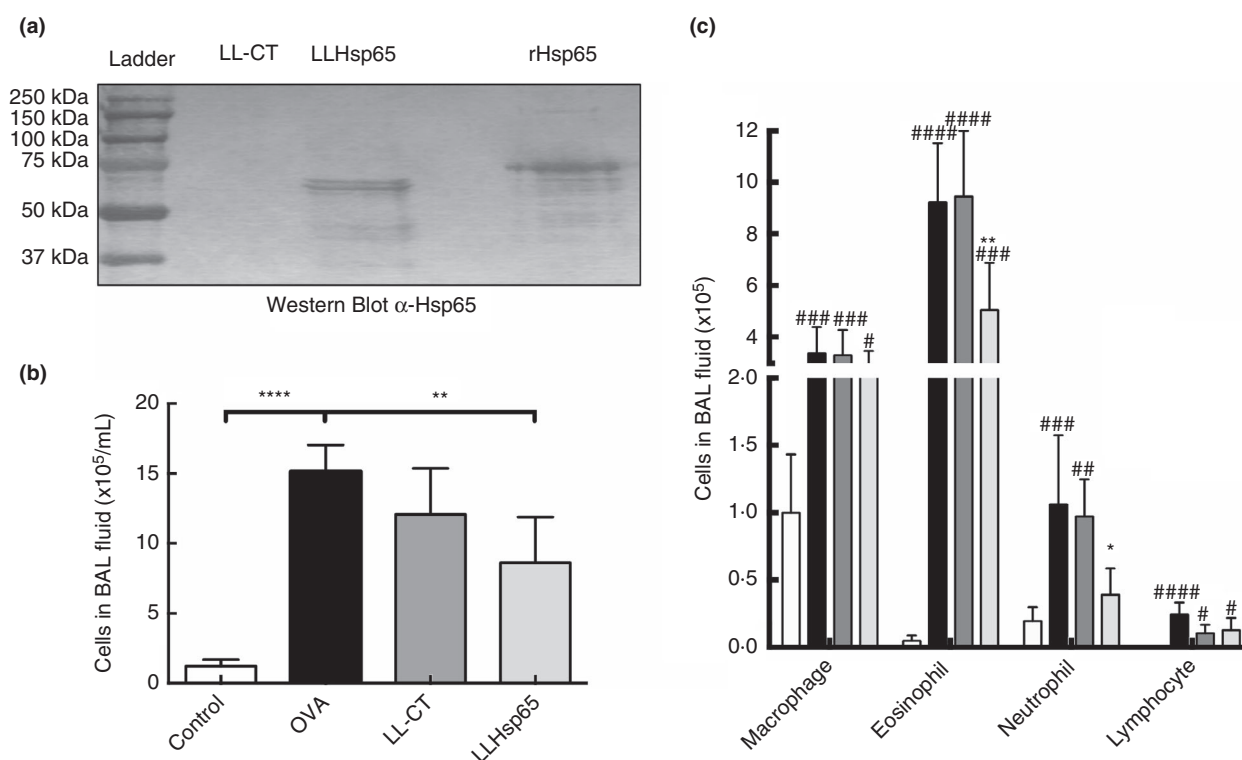


Figure 2 Western blot analysis of Hsp65 production and analysis of pulmonary cell infiltration. (a) Hsp65 production in *Lactococcus lactis*. LL-CT—protein extract of *L. lactis* NCDO2118; LLHsp65—protein extract of *L. lactis* with pXIES: SEC: Hsp65; rHsp65—recombinant *M. leprae* Hsp65 produced in *E. coli* ER2566. (b) Counts of total cells in BALF at the endpoint of assessment. (c) The cell population of macrophage, eosinophil, neutrophil, and lymphocyte in BALF. The results are expressed as the mean $\pm$ SD ($n = 6$). The data of a representative experiment were reproduced at least three times—*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. *—compared with the OVA group and #—compared with control group (□ control; ■ OVA; ▒ LL-CT; ▒ LLHsp65).

and two-way analysis of variance (ANOVA) followed by Bonferroni post-test. Values of $P < 0.05$ were considered statistically significant.

Results

Expression of mycobacterial Hsp65 in *L. lactis*

The Hsp65 expression in *L. lactis* was evaluated by western blot using the anti-Hsp65 polyclonal antibody. After xylose induction, a band of approximately 65 kDa was detected in the total protein extract of LLHsp65, similar to recombinant Hsp65 protein. Moreover, no signal was detected in LL-CT protein extract (Fig. 2a). These results indicate that strain LLhsp65 successfully produced mycobacterial Hsp65.

Oral administration of LLhsp65 reduces the influx of inflammatory cells in the BALF

The cellular influx into BALF was analysed to evaluate the effects of LLHsp65 on pulmonary inflammation. In the present study, the OVA-sensitized and OVA-

challenged mice were orally administered with 5×10^9 CFU of LL-CT or LLhsp65 for 10 consecutive days (Fig. 1). As shown in Fig. 2b, OVA-challenged mice develop significant airway inflammation with a predominance of eosinophils. The amount of migrated inflammatory cells in BALF of OVA and LL-CT groups was increased compared with the non-allergic (control) group. However, the LLHsp65 group showed an inhibitory effect on cell influx, including eosinophils and neutrophils in the lungs compared with the OVA group (Fig. 2c).

LLhsp65 attenuates allergic airway inflammation

In addition to inflammatory cell influx, the airway inflammation was evaluated by histological analysis. As shown in Fig. 3, upon H&E (Fig. 3a), the OVA group present an increased peribronchial infiltration of inflammatory cells, including eosinophils, accompanied to a higher thickness of epithelial cells and mucus production, scored as grade 2–3; which was also observed in the LL-CT group (Fig. 3b,c). LLHsp65 group revealed a marked decrease in the cellular infiltration and mucus

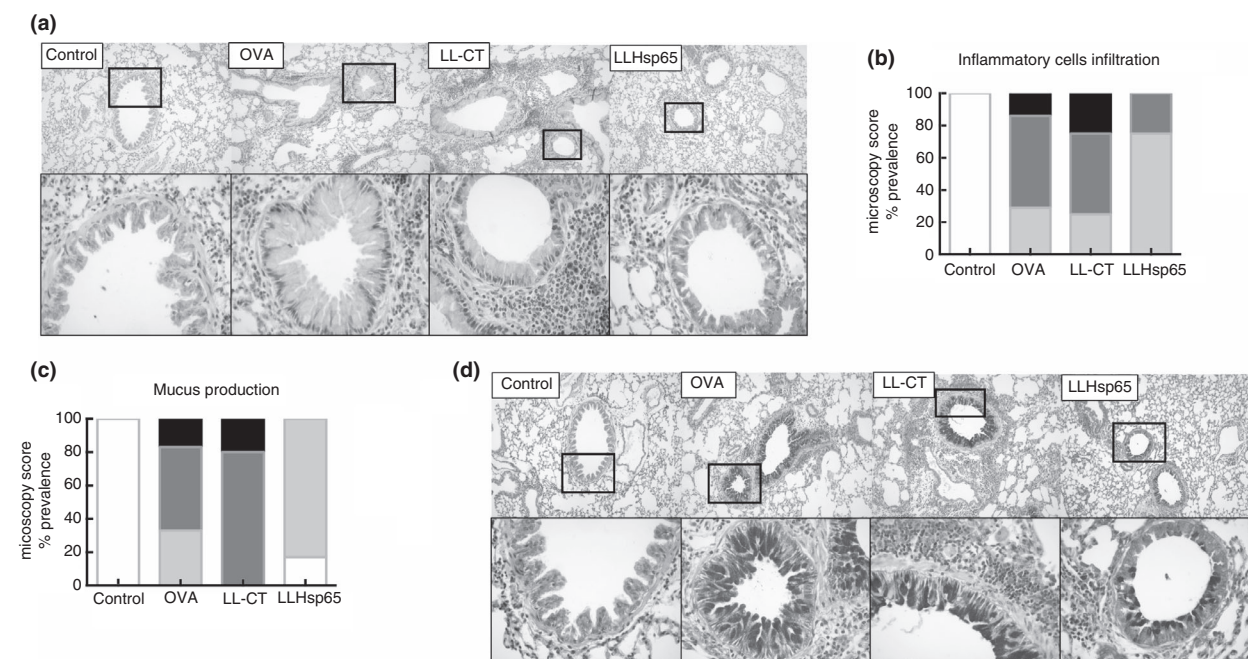


Figure 3 Histological analysis of inflammatory cell infiltration and mucus production in lung tissue. (a) Photomicrograph of representative lung sections stained with H&E of each group (magnification of 20× and 40×); (b) inflammatory scores from the different experimental groups; (c) score of mucus production from each group; (d) photomicrograph of representative lung sections stained with PAS of each group (magnification of 20× and 40×). The score was determined as follows: the absence of inflammation, no mucus production (grade 0); occasional peribronchial inflammation, focal bronchial mucus (grade 1); frequent peribronchial/perivascular inflammation, with eosinophilic infiltrate and recurrent bronchial mucus (grade 2); diffuse peribronchial/perivascular inflammation with a predominance of eosinophilic infiltrate and a spread of bronchial mucus (grade 3). The results are expressed as the percentage of grade score in each groups. The data of a representative experiment were reproduced at least three times (■;■;■;■;■;■;■;■;■;■).

production, and a thinner epithelial layer in airways (grades 1 and 2). The control group presented no inflammatory infiltrates or mucus production (grade 0). Moreover, LLHsp65 treatment can attenuate the critical features of airway inflammation.

LLHsp65 modulates OVA-specific antibody responses and cytokines profiles

The OVA group exhibited Th2-specific antibody responses with increased levels of OVA-specific IgE, IgG1 compared with the control group (Fig. 4a). The LLHsp65

treatment significantly decreased IgE, IgG1 levels compared with the OVA group, which was not observed in the LL-CT group. Moreover, the OVA-specific IgG2a levels were also increased in the OVA group compared with the control group. Surprisingly, IgG2a levels were diminished in LLHsp65 and LL-CT groups.

To investigate the effects of LLHsp65 on allergic responses, we evaluated Th1, Th2, and modulatory cytokines levels in BALF and lung homogenates. The OVA group showed a significant increase in Th2-cytokine levels, such as IL-4, IL-5 and IL-13 in BALF, compared with the control group. The administration of LL-CT or

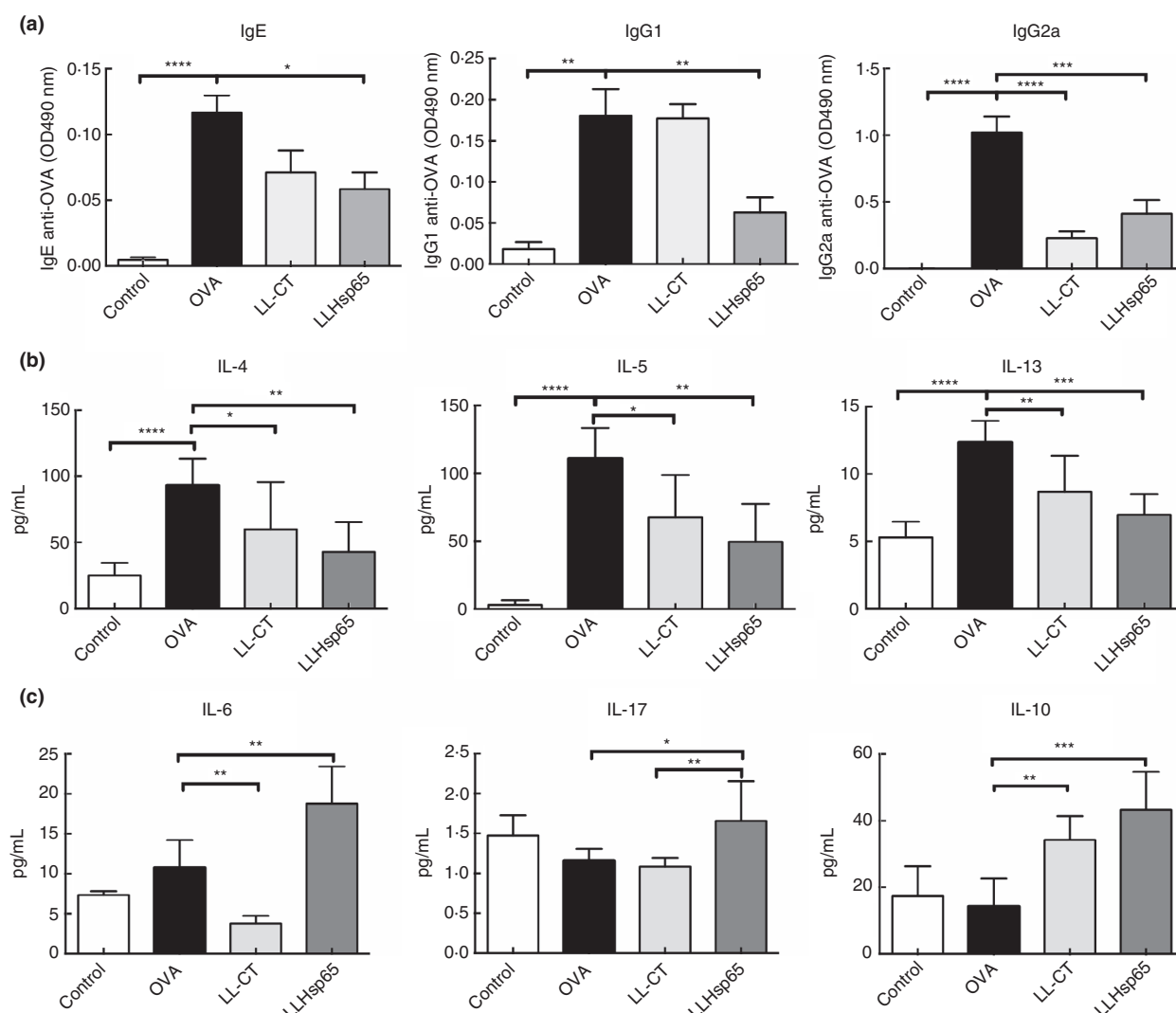


Figure 4 Evaluation of OVA-specific Igs and Th2-cytokine production. (a) Serum levels of OVA-specific IgE, IgG1 and IgG2a were determined by ELISA (b) Th2-cytokine levels in BALF. (c) IL-6, IL-10 and IL-17 levels in BALF. The results are expressed as the mean $\pm$ SD ($n = 6$). The data of a representative experiment were reproduced at least three times. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Table 1 Th1-Cytokine production in the lung tissue homogenates

	Control	OVA	LL-CT	LLHsp65
IFN- γ (pg ml ⁻¹)	38.40 $\pm$ 6.25	22.33 $\pm$ 9.10	36.96 $\pm$ 7.11*	39.65 $\pm$ 6.10**
IL-12 p70 (pg ml ⁻¹)	45.42 $\pm$ 16.40	29.80 $\pm$ 5.8	87.17 $\pm$ 23.7**	97.25 $\pm$ 29.20***
TNF- α (pg ml ⁻¹)	3.748 $\pm$ 1.3	3.860 $\pm$ 0.8	6.846 $\pm$ 2.29*	10.46 $\pm$ 0.95***

Results were expressed as the mean $\pm$ SD.

* $P > 0.05$;

** $P < 0.01$;

*** $P < 0.001$ compared with the OVA group.

LLHsp65 showed a prominent effect on lowering IL-4, IL-5, and IL-13 levels compared with the OVA group (Fig. 4b). In addition to the decrease in Th2-cytokine profile, the administration of LL-CT or LLHsp65 increased the IFN- γ , TNF- α , and IL-12p70 levels (Th1 cytokines) in lung tissue homogenates compared with the OVA group (Table 1). Furthermore, the production of IL-10, IL-6 and IL-17 was also significantly increased in BALF of LLHsp65 group compared with the OVA group (Fig. 4c).

Oral administration of LLhsp65 increases regulatory T cells in the lung tissue

The presence of CD4⁺CD25⁺Foxp3⁺ T cells in the lung was analysed to investigate whether LLHsp65 can induce regulatory cells at the inflammatory site. As shown in Fig. 5a, oral administration LL-CT increased the number of regulatory T cells (Tregs) in the lung tissue compared with the control group. However, this induction was intensified in the presence of Hsp65 protein, as observed in the LLHsp65-treated mice (Fig. 5b).

LLhsp65 treatment ameliorates airway hyperresponsiveness

Airway responsiveness to aerosolized MCh challenges was measured to ensure the effects of LLhsp65 on the pulmonary function. As shown in Fig. 5c, the OVA group showed a significant increase of hyperresponsiveness in all parameters measured: RRS (total resistance), ERS (total elastance), G (tissue resistance), and H (tissue elastance) compared with the control group. The administration of LL-CT did not significantly reduce neither parameters compared with the OVA group. In contrast, the administration of LLHsp65 presents a significant inhibitory effect on the development of AHR in each measured setting compared with the OVA group. Finally, our results demonstrate that the oral administration of LLHsp65 alleviates the principal features of allergic asthma, improving the lung function of allergic mice.

Discussion

In the present study, we evaluated the immunomodulatory effects of the oral administration of *L. lactis*-producing *M. leprae* Hsp65 on allergic asthma in a BALB/c mice model. Our results show that LLHsp65 treatment ameliorates the disease's overall burden by reducing bronchial hyperresponsiveness, pulmonary inflammation, and mucus production. Likewise, the treatment decreases the lung eosinophilia, in addition to lowering the OVA-specific IgE serum levels; deviate immune responses towards Th1-cytokine profile; increase regulatory T cells, and cytokines levels, such as IL-6 and IL-10.

Previous studies demonstrated that LAB strains are efficient as a prophylactic treatment of experimental asthma (Hong *et al.*, 2010; Mei *et al.*, 2012; Yu *et al.*, 2010). The oral treatment with LAB bacteria activates dendritic cells via NOD2 or TLR2 to produce IL-12, which directs a Th1 response, consequently, acting on the Th1/Th2 balance and preventing allergy development (Debarry *et al.* 2007). However, our data showed that LL-CT treatment did not attenuate the allergic inflammation, despite inducing this immunobalance.

Our group previously demonstrated that both Hsp65 protein with CpG adjuvant (Prado *et al.* 2015) and DNA vaccine (Hsp65-DNA) were able to attenuate experimental asthma (Fonseca *et al.* 2012, 2015). Likewise, Hsp65-producing *L. lactis* is described for the treatment of immunological disorders such as Type 1 diabetes (Liu *et al.* 2016; Ma *et al.* 2014), encephalomyelitis (Rezende *et al.* 2013), and colitis (Gomes-Santos *et al.* 2017). Herein, we demonstrated that Hsp65-producing *L. lactis* is also efficient as nonspecific immunotherapy for allergic asthma.

Many pharmaceutical therapies, such as omalizumab (anti-IgE antibody), reslizumab (anti-IL-5), lebrikizumab, and dupilumab (anti-IL-4 receptor) act neutralizing specific molecules of allergic Th2-response (Anderson and Szefer 2015). However, it is necessary to control the multiple factors of allergic responses to the achievement of effective treatment (Caminati *et al.* 2016). The LLHsp65 administration demonstrates a decreased levels

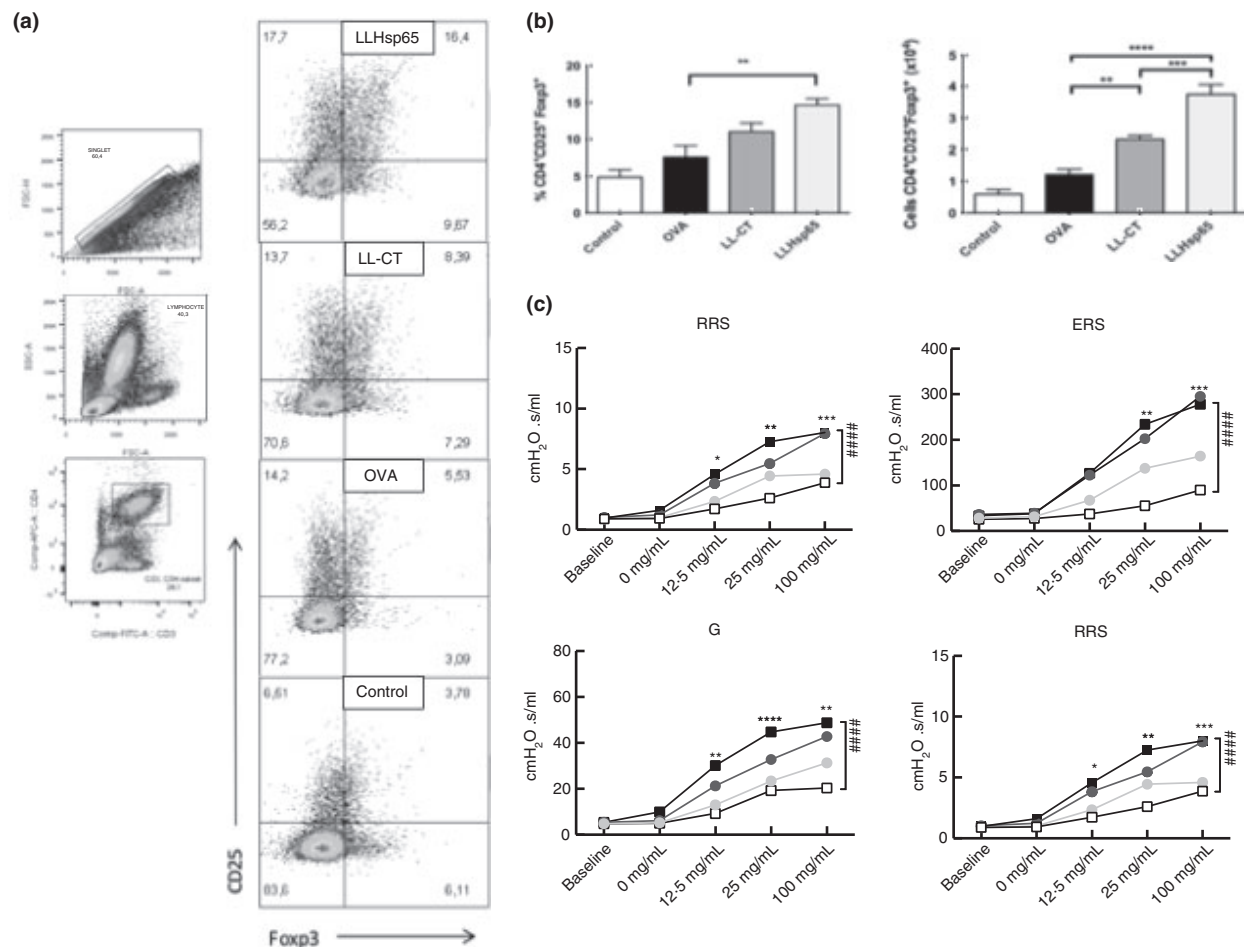


Figure 5 Flow cytometry analysis of pulmonary CD4⁺CD25⁺Foxp3⁺T cells and the assessment of airway hyperresponsiveness. (a) T regulatory cells in the lung tissue. Plots show cells expressing CD4, CD25 and intracellular Foxp3. (b) Relative proportion of CD4⁺CD25⁺Foxp3⁺Treg cells among total lung tissue cells. (c) Airway hyperresponsiveness in response to MCh challenges. Groups: Control (□), OVA (■), LL-CT (●) and LLHsp65 (●). Total resistance (RRS), total elastance (ERS), tissue resistance (G) and tissue elastance (H) were evaluated. The results were expressed as the mean $\pm$ SD ($n = 8$). The data of a representative experiment were reproduced at least twice: *,[#] $P < 0.05$; **,^{##} $P < 0.01$; ***,^{###} $P < 0.001$; ****,^{####} $P < 0.0001$. *—compared with the OVA group; and[#]—compared with control group.

of IL-4, IL-5, IL-13 cytokines, and OVA-specific IgE and IgG1, suggesting a potential candidate for allergic immunotherapy.

To understand how LLHsp65 suppresses airway allergic inflammation and improves pulmonary function, we investigated the mechanisms involved in this suppression. The oral administration of LLHsp65 reduces the serum levels of OVA-specific IgE, IgG1, and IgG2a. The high levels of antigen-specific IgE and IgG1 is a crucial feature of the allergic response. These are responsible for the activation of mast cells, basophils, and epithelial cells, which release pro-inflammatory mediators, that trigger smooth muscle contraction, mucus secretion, and airway remodelling (Bousquet *et al.* 2000; Lambrecht and Hammad 2014). The decrease in OVA-specific antibodies is an

important parameter that demonstrates the modulation of humoral allergic response.

The reduction of Th2 cytokines is also fundamental to allergic treatment, given its central role in asthma pathophysiology. The IL-4 cytokine is responsible for expanding Th2 cells, induction of allergen-specific IgE and IgG1, and recruitment of the eosinophils. IL-5 cytokine induces the eosinophils activation and airway remodelling, therefore increasing bronchial hyperresponsiveness. IL-13 is also related to hyperresponsiveness, mainly by inducing goblet cells to produce a thick mucus (Lambrecht and Hammad 2014).

The cytokine profile modulation in the airways is a fundamental factor to contains immunological dysfunctions (Sult *et al.* 2003; Romagnani 2004). The IFN- γ

production suppresses the allergic response through the induction of IL-12, which favors the Th1-cell differentiation, consequently, induce Th2-cells inhibition by Fas/Fas-L apoptosis (Fonseca *et al.* 2015). The Hsp65 protein boosts the Th1-pattern deviation by the induction of Delta1 expression in the dendritic cells, which activates lymphocytes to produce Th1 cytokines (Shin *et al.* 2012). In the present study, the LLHsp65 administration demonstrated that the increased Th1-cytokine level (IFN- γ , IL-12, and TNF- α) is associated with the lowering of Th2-cytokine levels in BALF.

In addition to increased Th1-cytokine levels, the LLHsp65 treatment also increases the production of modulatory cytokines, such as IL-6, IL-17, and IL-10. The IL-6 cytokine plays a controversial role. This cytokine can aggravate an inflammatory response due to leukocyte transmigration induction, and controversially, it is also associated with the mucosal repair, neutrophil apoptosis, and IgA secretion by naive B cells. Therefore, IL-6 might play a protective role in allergic diseases (Ozdemir 2010; Scheller *et al.* 2011; Luerce *et al.* 2014).

In the same perspective, IL-17 increased levels are usually related to the pathology aspects of allergic conditions, such as chronic bronchitis, chronic pulmonary obstruction, and cystic fibrosis (Akdis and Blaser 2001). However, Schnyder-Candrian *et al.* (2006) demonstrated the protective role of IL-17 in allergic asthma. This cytokine could inhibit the Th2 profile development in addition to downregulating TARC production by dendritic cells, which is responsible for the chemoattraction of Th2 cells (Schnyder-Candrian *et al.* 2006).

The IL-10 cytokine is an essential target for the control of allergic asthma. IL-10 can inhibit the production of antigen-specific IgE, suppress the eosinophils activation, and induce T-cell anergy (Akdis *et al.* 2005). The administration of Hsp65 (Rha *et al.* 2002; Shin *et al.* 2012; Fonseca *et al.* 2015) or LAB strains (Nonaka *et al.* 2008; Kwon *et al.* 2010; Jang *et al.* 2012; Lin *et al.* 2013) is capable of enhancing IL-10 production. In the present study, both treatments demonstrate IL-10 increased levels in BALF and pulmonary homogenates.

The regulatory T cells can suppress lung eosinophilia, mucus hypersecretion, and hyperresponsiveness (Lyons *et al.* 2010). The depletion of these cells reverses the suppressive activity of LAB strains in murine allergic models (Jang *et al.* 2012). In this study, the LLHsp65 treatment showed an increased Treg cells (CD4⁺CD25⁺Foxp3⁺) population in the inflammatory site. Furthermore, the Th1-/Th2-cytokine profile balance and the Tregs cell expansion in the pulmonary tissue suggests a possible mechanism for relieving experimental asthma.

The airway hyperresponsiveness is the main feature of allergic asthma and could be induced by increased

mucosal permeability, mucus production, airways remodelling, and muscle contractility (O'Byrne and Inman 2003). For this reason, the lung function assessment is essential for the evaluation of efficient therapies for asthma treatment (Wagers 2004). According to a decreased hyperresponsiveness compared with the OVA and LL-CT groups, the LLHsp65 treatment promotes pulmonary function recovery in allergic mice.

Although this research provides information on the possible mechanisms of the anti-allergic effects of LLHsp65, it is necessary to identify the specific mechanisms of this immune-modulatory response. Our data corroborate with studies that demonstrate a systemic immunomodulation effect from oral immunotherapy (Ganesh and Versalovic 2015; Prado *et al.* 2015). Accordingly, we suggest that allergen-free immunotherapy could be efficiently used to treat allergic disorders.

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