

Recombinant DNA immunotherapy ameliorate established airway allergy in a IL-10 dependent pathway

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Summary

Background Previous studies have established that mycobacterial infections ameliorate allergic inflammation. However, a non-infectious approach that controls allergic responses might represent a safer and more promising strategy. The 60–65 kDa heat shock protein (Hsp) family is endowed with anti-inflammatory properties, but it is still unclear whether and how single mycobacterial Hsp control allergic disorders.

Objective Therefore, in this study we determined whether the administration of *Mycobacterial leprae* Hsp65 expressed by recombinant a DNA plasmid could attenuate a previously established allergic response.

Methods We used an experimental model of airway allergic inflammation to test the effects of immunotherapy with DNA encoding Hsp65. Allergic mice, previously sensitized and challenged with ovalbumin, were treated with three intramuscular doses of recombinant DNA encoding Hsp65. After treatment, mice received a second allergen challenge and the allergic response was measured.

Results We found that immunotherapy attenuated eosinophilia, pulmonary inflammation, Th2 cytokine and mucus production. Moreover, we showed that the inhibition of allergic response is dependent on IL-10 production. Both Hsp65 and allergen-specific IL-10-producing cells contributed to this effect. Cells transferred from DNA-immunized mice to allergic mice migrated to allergic sites and down-modulated the Th2 response.

Conclusions and Clinical Relevance Our findings clearly show that immunotherapy with DNA encoding Hsp65 can attenuate an established Th2 allergic inflammation through an IL-10-dependent mechanism; moreover, the migration of allergen- and Hsp65-specific cells to the allergic sites exerts a fundamental role. This work represents a novel contribution to the understanding of immune regulation by Hsp65 in allergic diseases.

Keywords airway allergic inflammation, heat shock protein, immunotherapy, recombinant DNA

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Introduction

Since 1911, when Noon and co-workers successfully injected low doses of pollen extract to treat allergic disorders, a range of immunotherapy strategies have been developed to not only treat the acute reaction but also avoid or ameliorate new allergic episodes [1]. At that

time, the pathways that trigger the immunological mechanisms of allergy as well as the pathways that could modulate such responses were unknown. At present, it is well established that Th2-mediated immune responses that involve the production of IgE, IL-4, IL-5, IL-13, thymic stromal lymphopoietin (TSLP) and eotaxin and the activation of mast cells, eosinophils

and basophils are involved in allergic disorders [2]. Therefore, inhibition of the Th2 response has been the aim of immunotherapies.

Usually, specific immunotherapy (SIT) employs allergen extracts, recombinant proteins or modified proteins [1, 3]. However, specific immunotherapies require several allergen injections and long-term treatment. In addition, these therapies might not be efficient for all cases and for different types of allergic manifestations; in fact, they could contain potential associated risks such as systemic allergic reactions [4]. Recently, recombinant allergens such as Der p 1 or Der p 2 from house dust *Dermatophagoides pteronyssinus*, Bet v 1a from birch pollen or Ara h 1 from peanuts have been introduced in pre-clinical studies [5–7].

Another method to control allergic disorders is via microbial infections and/or products [4]. The rationale of this approach is based on the 'hygiene hypothesis', which postulates that exposure to microorganisms (whether pathogenic or not) stimulates the development of immune responses, such as those mediated by Th1 or regulatory T (Treg) cells, which dampen allergic inflammation [8, 9]. Heat shock proteins (Hsps) are prototypic microbial products that could be used for the down-modulation of allergic responses because they are highly conserved antigens that are secreted under stressful conditions, and they are immunodominant antigens in several types of infections [10, 11]. They act to promote inflammatory responses, or alternatively, they act in an opposing manner to reduce inflammatory disorders [11, 12]. Accumulating evidence indicates that the Hsps efficiently suppress inflammatory diseases, such as autoimmune diabetes, experimental arthritis, encephalomyelitis and atherosclerosis [13–15]. Although Hsps may play a regulatory function that could contribute to the down-modulation of the allergic response, these antigens have not been tested for the treatment of established airway allergic inflammation.

We have previously shown that a DNA encoding a 65 kDa Hsp from *Mycobacterium leprae* (DNA-HSP65) ameliorates experimental arthritis and diabetes [14, 15]. In both models, DNA-HSP65 induced both Hsp65-specific IL-10- and IFN- γ -producing cells.

Even though allergen-encoding DNA vaccines have been shown to be effective when administered before airway challenge, there is no evidence suggesting that a recombinant DNA encoding a microbial antigen could reverse an established allergic response. In addition, the DNA structure may contribute several advantages, including an adjuvant effect of the DNA backbone and a reduction in dose numbers because after injection, the DNA can express the antigen protein for a lengthy period of time [16].

In this work, we assessed for the first time whether it is possible to treat a previously established experimen-

tal allergic response by combining the adjuvant property of the DNA backbone with the immunoregulatory effects of a safe non-allergic antigen (Hsp65). Furthermore, using an experimental model of allergy, we investigated the possible mechanisms involved in the modulation of the allergic response by allergen-free immunotherapy.

Methods

Mice

Specific pathogen-free, 6- to 8-week-old female BALB/c, C57BL/6 and C57BL/6 green-fluorescent protein (GFP) transgenic mice were obtained from a local breeding facility at the School of Medicine of Ribeirão Preto, University of São Paulo, Brazil. IL-10 knockout (IL-10 KO) mice were provided by Dr João S. Silva (School of Medicine of Ribeirão Preto). The mice were housed under barrier conditions and provided with food and sterile water. Experiments were approved by the local Ethics Committee (Comissão Ética em Experimentação Animal – CETEA).

Recombinant DNA-HSP65 plasmid, Hsp65 protein and ovalbumin

The DNA-HSP65 recombinant plasmid was derived from the pcDNA3 vector (Invitrogen®, Carlsbad, CA, USA) with an insertion of the gene that encodes the Hsp65 protein from *M. leprae*; it was obtained as previously described [17]. The recombinant Hsp65 protein (r-Hsp65) was obtained from *Escherichia coli* ER2566 that was transformed with the Hsp65 gene, as previously described [18]. Chicken ovalbumin (OVA) was obtained from a commercial source (OVA – grade V; Sigma, St Louis, MO, USA).

Endotoxin concentrations were measured in all of reagents (recombinant DNA-HSP65, r-Hsp65, OVA, alum) using the Limulus amoebocyte lysate kit – QCL-1000 (BioWhittaker, Walkersville, MD, USA). The DNA-HSP65 or DNA-Vector plasmids had no detectable endotoxin, whereas OVA contained 0.2 EU endotoxin/ μ g of protein and Hsp65 contained 9.2 EU endotoxin/ μ g.

Experimental design

For the induction of airway allergic inflammation, we used a previously established protocol (Fig. 1a) [19]. BALB/c, IL-10 KO or C57BL/6 wild-type (WT) mice were sensitized with 100 μ g OVA administered subcutaneously with 1.6 mg alum, and after 14 days, they received a second dose of 50 μ g injected intraperitoneally. Seven days after sensitization, the mice were

challenged intranasally with 100 µg OVA. Seventy-two hours after the allergen challenge, when the allergic response was already established, the mice were treated with three intramuscular injections of 100 µg DNA-HSP65 in 12.5% sucrose solution at 15-day intervals. Control groups received saline (allergic untreated mice) or three doses of 100 µg DNA plasmid without the Hsp65 gene insert (DNA-Vector-treated mice). Two weeks after the last DNA injection, the mice received a second intranasal OVA challenge. Airway hyperresponsiveness (AHR) was evaluated after 24 h, and other parameters were assessed 72 h after this allergen challenge. In an alternate schedule of immunotherapy, DNA-HSP65-treated mice received 50 µg r-Hsp65 intranasally together with the last allergen challenge (as indicated in the legends of respective figures).

Cell counts in BALF and cytokine and antibody detection

Bronchoalveolar lavage fluid (BALF) was obtained by the injection of 1 mL RPMI-1640 medium into the trachea. This procedure was repeated five times. The first lavage was used for cytokine detection, and cells obtained from this and the other 4 mL of lavage were counted in a Neubauer chamber; 40 000 cells were centrifuged onto microscope slides and stained with panoptic (Clorzap; Hemogram Ind., Pinhais, PR, Brazil). The total numbers of eosinophils, lymphocytes, macrophages and neutrophils were obtained by multiplying the percentages of these cell types by the total number of cells obtained from each sample.

IL-4, IL-5, IL-13, TSLP, eotaxin, IFN- γ , IL-17, IL-10 and TNF- α levels in BALF, spleen and lung cell culture supernatants, and lung homogenates were determined by enzyme-linked immunosorbent assay (ELISA), according to manufacturer's instructions (BD Biosciences-PharMingen, San Diego, CA, USA) [20, 21]. Cytokine detection limits were as follows: 5 pg/mL for IL-13, TSLP and eotaxin; 9 pg/mL for IL-4, IL-17, IFN- γ and TNF- α ; and 19 pg/mL for IL-5 and IL-10.

Hsp65- and OVA-specific antibodies were assayed by sandwich ELISA, according to the manufacturer's recommended protocols (BD Biosciences-PharMingen) using biotin-conjugated anti-mouse IgG1 (A85-1), IgE (R35-118), and IgG2a (R19-15) antibodies.

Histological analyses

The left lung lobes were collected, fixed in 10% formol and stained with haematoxylin-eosin in five-micron sections. Perivascular and peribronchial inflammation present in all vessels and bronchi from each section were scored on a scale of 0–3, as previously described [22].

Lung tissues were also stained with periodic acid-Schiff (PAS)/haematoxylin for mucus detection. The mucus index was calculated using ImageJ software (NIH, Bethesda, MD, USA) by analysing all the bronchi micro-photographed in the lung section under a magnification of 200 $\times$. Results were expressed as the ratio of bronchi area to the PAS-stained area of each lung section.

Detection of Foxp3⁺ cells

For Foxp3 immunohistochemistry staining, the left lung lobes were collected and then frozen in O.C.T. compound (Sakura Finetek, Torrance, CA, USA). Lung sections were fixed with cold acetone, and endogenous peroxidase and biotin were blocked, respectively, by incubation with 3% H₂O₂ (Merck, Darmstadt, Germany) and the Avidin/Biotin Blocking kit (Vector Laboratories, Inc., Burlingame, CA, USA). Biotin-conjugated anti-mouse Foxp3 antibody (FJK-16, diluted 1 : 200 in 0.01% saponin; eBioscience) was incubated for 2 h at 37°C. The reaction was visualized by the addition of the Avidin/Biotin/Peroxidase-conjugated reagent Vectastain ABC kit (Vector Laboratories, Inc.), which was followed by the addition of 3,30-diaminobenzidine (Vector Laboratories, Inc.). Sections were counterstained with Mayer's haematoxylin. Controls were performed by incubating the slides with the IgG isotype control instead of primary antibodies and following the protocol described above. Forty different fields from the lung sections were micro-photographed (magnification, 400 $\times$), and positively brown-stained cells were counted using ImageJ software (NIH, Bethesda, MD, USA).

Spleen and lung cell cultures

When indicated in the figures, spleen cell cultures were performed and assayed. Briefly, spleens were collected and homogenized, and 5 $\times$ 10⁶ cells/mL was cultured in complete RPMI-1640 medium (with 10% bovine fetal serum, gentamicin, penicillin/streptomycin, and polymyxin B) at 37°C in 5% CO₂. Cells were stimulated with OVA (100 µg/mL) or r-Hsp65 (20 µg/mL). Supernatants were harvested after 48 h for cytokine detection. Lung cells were isolated as previously described [21], and they were cultured and stimulated in the same conditions as the spleen cells.

Cell transfer

Three doses of DNA-HSP65 were injected intramuscularly into GFP transgenic mice. Fifteen days after the third immunization, the mice received 50 µg r-Hsp65 intravenously. After 5 days, spleen cells (5 $\times$ 10⁶) from DNA-HSP65-immunized or non-immunized mice were

transferred intravenously to OVA-sensitized C57BL/6 WT mice. One day after the transfer, WT mice were intranasally challenged with OVA. BALF was collected 72 h after OVA-challenge for cytokine detection and cell counting. At this set point, lung, draining lymph nodes and spleen from WT recipient mice were analysed by flow cytometry to identify GFP⁺ cell migration. These cells were stained with anti-CD4 or anti-CD8 mAbs, according to the manufacturer's recommended protocol (BD Biosciences-PharMingen), and analysed with FlowJo Software.

Determination of AHR

Airway hyperresponsiveness was assessed in some experiments using a single-chamber, whole-body plethysmograph (Buxco Electronics Inc, Wilmington, NC, USA). Mice were nebulized with increasing metacholine doses for the determination of the Pehn index, as previously described [23].

Statistical analyses

All data were expressed as means $\pm$ SEM. The results were compared using analysis of variance (ANOVA) followed by Tukey's test with Prism software (Graphpad Software Inc., San Diego, CA, USA). Values of $P < 0.05$ were considered statistically significant.

Results

Immunotherapy with DNA-HSP65 attenuates allergic airway inflammation

To evaluate the therapeutic effects of DNA-HSP65, we detected cellular influx into BALF, lung inflammation and mucus production in allergic and control mice. Figure 1a shows the experimental design that was used. First, we confirmed that our allergic untreated mice exhibited the expected features, such as increased total cell and eosinophil counts in BALF, intense perivascular and peribronchial inflammation in the pulmonary parenchyma and mucus production (Fig. 1b–d). Mice that received DNA-HSP65 immunotherapy were characterized by reduced total cell and eosinophil counts in BALF, decreased lung inflammation and reduced mucus production compared to the control groups (allergic untreated mice and DNA-Vector-injected mice, Fig. 1b–d). In parallel, DNA-HSP65-treated mice contained increased numbers of lymphocytes and macrophages in BALF compared to the other groups.

After the evaluation of airway inflammation, we subsequently detected local cytokine production. DNA-HSP65 treatment down-modulated IL-4, IL-5, IL-13, TSLP and eotaxin production in BALF and lung

homogenates compared to the allergic untreated and DNA-Vector groups ($P < 0.05$; Fig. 2a and supporting information, Fig. S1). DNA-HSP65-treated mice exhibited increased IL-10 levels in BALF and lung homogenates compared to the other allergic groups ($P < 0.05$; Fig. 2a and supporting information, Fig. S1). In addition, there was also significantly increased TGF- β production in lung homogenates (supporting information, Fig. S1).

The modulation of local immune responses by DNA-HSP65 was accompanied by the modulation of allergen-specific systemic responses. We observed a decrease in IL-4 and IL-5 production by OVA-stimulated spleen cells obtained from DNA-HSP65-treated mice compared to cells from the other allergic groups ($P < 0.05$; Fig. 2b). In agreement with data obtained in BALF and lung homogenates (Fig. 2a and supporting information, Fig. S1), there was increased production of OVA-specific IL-10 and TGF- β by spleen cells of the DNA-HSP65-treated mice compared to the other groups ($P < 0.05$; Fig. 2b).

The immunotherapy did not modify the IFN- γ , IL-17 and TNF- α levels in BALF, lung homogenates or spleen cell cultures (data not shown). Treatment with DNA-HSP65 was also able to significantly reduce allergen-specific IgG1 and IgE antibody secretion and did not modify IgG2a production compared to the allergic groups (Fig. 2c and data not shown). These results indicate that DNA-HSP65 immunotherapy down-modulates allergic inflammation in the airway and the systemic allergen Th2 response. This treatment also increases IL-10 production locally and systemically by OVA-specific cells.

DNA-HSP65 therapy increased the number of Foxp3⁺ cells in the lungs

Because we observed increased IL-10 and TGF- β production after immunotherapy, we investigated whether it was due to the recruitment of Treg cells by detecting the presence of Foxp3⁺ cells in the lung parenchyma. Figure 3a shows an increased number of Foxp3⁺ cells in DNA-HSP65-treated mice compared to the other groups ($P < 0.05$).

Considering the increase in Foxp3⁺ and OVA-specific IL-10-producing cells, we determined whether the modulation of experimental allergy was associated with the inhibition of Th2 responses mediated by the OVA-specific or Hsp65-specific cells. To investigate this hypothesis, we analysed the Hsp65-specific cytokine production by spleen cells. We observed a significant increase in IL-10, TGF- β and IFN- γ secretion by Hsp65-stimulated spleen cells from DNA-HSP65-treated mice, but not by cells from the other groups (Fig. 3b). Therefore, immunotherapy with DNA-HSP65 induces an increase in Foxp3⁺ Treg cells in the lungs and stimulates an Hsp65-specific cellular immune response

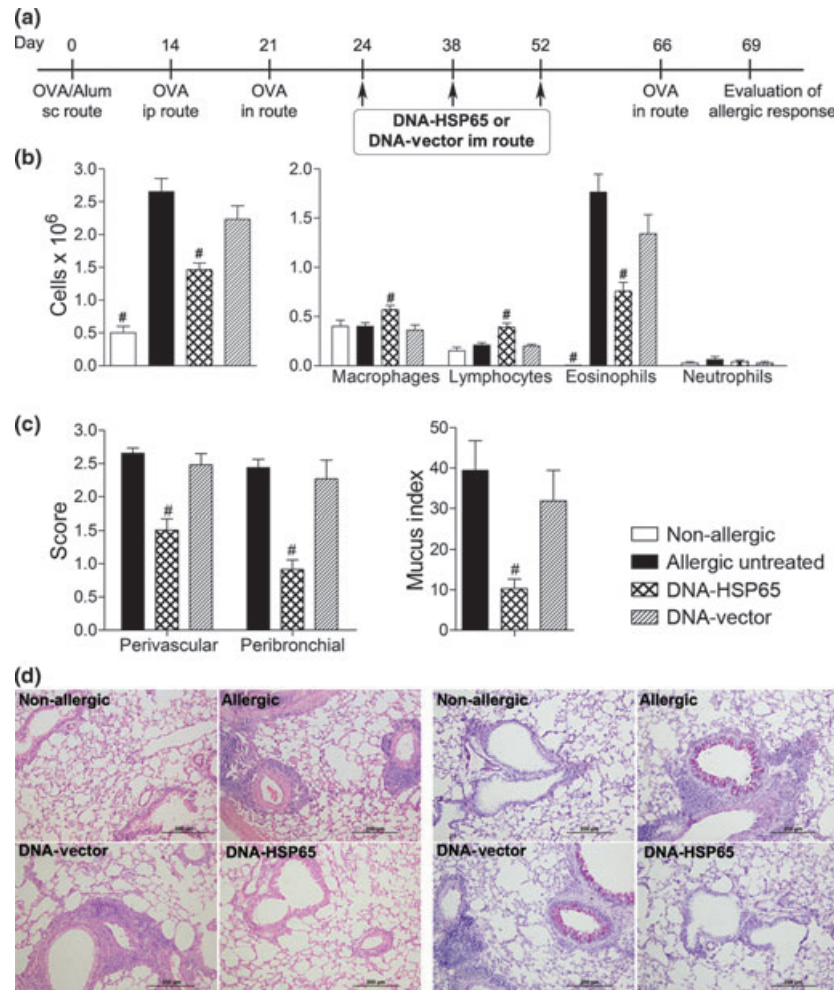


Fig. 1. Modulation of airway allergic inflammation by DNA-HSP65. (a) BALB/c mice were sensitized with OVA. Seven days after the sensitization, the mice received an intranasal OVA challenge, and after 72 h, the allergic mice were treated with three doses DNA-HSP65. As an experimental control, a group of mice received the same doses of DNA-Vector. Two weeks after treatment, mice received a second OVA challenge, and allergic airway inflammation was assessed by quantifying the cells in bronchoalveolar lavage fluid (BALF) (b), analysing lung histopathology, and quantifying of mucus production (c). Representative haematoxylin and eosin (left) and periodic acid-Schiff (right) staining of lung sections; magnification, 200× (d). Data are representative of three experiments ($n = 15$). $^{\#}P < 0.05$ as compared to the other groups; sc, subcutaneous; ip, intraperitoneal; in, intranasal; im, intramuscular.

characterized by IL-10 and TGF- β secretion, as we already observed (Fig. 2b) for OVA-specific spleen cells.

The efficacy of DNA-HSP65 immunotherapy was abrogated in IL-10 deficient mice

Considering that IL-10 is involved in the modulation of allergic responses and its induction is increased by our experimental immunotherapy, we evaluated the efficacy of DNA-HSP65 therapy in IL-10 deficient mice (IL-10 KO). We found that the DNA-HSP65 therapeutic effect was sustained in C57BL/6 WT mice. We observed decreased eosinophil counts; IL-4, IL-5, IL-13, TSLP and eotaxin levels in BALF; and IgG1 and IgE allergen-specific antibodies compared to allergic untreated WT and DNA-Vector WT groups ($P < 0.05$; Fig. 4a–c), as previ-

ously observed with BALB/c mice. Treated mice also had increased IL-10 production in BALF compared to the other groups (Fig. 4c). In the absence of IL-10, none of these effects were observed. Therefore, the DNA-HSP65 immunomodulatory effects are dependent on IL-10 production.

Cells activated after DNA-HSP65 immunization migrate to the allergic site and attenuate allergy

To evaluate the participation of Hsp65-specific cells in the modulation of allergy, we designed an experimental protocol to study the migration of these cells to the sites involved in the allergic immune response. Spleen cells of GFP transgenic mice previously immunized with DNA-HSP65 or cells from non-immunized mice

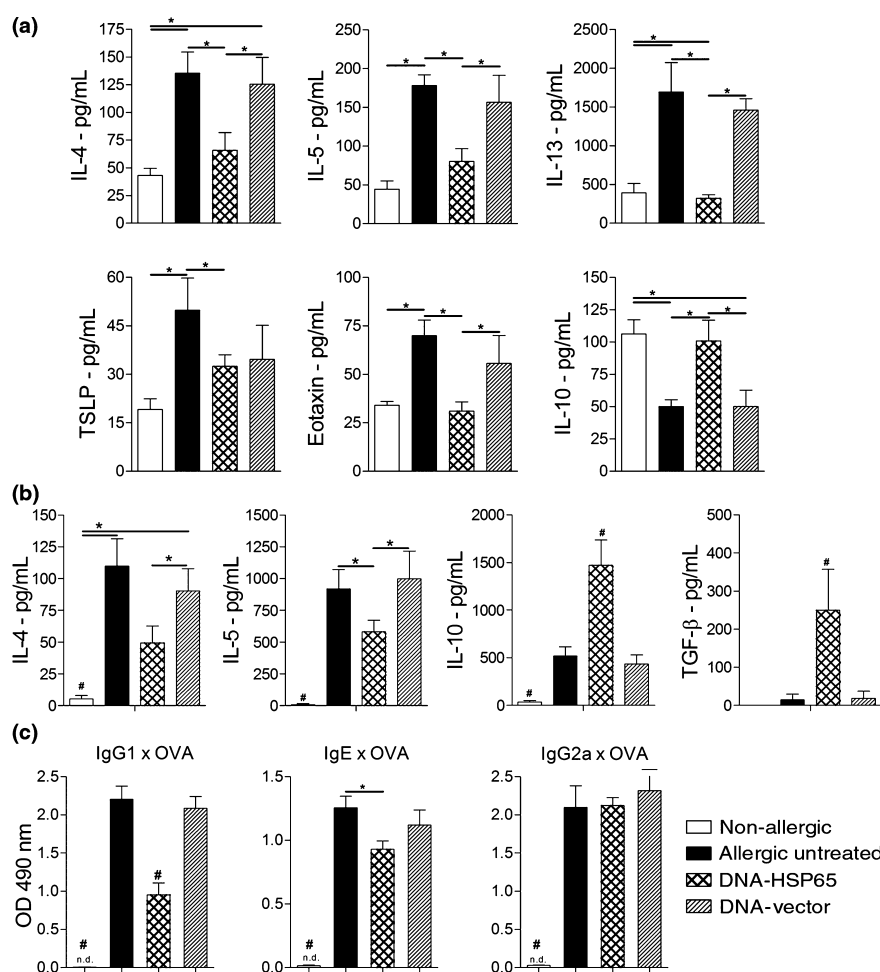


Fig. 2. Modulation of local cytokine secretion and systemic Th2 allergen-specific immune response by DNA-HSP65. Allergic BALB/c mice were treated with DNA-HSP65, as described in Fig. 1a. Seventy-two hours after the last OVA-challenge, cytokine levels were detected in bronchoalveolar lavage fluid (a) and in the culture, supernatants of OVA-stimulated spleen cells (b). OVA-specific IgG1, IgE and IgG2a antibodies were detected in serum at dilutions of 1 : 100 000, 1 : 10 and 1 : 10, respectively (c). Data are representative of three experiments ($n = 15$). * $P < 0.05$ between groups indicated by the line, # $P < 0.05$ as compared to the other groups; nd, not detectable; OD, optical density.

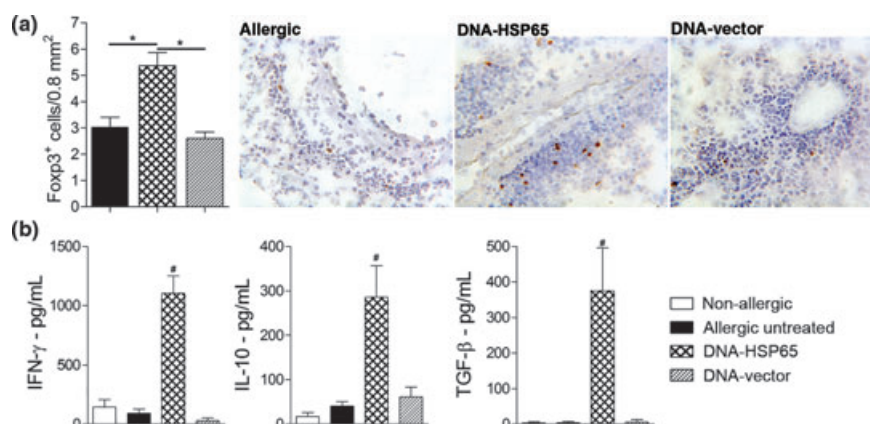


Fig. 3. Induction of regulatory T cells by immunotherapy and Hsp65-specific cellular responses. Allergic BALB/c mice were treated with DNA-HSP65, as described in Fig. 1a. Seventy-two hours after the last OVA-challenge Foxp3⁺ (arrows) cells were stained by immunohistochemistry, magnification, 400 $\times$. (a). Spleen cells from mice were cultured in the presence of r-Hsp65 for detection of cytokine production (b). * $P < 0.05$ between groups indicated by the line, # $P < 0.05$ as compared to the other groups.

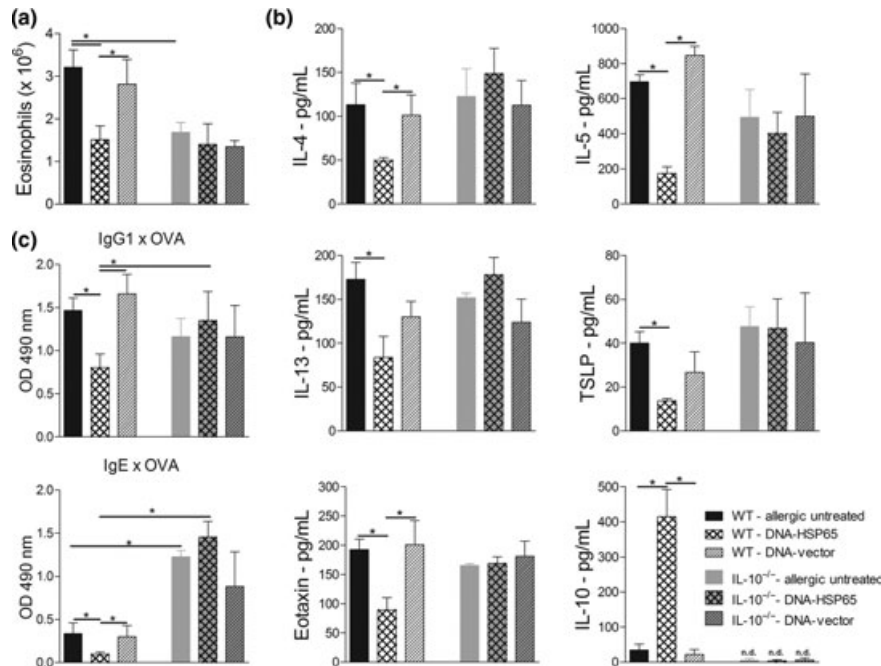


Fig. 4. IL-10 plays an essential role by mediating the effects of DNA-HSP65 immunotherapy. Allergic C57BL/6 wild-type (WT) and IL-10 knock-out (KO) mice were treated with DNA-HSP65, as described in Fig. 1a. Seventy-two hours after the last OVA-challenge, the allergic response was assessed by quantifying the number of cells (a) and detecting cytokine levels (b) in bronchoalveolar lavage fluid, and IgG1 and IgE OVA-specific antibody detection in serum (c) at dilutions of 1 : 100 000 and 1 : 10, respectively. * $P < 0.05$. OD, optical density.

were transferred to allergic mice, as described in Fig. 5a. A higher number and frequency of CD4⁺/GFP⁺ and CD8⁺/GFP⁺ cells was detected in BALF, lungs and draining lymph nodes from allergic mice that received total spleen cells from DNA-HSP65 immunized mice compared to allergic mice that received cells from non-immunized mice ($P < 0.05$; Fig. 5b–c and data not shown).

Because we observed that the GFP⁺ transferred cells migrated to the allergic site, we next evaluated if these cells could modulate the allergic response. In fact, we observed a significant reduction in eosinophil counts and IL-4 levels in BALF from mice that received cells from DNA-HSP65 immunized mice compared to mice that received cells from the other groups (Fig. 5d–e). The group that received cells from DNA-HSP65 immunized mice also exhibited increased lymphocyte counts and IL-10 secretion in BALF ($P < 0.05$; Fig. 5d–e). After the transfer of cells from DNA-HSP65 immunized mice, we also detected lower IL-4 and higher IL-10 secretion by OVA-stimulated spleen cells compared to those of mice that received cells from GFP non-immunized mice (data not shown). However, we did not observe a modulation in the OVA-specific humoral immune response in mice that received cell transfers (data not shown). These results indicate that cells activated by DNA-HSP65 immunization migrate to the allergic sites and mediate the immunomodulatory effects.

Intranasal administration of r-Hsp65 accentuated the effects of DNA-HSP65 intramuscular therapy

Because we observed that cells from DNA-HSP65 immunized mice migrated to the sites involved in allergic inflammation, we subsequently asked how these cells could modulate allergy because we hypothesize that Hsp65 is absent in the airways. Therefore, we administered an intranasal dose of r-Hsp65 protein to mice treated with DNA-HSP65 to induce the activation of specific Hsp65 cells that migrate to the airways and lungs (Fig. 6a).

DNA-HSP65-treated mice that received r-Hsp65 intranasally (DNA-HSP65/r-Hsp65 group) were characterized by a significant decrease in eosinophil counts compared to mice treated only with DNA-HSP65 (Fig. 6b). Instead of increasing lung inflammation through the activation of Hsp65-specific cells, intranasal administration of this protein reduced the perivascular and peribronchial inflammatory scores of these mice compared to allergic untreated mice (Fig. 6c–d). Together with the down-modulation of airway inflammation, we also observed an improvement in lung function that was characterized by a reduction in AHR in DNA-HSP65-treated mice that received r-Hsp65 intranasally compared to allergic untreated mice ($P < 0.05$; Fig. 6e).

Unexpectedly, we did not detect significant differences in cytokine production in BALF after the intranasal administration of r-Hsp65 protein in DNA-HSP65-treated

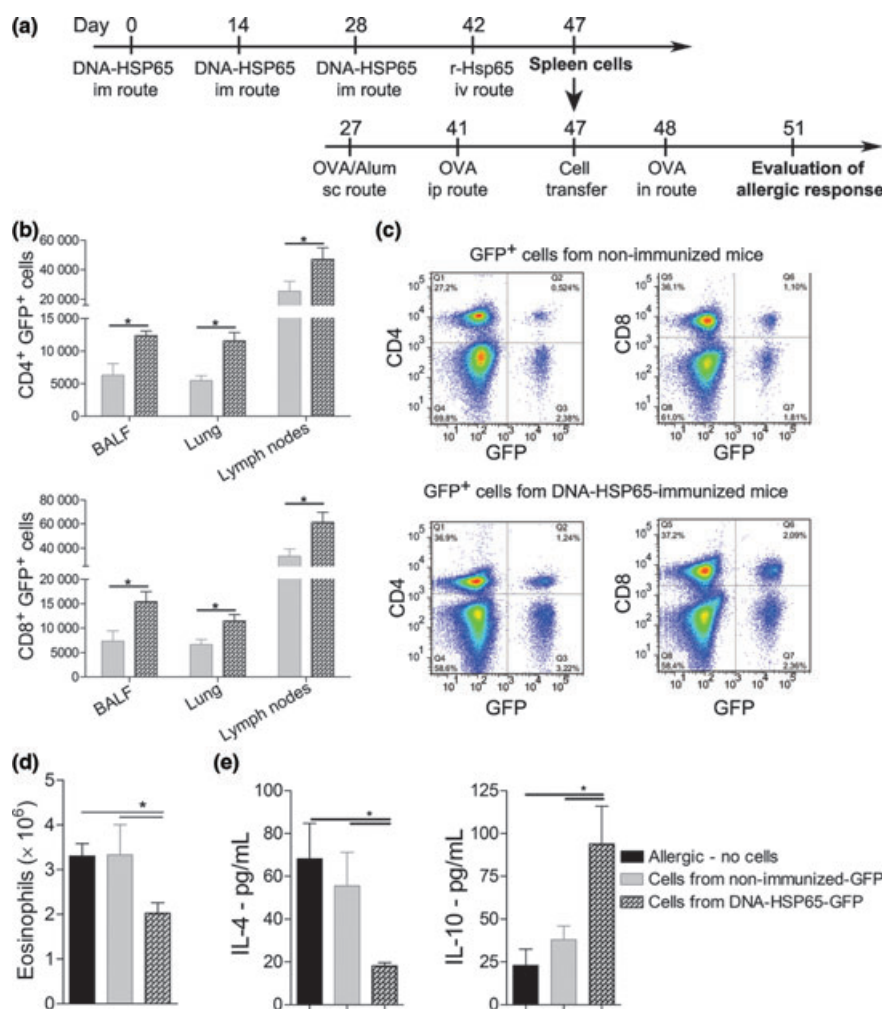


Fig. 5. Therapeutic effects and migration of spleen cells from DNA-HSP65 immunized mice. GFP transgenic mice were immunized with DNA-HSP65 and r-Hsp65 protein (a). Spleen cells from DNA-HSP65-immunized or non-immunized GFP transgenic mice were transferred to C57BL/6 WT mice, which were previously sensitized with OVA. One day after the cell transfer, these mice received an intranasal OVA challenge, and after 72 h, GFP⁺ cell migration in bronchoalveolar lavage fluid (BALF), lung and draining lymph nodes was evaluated by flow cytometry. (b) Number of GFP⁺ cells. (c) Representative plot of lymph node samples obtained from mice that had cells transferred from DNA-HSP65-immunized mice. The modulation of the allergic response was evaluated by quantifying the number of cells (d) and by detecting cytokine levels (e) in BALF. Results are representative of one experiment performed two times ($n = 7$). * $P < 0.05$ between groups indicated by the line. GFP, green-fluorescent protein; WT, wild-type; im, intramuscular.

mice compared to the group treated only with DNA-HSP65 (Fig. 6f). To understand why cytokine production in BALF remained unaffected by intranasal administration of r-Hsp65, and considering that cells from DNA-HSP65-immunized mice were able to reach the lungs (Fig. 5b), we examined lung cell cultures stimulated with OVA or r-Hsp65. Similarly to the results obtained with BALF, lung cells from both the DNA-HSP65 and DNA-HSP65/r-Hsp65 groups cultured with OVA exhibited similar reductions in IL-4, IL-5 and IL-13 levels compared to the cells from allergic untreated group (supporting information, Fig. S2). However, stimulation with r-Hsp65 resulted in a significant increase in secretion of IFN- γ , but not IL-10 or TGF- β , by

lung cells from the DNA-HSP65/r-Hsp65 group compared to the DNA-HSP65 group. Taken together, these results indicate that it is possible to strengthen the therapeutic effects of DNA-HSP65 by administering an intranasal dose of r-Hsp65; however, this new approach did not improve the modulation of Th2 cytokines.

Discussion

Therapeutic strategies designed to down-modulate allergic disorders include allergen SIT or allergen-free immunotherapy. An example of successful allergen-free immunotherapy in pre-clinical studies involved the administration of live or killed forms of non-pathogenic

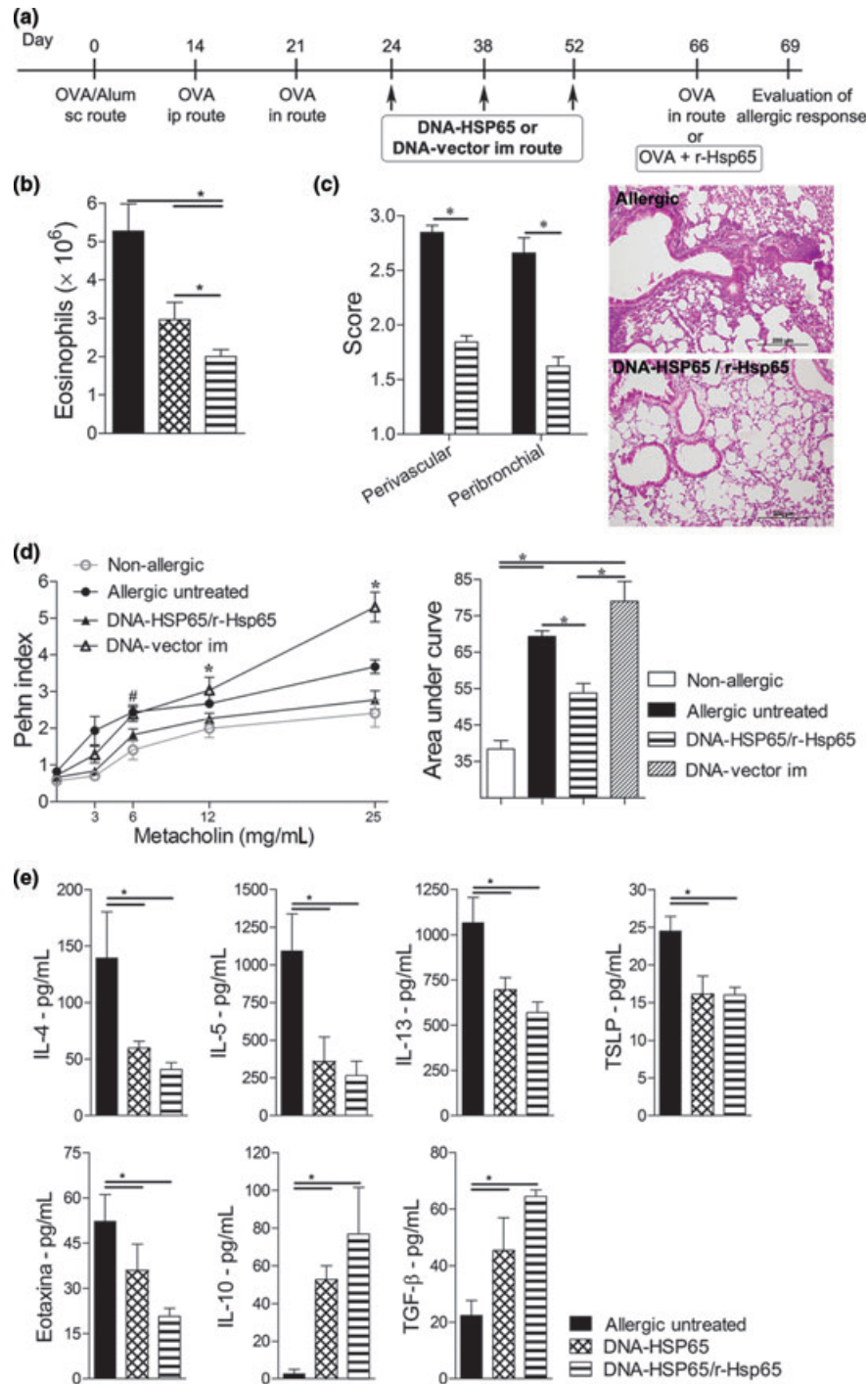


Fig. 6. Local administration of recombinant Hsp65 protein enhanced the effects of DNA-HSP65 parenteral therapy. Allergic BALB/c mice were treated with DNA-HSP65, as described in Fig. 1a. After treatment, mice received a second intranasal dose of OVA and one group of DNA-HSP66-treated mice received r-Hsp65 protein together with OVA during this challenge (DNA-HSP65/r-Hsp65 group) (a). After the challenge, airway allergic response was evaluated by quantifying the number of cells in the bronchoalveolar lavage fluid (BALF) (b), by determining the inflammatory score in lung tissue (c), by determining airway hyperresponsiveness (AHR) (d), and by detecting cytokine levels in BALF (e). AHR was measured using a single-chamber, whole-body plethysmograph, and the Pehn index was determined after nebulization with crescent doses of metacholine. Data are representative of two experiments ($n = 10$). * $P < 0.05$ between groups indicated by the line.

mycobacteria such as *Bacillus Calmette-Guérin* and *Mycobacterium vaccae* [24, 25]. However, there are still concerns regarding the safety of this type of immuno-

therapy because it can induce excessive inflammation. Therefore, a single and well-characterized antigen could be a promising strategy to be considered for human

application. Moreover, an antigen with adjuvant activity and anti-inflammatory properties would have an additional advantage. In this context, a recombinant DNA-encoding *M. leprae* Hsp65 appears to be a good candidate. Indeed, we showed in this study that allergen-free immunotherapy with DNA-HSP65 attenuates established airway allergic inflammation. In addition, we showed that a boosting dose of r-Hsp65 protein given intranasally optimizes the therapeutic effect of DNA-HSP65 treatment.

To understand how DNA-HSP65 immunotherapy suppresses airway allergic inflammation and improves pulmonary function, we investigated the mechanisms involved in this suppression. Although it is well known that IL-10 produced by Tr1 or Foxp3⁺ Treg cells is associated with the suppression of allergic responses after experimental and clinical protocols of immunotherapy [26, 27], it was unclear whether DNA vaccines for allergy utilize a similar mechanism. We found that both Hsp65- and OVA-specific IL-10-producing cells that migrate to site of allergic reaction appear to be relevant to this immunotherapeutic effect. Indeed, after stimulation with OVA or r-Hsp65, spleen or lung cells from treated mice secreted high levels of IL-10. More importantly, the therapeutic effect of DNA-HSP65 was abrogated in IL-10 deficient mice. Interestingly, IL-10 production appears to also be involved in the protective mechanism induced by SIT, which represents a clinically well-established method of treatment of allergic disorders [28]. In SIT, IL-10 can be secreted by Foxp3⁺ Treg cells, Tr1 cells, Th1 cells, dendritic cells and B cells [28]. In addition, IL-10 production is detected in cells from patients who have undergone allergen-immunotherapy as well as in healthy individuals chronically exposed to the allergen [26–28]. With regard to the modulation of allergic responses by helminthes, it was demonstrated that IL-10 plays a key role in dampening atopic disorders [29]. The induction of IL-10- and TGF- β -producing Treg cells was also described in an experimental murine model of oral tolerance induced by intragastric administration of an allergen conjugated to the B subunit of cholera toxin [30]. In this study, two distinct populations of Treg cells were found: one population was CD25⁺Foxp3⁺ and the other was CD25⁻Foxp3⁻.

The protective role that IL-10 secretion by Treg cells exerts has also been described after the administration of heat killed *M. vaccae* [25]. In this work, CD4⁺CD45RB^{lo} T cells down-modulated allergen-specific Th2 responses through mechanisms dependent on IL-10 and TGF- β secretion. In addition, mycobacterial immunization of allergic mice can also induce IL-10 production by dendritic cells [31].

Our results support the hypothesis that IL-10-producing cells play a key role in the suppression of allergic inflammation. It is possible that Foxp3⁺ T cells found in

the lungs of treated mice produce IL-10. However, in this work we could not conclude that the Foxp3⁺ T cells were the source of IL-10, because we did not perform a double staining for IL-10 and Foxp3. In addition, our results suggest that Hsp65-specific cells might also participate in the inhibition of Th2 allergic responses also by secreting TGF- β and/or IFN- γ , as we observed in cell cultures.

Although we established that spleen cells from DNA-HSP65-immunized mice migrated to the airways of allergic mice, we did not determine whether Hsp65-specific T cells modulate allergic Th2 responses directly in the lung. We hypothesized that by delivering Hsp65 antigen into the airways, we could re-stimulate recently migrated Hsp65-specific T cells and consequently increase the production of IL-10. Unexpectedly, although the administration of r-Hsp65 protein reduced the eosinophil counts, it did not alter IL-10 production, which suggests that Hsp65-specific cells are already primed for IL-10 production. However, we observed an increase in IFN- γ production after intranasal administration of r-Hsp65 protein. It remains to be determined why a challenge with r-Hsp65 protein increases IFN- γ but not IL-10.

As the r-Hp65 used during the intranasal treatment presented 9.2 EU of lipopolysaccharide (LPS) and this in itself could be an important factor in modulation of the allergic response, we evaluated if this effect was mediated by LPS. However, when allergic mice received the intranasal dose of r-Hsp65 without the previous administration of recombinant DNA-HSP65, the protein had no effect in the allergic response (data not shown). These data suggest that the induction of a previous Hsp65-specific immune response by the DNA-HSP65 is necessary for effect of the intranasal administration of r-Hsp65. In addition, when TLR4 deficient mice were treated, the immunomodulatory effects of intramuscular DNA-HSP65 and intranasal r-Hsp65 were sustained (data not shown), confirming that it was a Hsp65-mediated effect and not an LPS-mediated effect. Another important issue to be considered is that, despite the fact that Hsp is also TLR4 agonist, it is suggested that the regulatory properties of this protein could be mediated by TLR2 [12]. This hypothesis is under investigation by our group.

The fact that intranasal challenge with r-Hsp65 protein enhanced the suppressive effect on eosinophilic inflammation without increasing IL-10 levels suggests the presence of another mechanism involving IFN- γ production by Hsp65-specific cells might be operating. It is well established that IFN- γ suppresses airway eosinophilia [32]. In addition, IFN- γ secretion by Th1 cells can down-modulate the allergic response by directly inhibiting Th2 cells or by inducing FAS/FAS-L-mediated apoptosis, as has been previously described [33]. Using a cell transfer model, Cohn and colleagues

clearly demonstrated that the transfer of Th2 cells induced airway eosinophilia and mucus production [34]. Both were inhibited by the transfer of Th1 cells via a mechanism dependent on signalling through the IFN- γ receptor. This concept can be extrapolated to immunotherapy. Previous studies have shown a significant increase in allergen-specific production of IFN- γ in patients who have undergone SIT [26–28].

Our results are in line with previous studies showing that the mycobacterial Hsp65 protein was able to reduce eosinophilic inflammation, decrease Th2 cytokine production and ameliorate lung function [35, 36]. However, these studies lacked a mechanistic explanation for the suppressive effect of Hsp65 and employed recombinant protein for their experiments, whereas we used recombinant DNA that expressed Hsp65. Another important difference is the fact that we reversed an established airway allergic disease, whereas the other studies employed a protocol of Hsp65 treatment that was given before allergen challenge and during the phase of allergen sensitization. In this sense, our approach might have a broader clinical application.

Previous studies have shown that low concentrations of protein are translated from recombinant plasmid DNA after intramuscular injection [16]. Our results with mycobacterial Hsp administered as recombinant DNA indicate that the modulation of allergy can be achieved with reduced amounts of DNA. Despite the classical concerns about recombinant DNA in humans, our results and the results of other studies indicate that this type of formulation is safe for humans, does not integrate in the genomic DNA and does not induce autoimmune manifestations [37, 38]. In addition, we showed that the suppressive effect conferred by DNA could be enhanced by an additional injection of r-Hsp65 protein into the airway.

It is important to also mention the results pertaining to TGF- β production because this cytokine is involved in the induction of Treg cells, in the effector

function of Th3 cells, in the differentiation of Th9 cells in the presence of IL-4, and in the development of fibrosis [39–41]. Accordingly, TGF- β can control or exacerbate allergic response. Because we found a reduction in IL-4 secretion, we suggest that in our model, TGF- β promotes Treg cell differentiation instead of Th9 cell differentiation. In addition, it has been described that *M. leprae* DNA-HSP65 reduces fibrosis in an experimental model of bleomycin-induced lung injury [42].

Our work opens new perspectives regarding the therapeutic effect of DNA-HSP65 in allergic disorders. We have shown that allergen-free immunotherapy containing bacterial antigens can be efficiently used to treat airway allergic disorders.

Although we consider that DNA vaccination may represent a good strategy to treat allergic disease, the efficacy of these vaccines has not been shown to be as good in large animals as in mice. However, we consider that a regulatory response induced by DNA vaccination has not been widely evaluated in clinical trials. Because of this, the evaluation of allergic patient cellular responses after stimulation with this antigen may provide some clues for the effective and safe design of clinical trials.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Cytokine detection in lung homogenates.

Figure S2. Cytokine production by lung cells.

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