

Requirement of MyD88 and Fas pathways for the efficacy of allergen-free immunotherapy

D. M. Fonseca¹, P. F. Wowk^{1,2}, M. O. Paula¹, A. F. Gembre¹, M. D. Baruffi³, M. L. Fermino³, W. M. Turato¹, L. W. Campos¹, C. L. Silva¹, S. G. Ramos⁴, C. Horn⁵, G. Marchal⁶, L. K. Arruda⁷, M. Russo⁸ & V. L. D. Bonato¹

¹Department of Biochemistry and Immunology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto; ²Carlos Chagas Institute, Oswaldo Cruz Foundation, Curitiba; ³Department of Clinical Analyses, Toxicology and Food Sciences, Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo; ⁴Department of Pathology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto; ⁵Laboratory of Immunology and Immunogenetics, Evandro Chagas Clinical Research Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil; ⁶Immunotherapix Bio Top, Institute Pasteur, Paris, France; ⁷Department of Medicine, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto; ⁸Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil

To cite this article: Fonseca DM, Wowk PF, Paula MO, Gembre AF, Baruffi MD, Fermino ML, Turato WM, Campos LW, Silva CL, Ramos SG, Horn C, Marchal G, Arruda LK, Russo M, Bonato VLD. Requirement of MyD88 and Fas pathways for the efficacy of allergen-free immunotherapy. *Allergy* 2015; **70**: 275–284.

Keywords

allergen-free immunotherapy; CpG oligodeoxynucleotides; experimental allergy; mycobacterial antigens.

Correspondence

Vânia L. D. Bonato, PhD, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo. Av. Bandeirantes, 3900, Ribeirão Preto, São Paulo, 14049-900, Brazil.

Tel.: +55 16 3315 3089

Fax: +55 16 3315 0219

E-mail: vlbonato@fmrp.usp.br

Accepted for publication 2 December 2014

DOI:10.1111/all.12555

Edited by: Hans-Uwe Simon

Abstract

Background: We have shown that mycobacterial antigens and CpG oligodeoxynucleotides downmodulate airway allergic inflammation by mechanisms dependent on T-cell activation. Here, we investigated the participation of the innate response, particularly the role of MyD88 adaptor, and Fas molecules in the effectiveness of DNA-HSP65 or CpG/culture filtrate proteins (CFP) immunotherapy. **Methods:** Mice sensitized and challenged with Der p 1 allergen were treated with DNA-HSP65, CpG/CFP, or with adoptively transferred cells from immunized mice. The treatment efficacy was assessed by evaluating eosinophil recruitment, antibody, and cytokine production.

Results: In addition to downregulating the Th2 response, DNA-HSP65 and CpG/CFP promoted IL-10 and IFN- γ production. Adoptive transfer of cells from mice immunized with DNA-HSP65 or CpG/CFP to allergic recipients downmodulated the allergic response. Notably, transfer of cells from DNA-HSP65- or CpG/CFP-immunized MyD88^{-/-} mice failed to reduce allergy. Additionally, for effective reduction of allergy by cells from CpG/CFP-immunized mice, Fas molecules were required. Although DNA-HSP65 or CpG/CFP immunization stimulated antigen-specific production of IFN- γ and IL-10, the effect of DNA-HSP65 was associated with IL-10 while CpG/CFP was associated with IFN- γ . Moreover, after stimulation with mycobacterial antigens plus Der p 1 allergen, cells from mite-allergic patients with asthma exhibited similar patterns of cytokine production as those found in the lung of treated mice.

Conclusions: This study provides new insights on the mechanisms of allergen-free immunotherapy by showing that both DNA-HSP65 and CpG/CFP downregulated house dust mite-induced allergic airway inflammation via distinct pathways that involve not only induction of mycobacterial-specific adaptive responses but also signaling via MyD88 and Fas molecules.

Asthma and other atopic disorders are on rise in industrialized countries, the cause of which is still a matter of debate. The hygiene hypothesis postulates that the lack of exposure to microbial agents (e.g., tuberculosis and measles) contrib-

utes to increased susceptibility to allergy (1). Indeed, Shirakawa and colleagues indicated an association between exposure to *Mycobacterium tuberculosis* and reduced risk of developing asthma (2). Airway inflammation and hyperactivity (AHR)

driven by T-helper (Th) 2 cells are hallmarks of allergic asthma. Experimentally, it has been shown that mycobacterial infections or products downregulate the establishment of allergen-induced Th2 immunity (3–5).

Mycobacterial-derived components such as lipoarabinomannan induce pro-inflammatory cytokine production by macrophages via Toll-like receptor 2 (TLR) (6) while mannosyl-lipoarabinomannan can induce IL-10 secretion by dendritic cells when interacting with DC-SIGN (7). The *M. tuberculosis* 19 kDa-secreted lipoprotein also induces pro-inflammatory or anti-inflammatory responses, via TLR2 (8, 9). Mycobacterial heat-shock proteins (Hsp) are known to modulate the host immune system (10). As chaperonins, they act as adjuvants to promote antigen presentation and induce NF κ B-dependent secretion of pro-inflammatory cytokines by interacting with TLR2 and TLR4 (10, 11). Because Hsp are immunodominant antigens during mycobacterial infections, they have been considered for vaccine formulations against infectious diseases (11). The mycobacterial Hsp65 has also been the focus of tolerogenic immune interventions aimed at preventing and treating immune-mediated diseases such as experimental autoimmune encephalitis, arthritis, atherosclerosis, and diabetes (10, 12).

According to the hygiene hypothesis (1, 13), two major immunologic pathways could be involved in the control of Th2 responses by mycobacterial infections: (i) generation of regulatory T cells (Treg) and/or induction of suppressive molecules, which prevent the development or block the activities of Th2 effector cells and/or (ii) development of allergen-specific Th1 cells or Th1-immune deviation, which antagonizes Th2 responses via type 1 cytokines (13).

We have previously shown using a classic model of ovalbumin (OVA)-induced asthma, that airway allergic inflammation was reversed by treating mice with a DNA plasmid encoding the *M. leprae* 65 kDa Hsp (DNA-HSP65) (14). The immunomodulatory effect of DNA-HSP65 was dependent on IL-10 production and on Hsp65-specific immune response. In addition, culture filtrated proteins (CFP) from *M. tuberculosis* delivered in the presence of the TLR9 agonist CpG (CpG/CFP) also downmodulated experimental allergy, in an antigen- and IFN- γ -dependent manner (15).

In this study, we have evaluated the efficacy and the additional mechanisms of DNA-HSP65 and CpG/CFP in modulating the Th2 response induced by the major *Dermatophagoides pteronyssinus* house dust mite (HDM) allergen Der p 1 in an experimental model of asthma. We have also studied the effect of these mycobacterial products on cultured human mononuclear cells from mite-allergic patients with asthma. Our results showed that therapy with mycobacterial antigens was effective in downmodulating the Th2 response in a Der p 1 murine model of asthma, and revealed that MyD88 adaptor molecule is critically involved in this immunotherapeutic process, while Fas molecule is also important in CpG/CFP immunotherapy. In addition, mycobacterial antigens reduced IL-4 production by peripheral blood mononuclear cells from mite-allergic patients. Taken together, these results highlight the importance of mycobacterial products in the reversal of Th2 responses.

Methods

Animals

Female C57BL/6 wild-type (WT), MyD88^{-/-}, and Fas^{-/-} mice at 6–8 weeks old were obtained from the local breeding facility at the Ribeirão Preto Medical School, University of São Paulo, Brazil, and housed under specific pathogen-free conditions. All knockout mice were C57BL/6 genetic background. Mice were treated according to the Animal Welfare Guidelines of the Ribeirão Preto Medical School, University of São Paulo. The local Animal Research Ethics Committee approved all of the procedures (Process number 071/2006).

Experimental design

For the induction of allergic airway response, BALB/c mice were sensitized with two doses of 10 μ g HDM allergen Der p 1. The first dose was administered via subcutaneous injection in the presence of 1.6 mg alum, and the second dose was administered after 14 days by intraperitoneal injection. Seven days after sensitization, mice were challenged intranasally with 10 μ g Der p 1/50 μ l PBS and, after 72 h, mice were treated with the different mycobacterial antigens as previously described (14, 15). Mice treated with DNA-HSP65 or DNA-vector control (pcDNA3) received three intramuscular doses of 100 μ g DNA in the presence of 12.5% sucrose at 2 weeks intervals. Mice treated with CpG/CFP or CpG alone received three doses of 50 μ g CpG1826 and 50 μ g CFP by subcutaneous injection at 7-day intervals. At 2 weeks post-treatment, mice received a second allergen challenge in the presence of 50 μ g Hsp65 or CFP, according to the treatment (Fig. 1A). Airway hyperresponsiveness (AHR) was evaluated after 24 h, and eosinophil recruitment, lung inflammation, and cytokine production were evaluated 72 h after the allergen challenge.

For the adoptive cell transfer experiments, C57BL/6, MyD88^{-/-}, and Fas^{-/-} mice were immunized with three doses of DNA-HSP65, DNA-vector, CpG/CFP, or CFP and boosted with 50 μ g Hsp65 or CFP, according to the immunization, 1 week after the last immunization. Five days post-boost, spleen cells from immunized or naïve mice were isolated and 5×10^6 cells were intravenously transferred to C57BL/6 mice, previously sensitized with Der p 1. Twenty-four hours after the cell transfer, mice received an allergen challenge in the presence of Hsp65 or CFP and eosinophil number, IL-4, and IL-5 were evaluated after 72 h.

Statistical analysis

The data were expressed as the mean $\pm$ SEM. The results were compared using analysis of variance (ANOVA) followed by a Tukey's test with Prism software (GraphPad Software Inc., San Diego, CA, USA). For human samples, statistical significance was determined by a paired nonparametric test (Wilcoxon) among different stimulations in the same group and an unpaired nonparametric test (Mann–Whitney) for different groups. Values of $P < 0.05$ were considered statistically significant.

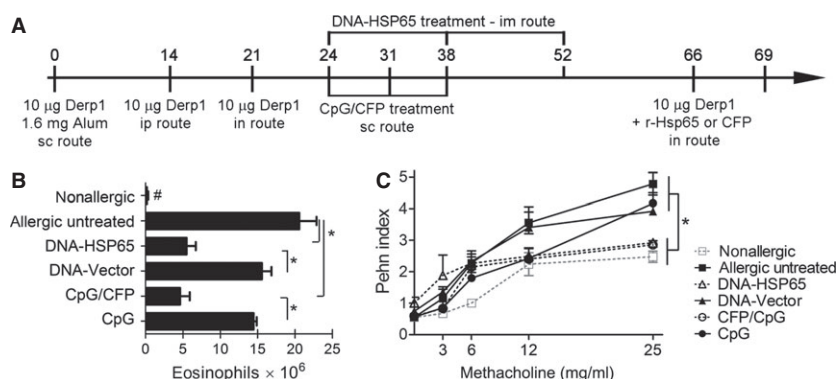


Figure 1 Modulation of Der p 1-induced allergic airway inflammation by mycobacterial antigens. BALB/c mice were sensitized and challenged with Der p 1, and 72 h after challenge, mice were treated with three doses of DNA-HSP65 or CpG/ culture filtrated proteins (CFP) (A). Control groups were untreated or received the empty DNA-vector or CpG motifs. Two weeks after treatment, mice received a second challenge with Der p 1 and, after 72 h,

total eosinophils were assessed in bronchoalveolar lavage fluid (BALF) (B). The AHR was measured using a single-chamber, whole-body plethysmograph 24 h after the allergen challenge, and the Pehn index was determined after nebulization with increasing doses of methacholine (C). Data are representative of two experiments ($n = 4-8$) and expressed as the mean $\pm$ SEM. * $P < 0.05$, # $P < 0.05$ compared to the other groups.

For more detailed information, please consult the Appendix S1.

Results

Reversal of Der p 1-induced airway allergic inflammation by mycobacterial antigens

To test efficacy of mycobacterial antigens in modulation of allergic inflammation, mice were sensitized and challenged with recombinant Der p 1 from *Dermatophagoides pteronyssinus*, a major mite allergen (16), to establish airway inflammation. After that, mice were treated with DNA-HSP65 or CpG/CFP as depicted in Fig. 1A. Mice sensitized and challenged with Der p 1 showed an increase in total cell counts and eosinophil recruitment to bronchoalveolar lavage fluid (BALF), peribronchial lung inflammation, mucus production, airway responsiveness to methacholine, and Der p 1-specific antibodies, when compared with control mice (nonallergic mice) (Fig. 1B,C and Figs S1 and S2). Notably, treatment with DNA-HSP65 or CFP/CpG significantly decreased all parameters of airway allergic inflammation, including type 2 cytokine production such as IL-4, IL-5, IL-13, and TSLP in BALF and lung homogenates (Fig. S1D,E). DNA-HSP65 treatment significantly increased IL-10 secretion in lung homogenates, whereas CpG/CFP treatment increased IFN- γ levels in BALF and lung homogenates (Fig. S1D,E). Administration of the empty DNA-vector had no effect on airway inflammation, whereas CpG treatment induced a mild reduction in Th2 responses (Fig. 1, Figs S1 and S2).

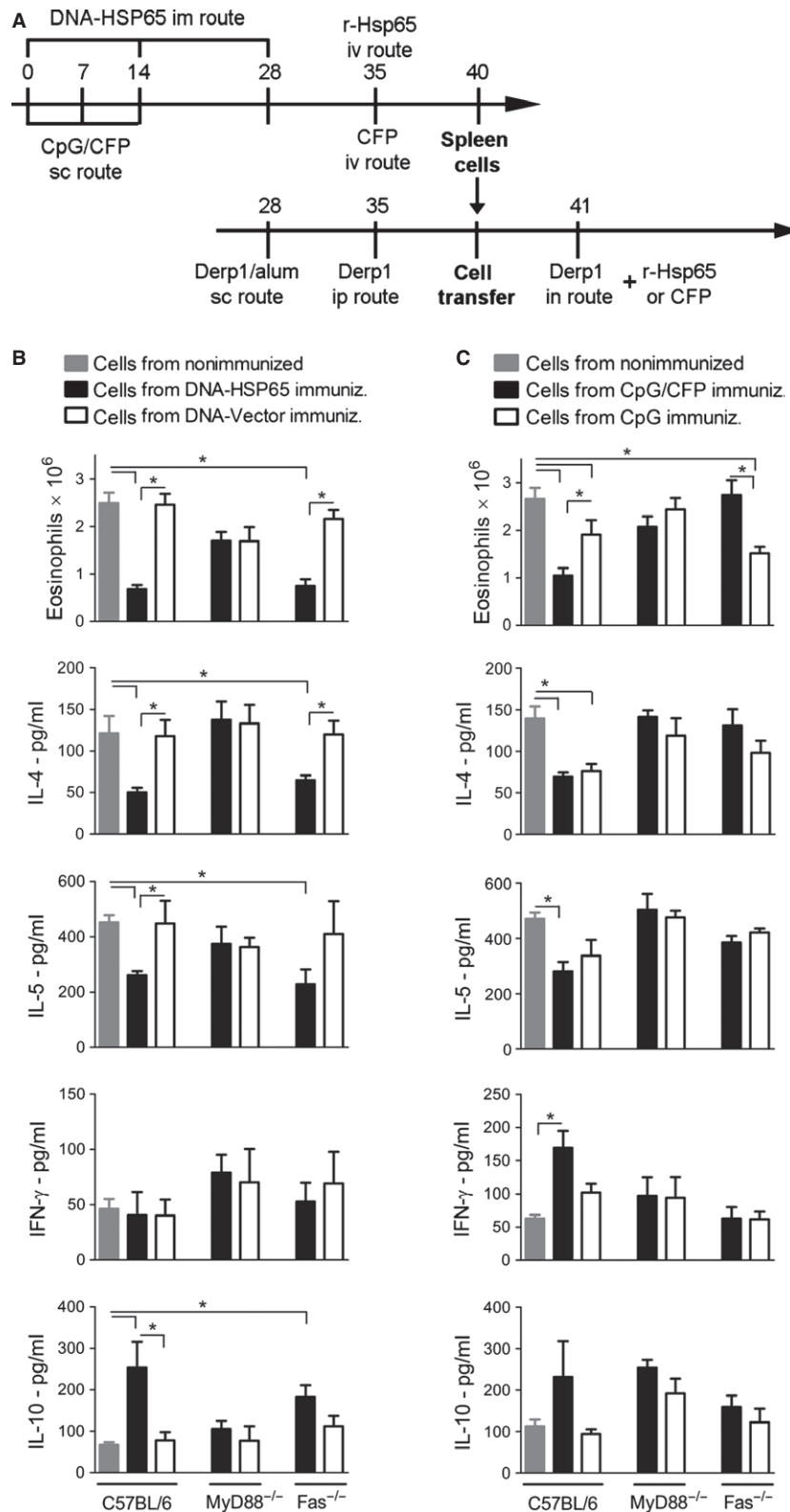
Reversal of allergic inflammation by DNA-HSP65 or CpG/ CFP immunotherapy is dependent on MyD88, while CpG/ CFP effect is also dependent on Fas

To characterize the immunologic pathways associated with reversal of allergic inflammation by mycobacterial immuno-

therapy, we focused on two mechanisms known to control allergic inflammation: emergence of Treg cells and apoptosis of effector cells (3, 13, 17, 18). We found a significant increase of Foxp3⁺ Tregs cells in BALF and lungs of DNA-HSP65-treated mice, but not in lungs of CpG/CFP group, when compared to other groups (Fig. S3A,B). Treatment with CpG/CFP increased the frequency of Annexin-V⁺ BALF cells when compared with other groups, indicating that the therapy could promote phosphatidylserine (PS) exposure on leukocytes, a marker of apoptosis (19) (Fig. S3C,D). Therefore, our data indicate that both strategies of immunotherapy tested are efficient in reversing an established allergic inflammation, but encompass different immunomodulatory pathways: DNA-HSP65 increased IL-10 production and Foxp3⁺ cells, while CpG/CFP increased IFN- γ production and frequency of Annexin-V⁺ cells. Therefore, we used WT, MyD88^{-/-}, and Fas^{-/-} mice to study the mechanisms associated with mycobacterial immunotherapy as MyD88 participates in multiple TLR-dependent mechanisms and Fas is associated with apoptotic and nonapoptotic immune activities (20).

We have previously shown that spleen cells from mice immunized with DNA-HSP65 or CpG/CFP could adoptively transfer suppression of ongoing allergic inflammation. Therefore, we performed cell transfer experiments to circumvent the deficiency of MyD88 or Fas during the induction of allergy. Spleen cells from C57BL/6 (WT), MyD88^{-/-}, Fas^{-/-} mice, immunized with DNA-HSP65 or CpG/CFP, were adoptively transferred to allergic WT mice, which were later challenged with Der p 1 (Fig. 2A).

Allergic mice that received spleen cells from DNA-HSP65 or CpG/CFP-immunized WT mice showed a significant decrease in eosinophil counts and IL-4 and IL-5 secretion in BALF, as compared to allergic mice transferred with cells from naïve WT mice or from mice that received DNA-vector or CpG (Fig. 2B,C). Notably, transfer of spleen cells from CpG/CFP- or DNA-HSP65-immunized MyD88^{-/-} mice did not affect eosinophil recruitment or cytokine production



(Fig. 2B,C). Additionally, transfer of spleen cells from CpG/CFP-immunized Fas^{-/-} mice had no effect on the downmodulation of Th2 allergic response (Fig. 2C). We found

increased levels of IL-10, but not IFN- γ , in BALF of allergic WT mice treated with spleen cells from DNA-HSP65-immunized WT mice or Fas^{-/-}, but not MyD88^{-/-} (Fig. 2B). In

Figure 2 Modulation of the allergic response by cells from DNA-HSP65 or CpG/culture filtrated proteins (CFP)-immunized mice. (A) C57BL/6 wild-type, MyD88^{-/-}, and Fas^{-/-} mice were immunized with three doses of DNAHSP65 or CpG/CFP. Control groups were nonimmunized or received DNA-vector or CpG. Two weeks after the last immunization, spleen cells from DNA-HSP65- or CpG/CFP-immunized mice (black bars in B and C, respectively) or nonimmunized (gray bars) mice, or cells from DNA-vector administered mice

or CpG administered mice (white bars) were transferred to allergic C57BL/6 mice. Twenty-four hours after the cell transfer, mice received an intranasal allergen challenge in the presence of recombinant Hsp65 or CFP antigens. After 72 h, modulation of the allergic response was evaluated by quantifying the number of eosinophils and by measuring cytokine levels in bronchoalveolar lavage fluid. Results are representative of two experiments ($n = 7$) and expressed as mean $\pm$ SEM. * $P < 0.05$.

contrast, we found increased levels of IFN- γ , but not IL-10, in BALF of allergic WT mice treated with spleen cells from CpG/CFP-immunized WT, but not MyD88^{-/-} or Fas^{-/-} mice (Fig. 2C). These data suggest that the effect of DNA-HSP65 is dependent on MyD88 signaling and CpG/CFP is mediated by both MyD88 and Fas-dependent pathways.

As Fas signaling exerts other functions on the immune system beyond cell apoptosis (20) and in an attempt to find additional evidences to support apoptotic process in CpG/CFP-treated mice, we determined other biomarkers of apoptosis: the cleavage of PARP and caspase 3 in lung homogenates. Surprisingly, we did not detect any cleavage of the caspase common substrate PARP or caspase 3 by Western blotting analysis (data not shown) (21). Therefore, the increase of Annexin-V+ in BALF cells might indicate that the PS externalization is regulating other cellular processes not involved in apoptosis (22–24).

Altogether, our data indicate that both strategies of immunotherapy tested are efficient in reversing an established HDM-induced allergic inflammation, but encompass different immunomodulatory pathways.

Cytokine production by spleen cells of DNA-HSP65- or CpG/CFP-immunized animals

Next, we determined the cytokine production by spleen cells of WT, MyD88^{-/-}, and Fas^{-/-} mice immunized with DNA-HSP65 or CpG/CFP. We found that spleen cells from WT mice immunized with DNA-HSP65 or CpG/CFP produced significant levels of IL-10 or IFN- γ after *in vitro* restimulation with rHsp65 or CFP, respectively (Fig. 3A,B). The IL-10 or IFN- γ production was dependent on MyD88 expression because the deficiency in expression of MyD88 impaired either IFN- γ or IL-10 secretion by spleen cells obtained from DNA-HSP65- or CpG/CFP-immunized mice (Fig. 3A,B). Importantly, IL-10 or IFN- γ production was also dependent on Fas expression because spleen cells of DNA-HSP65- or CpG/CFP-immunized Fas^{-/-} mice secreted lower levels of both cytokines than cells from WT counterparts (Fig. 3A,B).

Therefore, MyD88 and Fas signaling are crucial for IL-10 and IFN- γ secretion induced by DNA-HSP65- and by CpG/CFP-immunized mice.

We also detected similar levels of anti-Hsp65 and anti-CFP IgG1 antibodies in the serum of WT, My88^{-/-}, and Fas^{-/-} immunized mice (Fig. 3C,D). The anti-Hsp65 IgG2a production was partially dependent on MyD88 for mice immunized with DNA-HSP65, while Fas was not required for anti-

Hsp65 IgG2a production. However, anti-CFP IgG2a production was completely dependent on MyD88 and Fas for animals immunized with CpG/CFP (Fig. 3C,D).

Effect of mycobacterial antigens on cytokine production by Der p 1-stimulated peripheral blood mononuclear cells of mite-allergic patients with asthma

To determine whether mycobacterial compounds also affect Der p 1-specific responses in humans, we studied the cytokine production of PBMC from patients with asthma and healthy controls stimulated *in vitro* with Der p 1 in the presence or absence of mycobacterial antigens. We found an increased secretion of IL-4, but not IFN- γ or IL-10, in the supernatants of PBMC from patients with asthma stimulated with Der p 1 compared to nonstimulated cells or to healthy controls (Fig. 4A). Stimulation of PBMC from patients with asthma with Der p 1 plus rHsp65 or CpG/CFP resulted in reduction of IL-4 secretion. PBMC cultures from patients with asthma produced IFN- γ in response to Der p 1 plus rHsp65 ($P > 0.05$) or Der p 1 plus CpG/CFP ($P < 0.05$), whereas Der p 1 plus rHsp65 induced IL-10 production ($P < 0.05$), compared to PBMC from patients with asthma stimulated with Der p 1 alone. PHA polyclonal stimulus was able to induce significant production of all evaluated cytokines, but IL-4 production was higher in supernatants from patients with asthma (Fig. 4B).

Taken together, these data show that, like the experimental model, cells from mite-allergic patients with asthma produce similar pattern of cytokines in response to mycobacterial antigens.

Discussion

Allergen-free strategies of immunotherapy have proven promising in experimental models and clinical trials (3, 4, 13, 14, 25). Although some of the mechanisms underlying the therapeutic effects of those formulations have been clarified in the classic OVA murine model, it is still unclear whether these events could be induced during treatment of patients. Here, we show that mycobacterial antigens controlled HDM-induced airway allergic inflammation by modulating the adaptive immune response through MyD88- and Fas-dependent pathways. In addition, we observed that cells from mite-allergic patients with asthma respond to mycobacterial antigens by producing a pattern of cytokines similar to that observed in allergic-treated mice.

Using an experimental model of asthma with OVA, we have shown that treatment with DNA-HSP65 or CpG/CFP

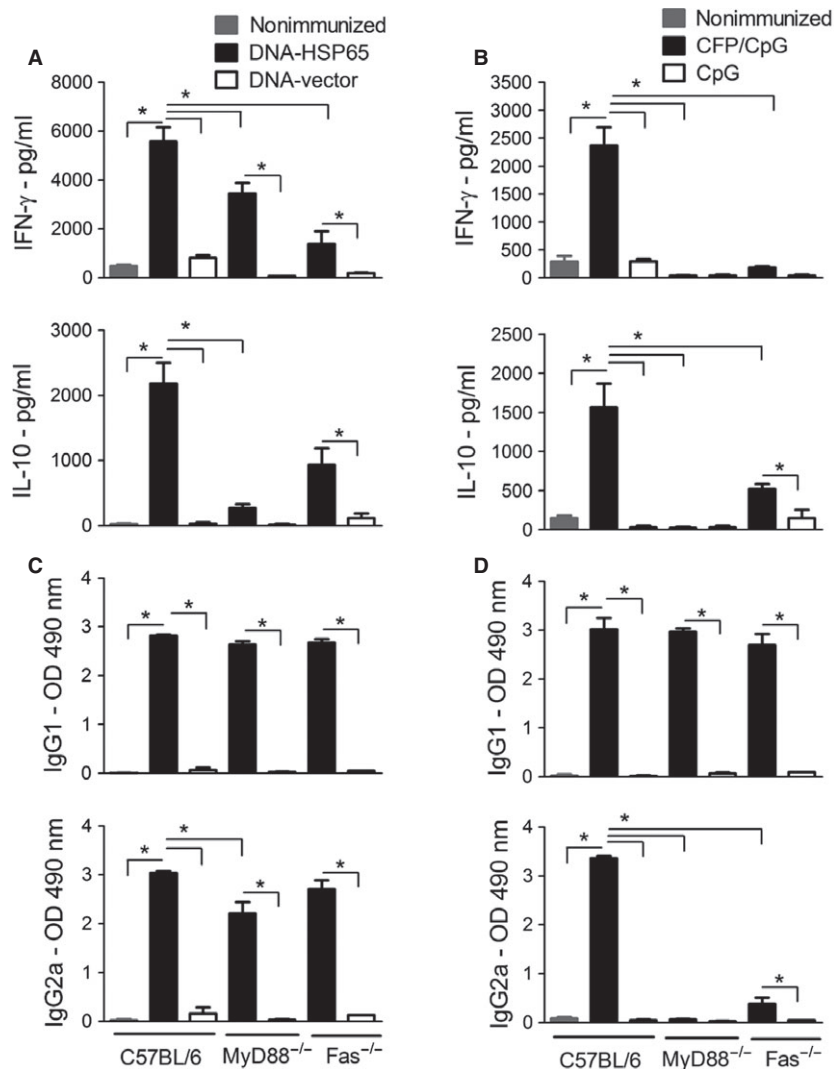


Figure 3 Role of MyD88 and Fas in the pattern of the immune response induced by DNA-HSP65 or CpG/culture filtrated proteins (CFP) immunization. C57BL/6 wild-type or MyD88^{-/-} mice were immunized with three doses of DNA-HSP65 or CpG/CFP. Control groups were nonimmunized or received DNA-vector or CpG. Two weeks after the last immunization, spleen cells from DNA-HSP65- or CpG/CFP-immunized (black bars in A and B, respectively) or non-immunized (gray bars), or cells from DNA-vector administered mice

or CpG administered mice (white bars) were cultured for 48 h in the presence of recombinant Hsp65 or CFP, respectively. The levels of IFN-γ and IL-10 were detected in culture supernatants by ELISA. In parallel, anti-Hsp65 and anti-CFP antibodies were detected in sera of DNA-HSP65- (C) or CpG/CFP- (D) immunized or nonimmunized mice. Results are representative of two experiments ($n = 7$) and expressed as mean $\pm$ SEM. * $P < 0.05$.

reversed previously established allergic response (14, 15). However, although the classical model of OVA-induced allergy resembles several features of human disease, intrinsic characteristics of the natural allergens might interfere with the efficacy of immunotherapy. In addition to the fact that HDM is one of the most prevalent allergen sources worldwide, some of the mite allergens exhibit enzymatic activity that could potentiate allergic sensitization (16, 26). Therefore, it is reasonable to test the efficacy of mycobacterial formulations in the treatment of mice sensitized and challenged with Der p 1, a major HDM allergen (16). In the present study,

we show that the DNA-HSP65 or CpG/CFP immunotherapy is effective in Der p 1 allergic mice.

Most of the studies with allergen-free immunotherapy have focused on the ability of microbial antigens to modulate the adaptive Th2 response by interfering with antigen-presenting cell (APC) function, inducing Treg cells or boosting Th1 responses (3, 13, 27). According to our previous data, the main mechanism mediating the efficacy of DNA-HSP65 and CpG/CFP depends on IL-10 and IFN-γ, respectively, produced by T cells specific for either the mycobacterial antigen or the allergen (14, 15). However, we did not identify how IL-10 and

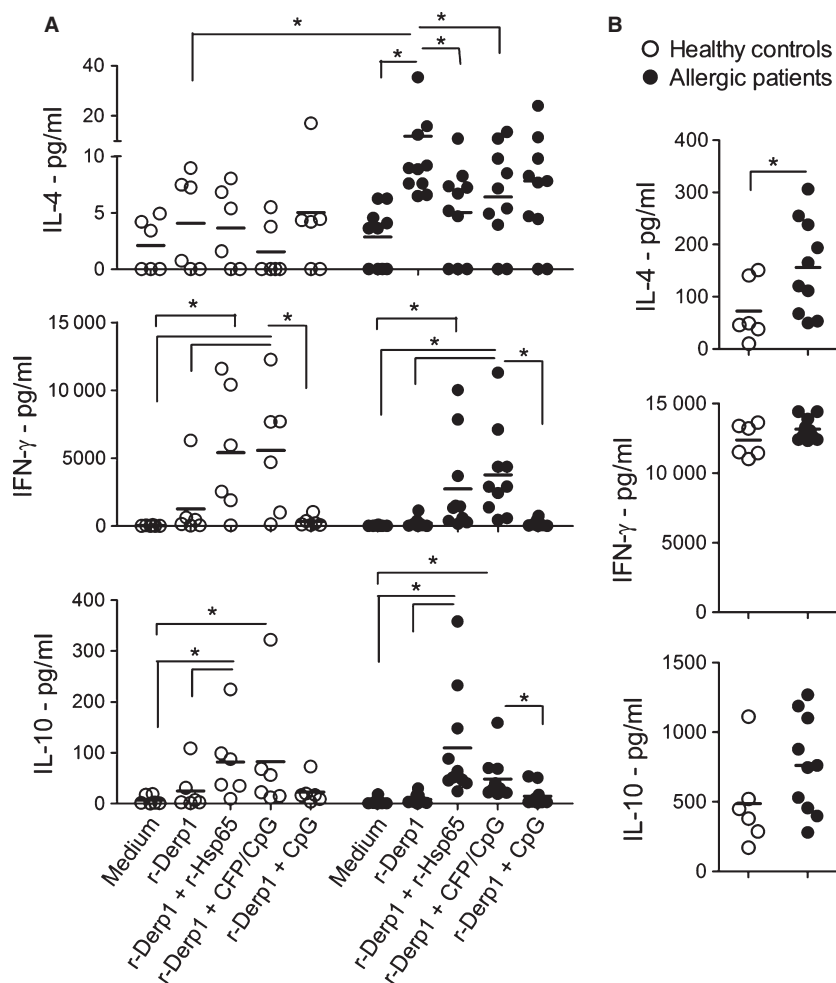


Figure 4 Modulation of cytokine production by PBMC from allergic patients in response to mycobacterial antigen stimulation. PBMC from allergic patients and healthy controls were stimulated with allergen Der p 1 in the presence of mycobacterial antigens (A). Phytohe-

magglutinin (PHA) was used as positive control (B). Supernatants of cultures stimulated with recombinant-Hsp65 (r-Hsp65), culture filtrated proteins, or CpG were collected after 48 h. Cytokines were detected by ELISA (IFN- γ) or CBA (IL-4 and IL-10). * $P < 0.05$.

IFN- γ responses are induced or the main pathways used by these cytokines to modulate the allergic response.

Considering that the innate cells activated by TLR agonists represent an important source of IL-10 and IL-12, which drive the activation of regulatory and Th1 patterns, respectively, we tested the role of MyD88 signaling in mediating the efficacy of DNA-HSP65 or CpG/CFP immunotherapy. Because we have previously shown that the effect of the mycobacterial-based allergen-free immunotherapy is cell-mediated and can be adoptively transferred, we used cell transfer experiments instead of directly treating the deficient mice, to avoid the effects of the absence of MyD88 and Fas signaling during the induction of allergic response by the enzymatically active allergen. Our data show that the mechanisms behind of IFN- γ and IL-10 production by DNA-HSP65 and CpG/CFP have a common pathway dependent on MyD88 signaling. In fact, it is well known that CpG motifs activate IL-12 production by innate cells, such as dendritic cells and macrophages, via TLR9-induced MyD88 signaling, driving the

differentiation of Th1 cells (25, 27). In addition to CpG, components of CFP, such as the 19 kDa lipoprotein which interacts with TLR2, could stimulate cytokine secretion via MyD88. However, in contrast to cells from CpG/CFP-immunized MyD88 $^{-/-}$ mice, cells from CpG/CFP-immunized TLR2 $^{-/-}$ mice were still able to produce IFN- γ and to protect allergic mice (data not shown). This suggests that CFP exclusively play an antigen-specific role whose pattern of induced adaptive immune response is probably driven by CpG. Therefore, we suggest that activation of TLR9 by CpG motifs induces IL-12 production, which drives the differentiation of CFP and allergen-specific Th1 cells. IFN- γ production by Th1 cells could downregulate the allergic responses via: (i) direct inhibition of Th2 cell proliferation, (ii) potentiating IL-12 production, which favors differentiation of Th1 cells, (iii) by inducing apoptosis via Fas/Fas-L pathway (13, 17, 18, 27, 28). Indeed, the modulatory effect of CpG/CFP is impaired in the absence of Fas expression. However, the increase in the frequency of Annexin-V $^{+}$ BALF cells suggests other

functions than apoptotic cell death for Fas receptor, as described for other cellular events (23, 24). Different components of Fas signaling cascade have been described in events such as cell proliferation, activation, and differentiation (20). Enzymatic inhibitors of caspase-8 impaired mitogen- or antigen-induced T-cell proliferation (29), while the blockade of FAAD (Fas-interacting death domain) function also reduced specific and polyclonal T-cell activation and proliferation (30). Considering that Th2 cells are more resistant to CD95L-induced apoptosis than Th1 cells (31) and consistent with our findings showing that the absence of Fas impaired either IFN- γ or IL-10 production by spleen cells of DNA-HSP65- or CpG/CFP-immunized mice, we suggest that Fas pathway induces IFN- γ which promotes an immune deviation of allergic Th2 response. Actually, a previous study showed that during dendritic cell and T-cell interaction, the production of IL-1 β and IFN- γ was reduced by blocking Fas and Fas-L signaling (32). Therefore, we favor the notion that Fas deficiency impaired cytokine production that in turn appears to be responsible for the suppressive effect of CpG/CFP. Although we did not find an increase of IL-10 levels in BALF after CpG/CFP immunotherapy, the ability of CpG/CFP immunization to induce IL-10 production may contribute to protection against tissue damage as confirmed by the reduced lung inflammation in CpG/CFP-treated animals.

In the same way, we hypothesized that the role of MyD88 in efficacy of DNA-HSP65 therapy could be attributed to the expression of Hsp65 protein, after transfection of host cells, and interaction with TLRs, inducing proinflammatory cytokine production. However, the therapeutic effect of DNA-HSP65 was mediated by IL-10 rather than by proinflammatory cytokines. Moreover, we have shown that the effect of DNA-HSP65 is independent of TLR4 (14) and that cells from DNA-HSP65 or CpG/CFP-immunized TRL2^{-/-} mice are still able to produce IL-10 and protect allergic mice (data not shown).

In the absence of MyD88 signaling, IL-10 production, but not IFN- γ secretion, was impaired after DNA-HSP65 immunization. The protective effect of cells from DNA-HSP65 immunized mice was significantly reduced with cells obtained from MyD88^{-/-} mice. Although we did not identify a possible MyD88-signaling TLR ligand, it is possible that the plasmid backbone could signal via PRR-MyD88 and induce IL-10 production. Some immune regulatory CpG motifs have been identified in bacterial DNA, including plasmids (33). We suppose that the presentation of Hsp65 antigen in the context of an anti-inflammatory environment could drive the differentiation of Treg cells. Indeed, we found a significant increase in local Treg cell population of DNA-HSP65-treated mice. Moreover, the induction of an anti-inflammatory response by Hsp has been addressed for the development of new therapeutic strategies against inflammatory disorders (10, 12, 34). Considering this, a protective role for Treg cells during allergen-specific and allergen-free immunotherapy has been shown in different studies, making them an interesting target for development of new treatments (13). Therefore, the association of Treg induction and safety of an allergen-free treatment opens a new perspective for controlling allergic disease.

In this context, it is essential to consider whether cells from allergic patients would exhibit similar pattern of immune response to mycobacterial antigens to cells from allergic mice. In fact, we confirmed that PBMC from mite-allergic patients with asthma and healthy individuals respond similarly to mice when stimulated with mycobacterial antigens. Therefore, the data presented in this study show that the use of allergen-free therapy for controlling airway allergic disease using antigens that modulate the immune system at the same time by common (MyD88) and distinct pathways is a promising strategy.

Taken together, these results suggest that MyD88 signaling could be a common pathway in the distinct mechanisms of therapeutic efficacy of allergen-free immunotherapy. During immunotherapy with CpG/CFP, IFN- γ secretion induced via MyD88 signaling could reverse the allergic response by downregulating Th2 cells. In addition, immunotherapy with CpG/CFP activates nonapoptotic Fas signaling and possibly by IFN- γ and IL-10 production reduces allergy. In parallel, MyD88 could mediate IL-10 production after DNA-HSP65 immunotherapy, and this IL-10 production could be responsible for the therapeutic effect. These data indicate the promising potential of mycobacterial antigens for clinical trials.

Acknowledgments

We would like to thank Izaíra Tincani Brandão, Ana Paula Masson, and Elaine Medeiros Floriano (Ribeirão Preto Medical School—University of São Paulo) for technical assistance.

Author contributions

DMF and VLDB designed the study, analyzed the data, and prepared the manuscript. MR and LKA contributed to the interpretation and discussion of the data and drafting article. LKA made contributions to design the experiments with patient samples. DMF, PFW, MOP, AFG, and LWC performed the experiments. WMT acquired and analyzed the cells for the flow cytometry experiments. SGR performed the histological analyses of the lung sections. CH and GM produced and provided the CFP antigens. CLS provided the DNA-HSP65 vaccine.

Funding

The present study received financial support from 'Fundação de Amparo à Pesquisa do Estado de São Paulo—FAPESP' (Foundation for Research Support of the State of São Paulo) Grants Nos. 05/59198-8 and 05/01995-0 and 'Conselho Nacional de Desenvolvimento Científico e Tecnológico'—CNPq (National Council for Scientific and Technological Development).

Conflicts of interest

The authors declare that they have no conflicts of interest.

Supporting Information

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

Figure S1. Modulation of Der p 1-induced allergic airway inflammation by allergen-free immunotherapy.

Figure S2. Mycobacterial antigen treatment modulates allergen-specific antibody production.

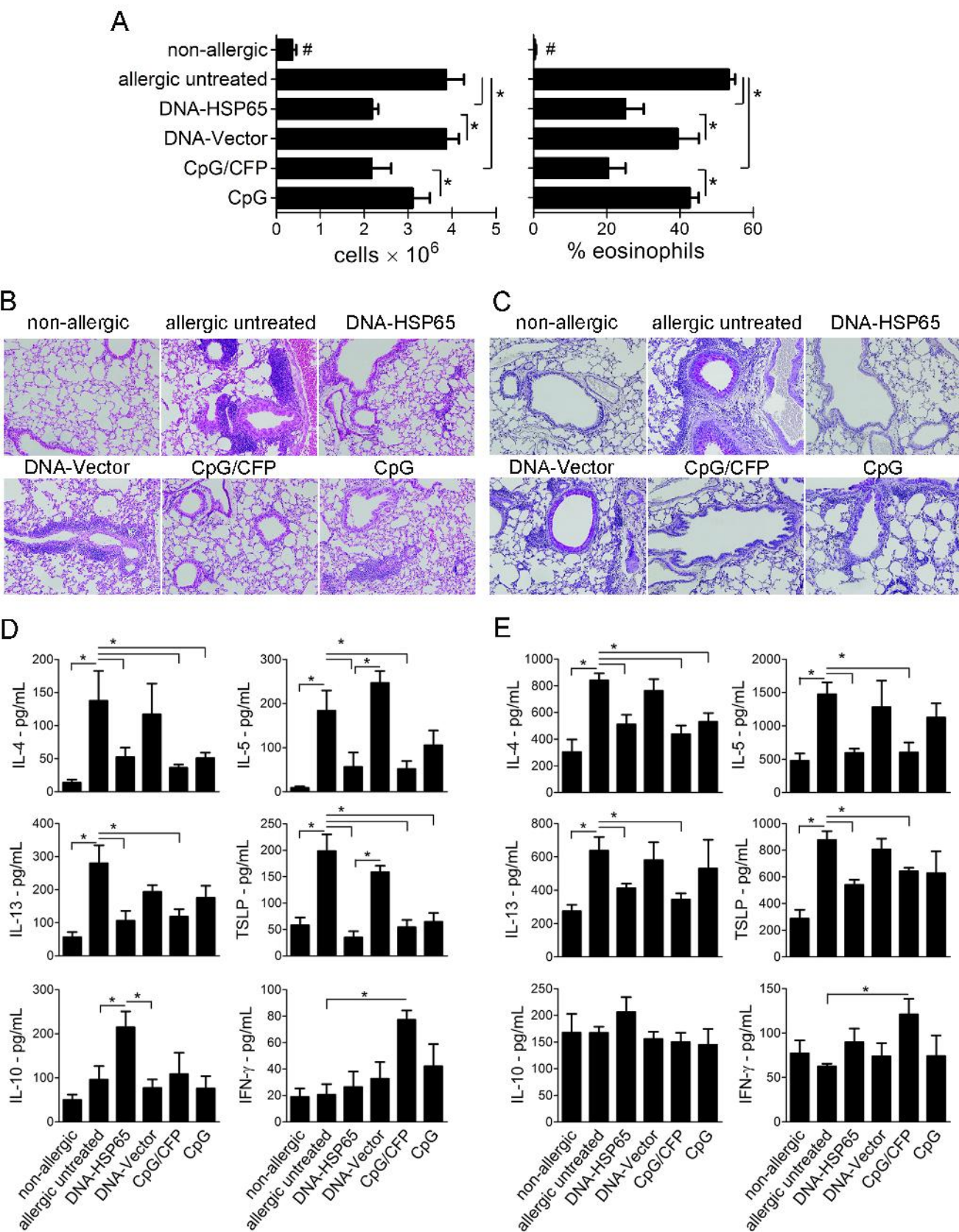
Figure S3. Induction of Treg cells and apoptosis in allergen-treated mice depends on the mycobacterial antigen used during the immunotherapy.

Appendix S1. Supplementary Methods.

References

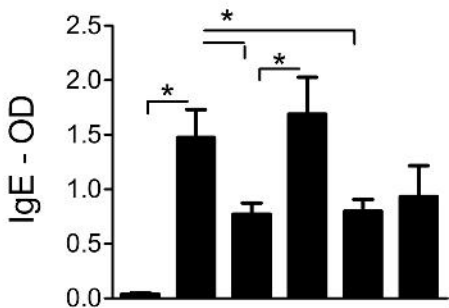
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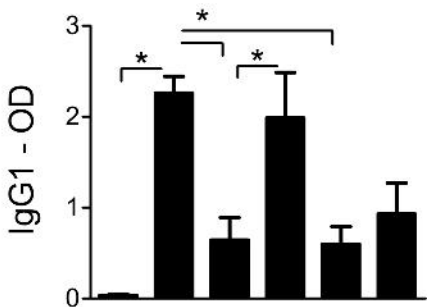


Supplementary Figure 2

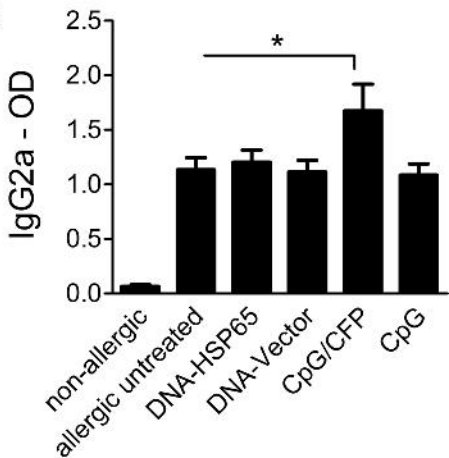
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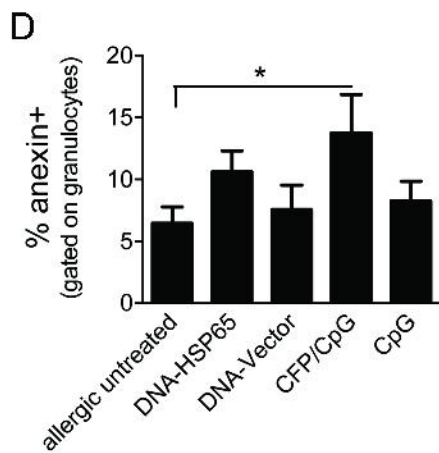
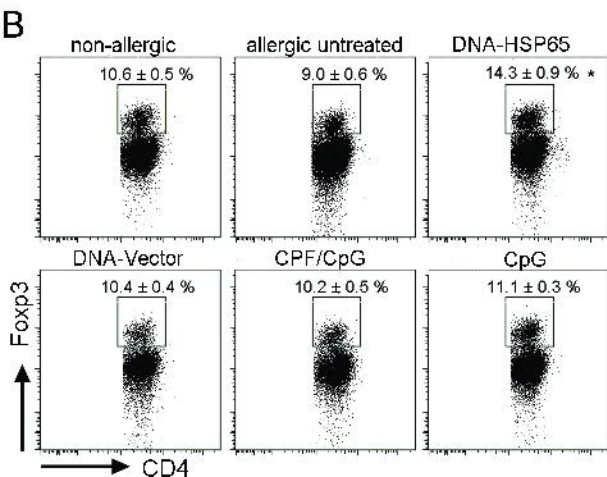
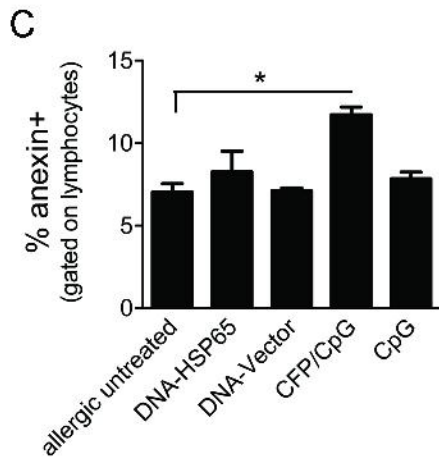
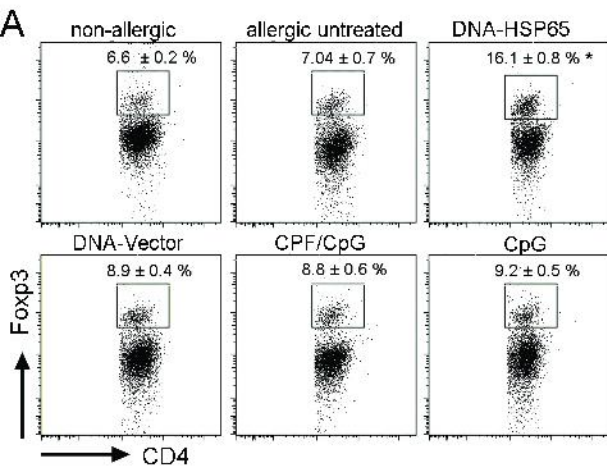


B



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Supplementary Methods

Mycobacterial antigens, CpG-ODNs and Der p 1 allergen

The gene that encodes the Hsp65 protein from *M. leprae* was inserted into the pcDNA3 plasmid vector (Invitrogen®, Carlsbad, CA, USA) as previously described (14). Recombinant Hsp65 protein (rHsp65) was obtained from *Escherichia coli* ER2566 transformed with the Hsp65 gene, according to methods previously described (14).

The *M. tuberculosis* CFP antigens were obtained using supernatant from a *M. tuberculosis* 14-day culture, as previously described (15).

CpG-ODNs were synthesized using custom primers (Invitrogen, San Diego, CA, USA). The CpG1826 was used in mouse experiments and the CpG2006 was used to stimulate human PBMC.

Recombinant Der p 1 allergen (rDer p 1) was kindly provided by Dr. Martin Chapman (Indoor Biotechnologies, Inc, Charlottesville, VA, USA). Briefly, the allergen was expressed in *Pichia pastoris*, and the recombinant protein was purified from culture supernatant using gel filtration chromatography. For stimulation of human PBMC, we used a purified natural Der p 1, nDer p 1 (Indoor Biotechnologies, Inc., Charlottesville, USA).

Endotoxin concentrations were measured in all reagents (DNA-HSP65, DNA-vector, rHsp65, CFP, CpG, rDer p 1) using the Limulus amoebocyte lysate kit – QCL 1000 (BioWhittaker, Walkersville, MD, USA). DNA plasmids, CFP antigens, CpG and rDer p 1 had no detectable endotoxin, whereas rHsp65 contained 9.2 EU endotoxin/μg of protein.

Bronchoalveolar lavage fluid (BALF) and lung cell isolation

Mice were euthanized, the trachea was cannulated, and bronchial lavage was carried out with 1 ml cold incomplete RPMI (Sigma St Louis – MO, USA); this procedure was repeated 4 times. The supernatant of the first lavage was collected for cytokine detection, and cells obtained in the total bronchoalveolar lavage fluid (BALF) were counted in a Newbauer chamber. Differential cell counts of BALF were determined in cytopsin preparations stained with Panoptic (Clozap-Hemogram, Inc., Curitiba, Brazil). After collection of BALF, lungs were perfused with 5 ml ice-cold PBS and removed. Lungs were digested as previously described, (5, 14, 15) and viable lung cells were counted in a Newbauer chamber via trypan blue exclusion.

Flow cytometry

Single-cell suspensions obtained from BALF and lungs were pre-incubated with FcBlock (2.4G2) (BD Biosciences). Cells were stained with fluorochrome-conjugated Abs against surface markers CD3 (145-2C11) and CD4 (RM4-5) in staining buffer (PBS containing 2% FCS) for 30 min at 4°C and then washed. For Foxp3 (FJK-16s clone) staining, cells were permeabilized and subsequently stained using the Foxp3 Staining Buffer Set (BD-Biosciences) according to the manufacturer's protocol. Cell acquisition was performed on a FACSCanto II instrument using FACSDiva software (BD Biosciences). For each sample, at least 300,000 events were collected. Data were analyzed using FlowJo software (Tree Star).

Isolation of spleen cells and culture

Spleens were collected and homogenized, and 5×10^6 cells/mL were 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 rHsp65 or CFP (10 µg/mL). Supernatants were harvested after 48 h for cytokine detection.

Airway hyperresponsiveness (AHR)

AHR was determined by 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 (20, 21).

Cytokine and antibody detection

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

Der p 1, Hsp65 and CFP-specific antibodies were assayed by sandwich ELISA in serum of mice, 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.

Histology

The left lung lobes were collected, fixed in 10% formol and stained with hematoxylin–eosin or hematoxylin/periodic acid–Schiff (PAS) in five-micron sections for analysis of cellular inflammation or mucus production, respectively.

Peripheral blood mononuclear cell isolation and cell cultures

Mite allergic asthmatic patients were selected from those attending the Allergy Clinic of the Clinical Hospital of Ribeirão Preto Medical School. All patients presented positive skin prick tests to HDM (*Dermatophagoides pteronyssinus*) and serum IgE antibodies to Der p 1, as measured by chimeric ELISA (35). Control non-allergic, non-asthmatic individuals were selected from the Medical staff of the Clinical Hospital of Ribeirão Preto Medical School and laboratory personnel. This section of the study was approved by the Ethics Committee of the Clinical Hospital of Ribeirão Preto Medical School, and all patients sign informed consent.

Peripheral blood mononuclear cells (PBMC) from allergic patients or healthy controls were isolated by density gradient centrifugation using Ficoll-Paque (GE Life Sciences, Uppsala, Sweden). Cells were stimulated with purified natural Der p 1 allergen (10 µg/mL) in the presence of 10 µg/mL of the mycobacterial antigens Hsp65, DNA-HSP65, CFP or CpG-2006. As positive control, we have used phytohemagglutinin (PHA) for stimulating the cells. Supernatants of cultures stimulated with rHsp65, CFP or CpG were collected after 48 hours, and the supernatants of cells stimulated with DNA plasmid were collected after 6 days of culture. Cytokines were detected by sandwich ELISA (IFN-γ – BD-Bioscience), and IL-4 and IL-10 were detected by Cytometric Bead Array (CBA – BD Biosciences), according to the manufacture instructions.