Immunology and Cell Biology (2011) 89, 777–785
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ORIGINAL ARTICLE

IFN-γ-mediated efficacy of allergen-free immunotherapy using mycobacterial antigens and CpG-ODN

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Epidemiological and experimental evidence supports the notion that microbial infections that are known to induce Th1-type immune responses can suppress Th2 immune responses, which are characteristics of allergic disorders. However, live microbial immunization might not be feasible for human immunotherapy. Here, we evaluated whether induction of Th1 immunity by the immunostimulatory sequences of CpG-oligodeoxynucleotides (CpG-ODN), with or without culture filtrate proteins (CFP), from *Mycobacterium tuberculosis* would suppress ongoing allergic lung disease. Presensitized and ovalbumin (OVA)-challenged mice were treated subcutaneously with CpG, or CpG in combination with CFP (CpG/CFP). After 15 days of treatment, airway inflammation and specific T- and B-cell responses were determined. Cell transfer experiments were also performed. CpG treatment attenuated airway allergic disease; however, the combination CpG/CFP treatment was significantly more effective in decreasing airway hyperresponsiveness, eosinophilia and Th2 response. When an additional intranasal dose of CFP was given, allergy was even more attenuated. The CpG/CFP therapy also reduced allergen-specific IgG1 and IgE antibodies and increased IgG2a. Transfer of spleen cells from mice immunized with CpG/CFP also reduced allergic lung inflammation. CpG/CFP treatment induced CFP-specific production of IFN-γ and IL-10 by spleen cells and increased production of IFN-γ in response to OVA. The essential role of IFN-γ for the therapeutic effect of CpG/CFP was evidenced in IFN-γ knockout mice. These results show that CpG/CFP treatment reverses established Th2 allergic responses by an IFN-γ-dependent mechanism that seems to act both locally in the lung and systemically to decrease allergen-specific Th2 responses.
Immunology and Cell Biology (2011) **89,** 777–785; doi:10.1038/icb.2011.9; published online 15 March 2011

Keywords: experimental asthma; *M. tuberculosis*; culture filtrate proteins; CpG-oligodeoxynucleotides; allergen free immunotherapy; IFN-gamma

Allergic asthma is a chronic inflammatory lung disease characterized by a prominent allergen-specific Th2 immune response, which promotes IgE synthesis, eosinophilic inflammation and airway hyperresponsiveness (AHR).[1,2] Allergen-specific immunotherapy has been shown to decrease symptoms and medication usage in patients with asthma.[3] However, currently available modalities of immunotherapy have limitations, including lack of efficacy in some patients, long time to induce the effects and risk of side-effects, which may present as life-threatening systemic allergic reactions.[3] Therefore, improved forms of immunotherapy are necessary to downmodulate allergic responses and prevent new hypersensitivity reactions.

It has been suggested that allergen-free strategies of immunotherapy could be used to treat asthma.[4,5] Infection or immunization with microorganisms, including mycobacterial species, have been shown to modulate allergic responses.[6–10] The bacillus Calmette–Guérin (BCG) has been widely studied as a strategy to downmodulate airway experimental allergy. In addition to live BCG, it has been described that BCG killed by extensive freeze-drying reduced eosinophilia, lung inflammation and AHR in murine and guinea pig models of asthma.[11] On the other hand, BCG administration, in conjunction with allergen-specific immunotherapy has not resulted in increased efficacy in human patients.[12]

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Received 21 October 2010; revised 20 December 2010; accepted 18 January 2011; published online 15 March 2011

The use of toll-like receptor (TLR) agonists represents a promising additional strategy of allergen-free therapy, because TLR-induced cytokines, such as IL-12, cooperate in induction of Th1 immune responses.[3–5,13] In experimental models, studies demonstrated that CpG-oligodeoxynucleotides (CpG-ODN), a TLR9 agonist, effectively controlled allergic acute inflammation not only in IFN-γ-dependent, but also in IFN-γ-independent ways.[14–17] Studies in human subjects have shown promising results with the use of CpG-ODNs or CpG-ODN plus allergens.[18–20] These treatments induced a Th1 immune response that downmodulated allergic response. However, in some cases, the use of CpG-ODN did not show a significant efficacy against severe cases of asthma.[21]

We have previously described that immunization of mice with *M. tuberculosis* culture filtrate proteins (CFP), in the presence of CpG-ODN, induced a Th1-biased specific immune response.[22] The CFP are immunodominant antigens, actively secreted by *M. tuberculosis* during bacterial growth. These proteins are highly immunogenic and have been intensively studied for the development of tuberculosis vaccines.[23,24]

Considering the data on modulation of allergic responses by mycobacterial infection/immunization, or by TLR9 agonist (CpG), and the IFN-γ-inducing ability of CFP antigens, we proposed an allergen-free immunotherapy, using CpG-ODN in conjunction with CFP antigens (CpG/CFP). Avoiding the use of whole mycobacteria would eliminate the risk of developing an active infection in immunocompromised patients, such as those exposed to long-term corticosteroid treatment. In addition, immunotherapy with CpG/CFP could have advantages over CpG alone, because although the TLR agonist stimulates mainly the innate system, the mycobacterial antigens could induce a specific immune response driven by the TLR agonist, which could be sustained for a long time and elicited at any time by memory cells. In the present study, we observed that subcutaneous CpG/CFP treatment downmodulated eosinophil accumulation, lung inflammation and AHR. In contrast to CpG alone, CpG/CFP co-administration induced a Th1-adaptive immune response. This antigen-specific immune response was boosted locally by intranasal administration of CFP, and this treatment was associated with an accentuated downmodulation of allergy. Finally, it was also observed that the IFN-γ-induced immune response by CFP antigens was crucial for the modulation of allergy in this model of allergen-free immunotherapy.

RESULTS

CpG/CFP treatment modulated local allergic Th2 response

Allergic mice treated subcutaneously with CpG/CFP, but not with CpG only, exhibited a significant reduction in eosinophil counts and increase in lymphocyte number in bronchoalveolar lavage fluid (BALF), as compared with allergic-untreated mice (Figure 1b). Levels of IL-4, IL-5 and IL-13 were reduced in BALF after treatment with CpG/CFP or CpG, as compared with allergic-untreated group; however, reduction of IL-13 and IL-5 was more pronounced with CpG/CFP treatment (Figure 1c). On the other hand, decrease in levels of TSLP, eotaxin and IL-17, and increased secretion of IFN-γ were found only in CpG/CFP-treated mice (Figure 1c).

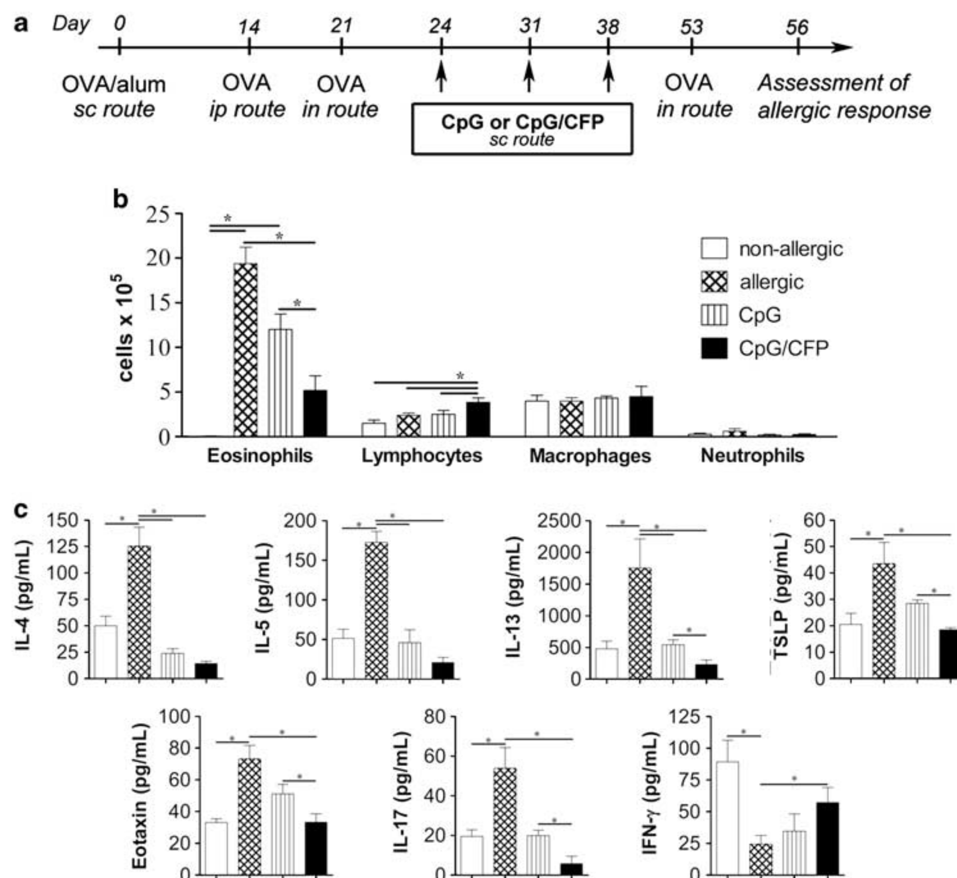

Figure 1 Cell profile and cytokine production in BALF, following treatment with CpG/CFP. OVA-sensitized and challenged BALB/c mice were treated with CpG or CpG/CFP sc, according to the study protocol (**a**). At 72 h after the second OVA-challenge, cells (**b**) and cytokine secretion (**c**) were quantified in BALF. $*P<0.05$. Representative data of two experiments (n=8). sc, subcutaneous; ip, intraperitoneal; in, intranasal.

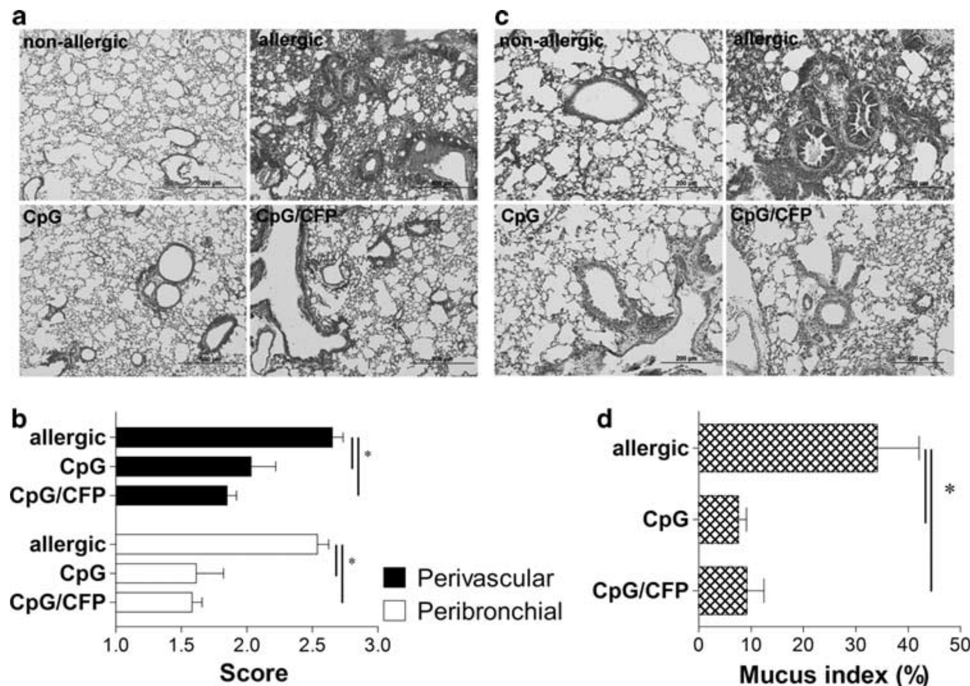

Figure 2 Inflammation and mucus production in the lungs, following treatment with CpG/CFP. OVA-sensitized and challenged BALB/c mice were treated with CpG or CpG/CFP sc, according to Figure 1a. At 72 h after the second OVA-challenge, lung inflammation (**a**, **b**) and mucus production (**c**, **d**) were assessed by established scores. Lung sections were stained with HE (**a**) or periodic acid-Schiff (**c**) and analyzed at ×100 or ×200 magnification, respectively. $*P<0.05$. Representative data of two experiments (n=8).

No significant differences were seen for IL-10 production (data not shown). Immune modulation by CpG/CFP treatment was accompanied by decrease of inflammation scores and mucus staining in lung tissue (Figure 2).

CpG/CFP treatment induced systemic Th1 allergen- and CFP-specific responses

As the CpG/CFP-treatment downmodulated local allergic inflammation, we next evaluated the systemic humoral and cellular allergen-specific immune responses. Both treatments with CpG/CFP and CpG effectively reduced production of ovalbumin (OVA)-specific IgE. On the other hand, decrease in IgG1 and increase in IgG2a antibodies to OVA were observed only in the CpG/CFP-treated group, suggesting an immune modulation of the allergen-specific response from a Th2 to a Th1 pattern (Figure 3a). In keeping with results in BALF, lower concentrations of IL-4 and IL-5 were found in spleen cell cultures stimulated with OVA in both CpG/CFP- and CpG-treated mice, as compared with allergic-untreated animals; however, only cells from CpG/CFP-treated mice produced increased levels of IFN-γ (Figure 3b). No significant changes on IL-10 production were observed for both treatment groups, as compared with allergic-untreated mice (data not shown). These results suggest that immune therapy with CpG/CFP induced an allergen-specific immune modulation from a Th2 to Th1 pattern, both locally and systemically.

As we previously described that CpG/CFP immunization induced a strong Th1 immune response in mice,[22] we attempted to correlate these findings to the pattern of the CFP-specific immune response induced by the treatment, by detecting CFP-specific antibodies and cytokine secretion. CpG/CFP-treated mice showed production of CFP-specific IgG1 and IgG2a, but not IgE, and spleen cells of CpG/CFP-treated mice stimulated with CFP secreted IFN-γ ($2082.9 \pm 409.7\,\text{pg ml}^{-1}$) and IL-10 ($491.7 \pm 129.9\,\text{pg ml}^{-1}$), but not IL-4 or IL-5 (data not shown).

Therefore, we suggest that treatment with CpG/CFP induced a CFP-specific Th1 immune response that was able to skew the Th2 allergic response to an allergen-specific Th1 response.

Modulation of allergic response by CpG/CFP could be adoptively transferred

In an attempt to study the role of CFP-specific cells during the modulation of allergic responses, we performed a cell transfer experiment. Spleen cells transferred from C57BL/6 green-fluorescent protein (GFP) transgenic mice immunized with CpG/CFP downmodulated eosinophil recruitment and IL-4 secretion in BALF, as well as increased lymphocyte counts and IFN-γ production, in WT C57BL/6 mice, following sensitization and intranasal challenge with OVA (Figures 4a-c). $CD4^+$- and $CD8^+$-transferred GFP^+ cells were found in increased numbers in BALF, lungs and draining lymph nodes from WT-allergic mice that received cells from CpG/CFP-immunized transgenic mice (Figure 4d and Supplementary Figure 1). In the spleen, we found a similar number of GFP^+ cells in mice that received cells either from non-immunized or CpG/CFP-immunized mice (data not shown).

From the data above, we hypothesized that the lymphocytes from CpG/CFP-immunized mice were able to migrate to the allergic sites and downmodulate the allergic Th2 response, in addition to inducing Th1 cytokine secretion.

Indeed, a cell culture of transferred cells showed that, before the transfer, cells from CpG/CFP-immunized mice showed secretion of IFN-γ, after stimulation in culture with CFP ($5095.0 \pm 368.7\,\text{pg ml}^{-1}$) and no secretion of IL-4 and IL-5.

At 4 days following cell transfer, recipient mice had detectable CFP-specific IgG1 and IgG2a antibodies (OD 0.15 ± 0.04 and 1.12 ± 0.10,

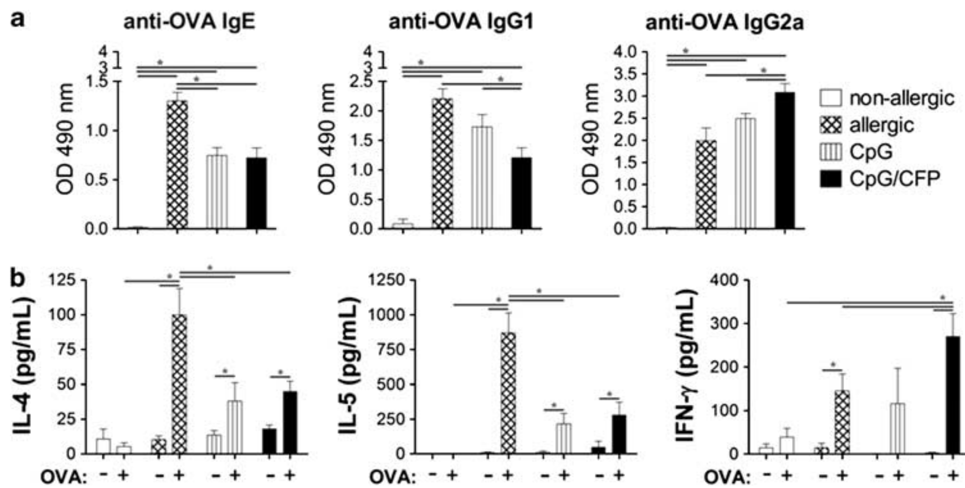

Figure 3 Serum antibody and cytokine production by spleen cells, following treatment with CpG/CFP. OVA-sensitized and challenged BALB/c mice were treated with CpG or CpG/CFP sc, according to Figure 1a. At 72 h after the second OVA-challenge, serum OVA-specific antibodies (**a**) and cytokine production by spleen cells stimulated with OVA (**b**) were detected by ELISA. $^*P<0.05$. Representative data of two experiments (n=8). Sera were analyzed at 1:10, 1:1 00 000 and 1:10 dilutions for IgE, IgG1 and IgG2a assays, respectively.

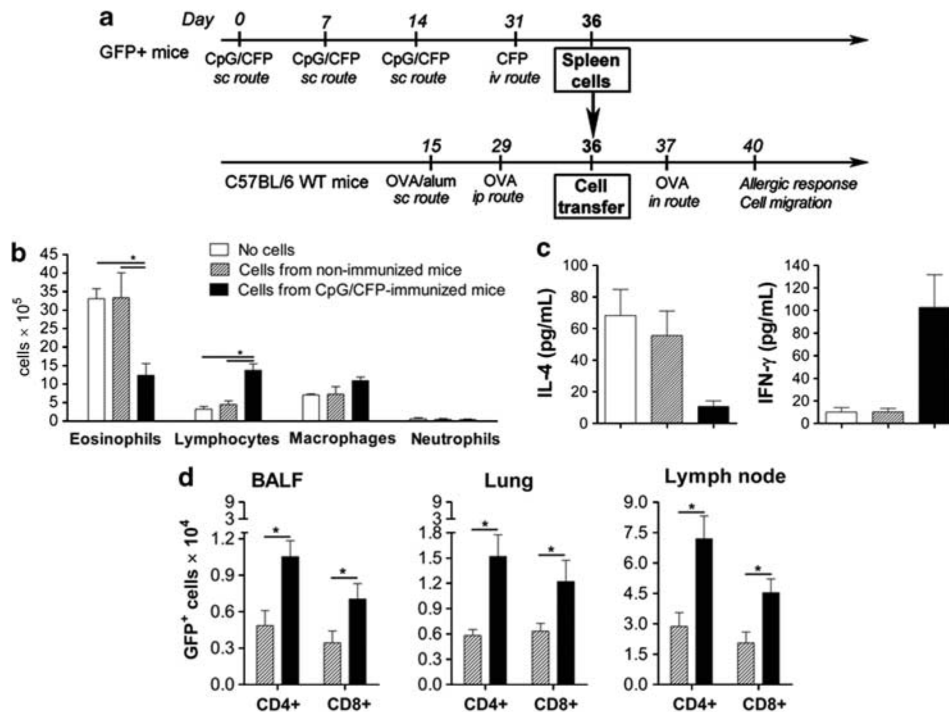

Figure 4 Adoptive transference of modulatory effects of CpG/CFP on airway inflammation. Spleen cells from C57BL/6 GFP transgenic mice that were immunized with CpG/CFP sc and boosted with CFP iv, or cells from non-immunized mice were transferred to allergic WT mice, according to the study protocol (**a**). After 72 h, cells (**b**) and cytokine secretion (**c**) were quantified on BALF. GFP$^+$ cells were detected in distinct organs by flow cytometry (**d**). $^*P<0.05$. Representative data of one experiment performed twice (n=7). sc, subcutaneous; ip, intraperitoneal; in, intranasal; iv, intravenous.

respectively), and showed production of IFN-γ by spleen cells in cultures stimulated with CFP ($631.7 \pm 151.5\,\mathrm{pg\,ml^{-1}}$), indicating that the transferred cells were viable and presented a Th1 profile.

IFN-γ was crucial for the immunomodulatory effects of CpG/CPF treatment

To confirm the role of IFN-γ in the modulation of experimental asthma by CpG/CFP treatment, we used allergic-treated IFN-γ knock-out (KO) mice. Overall, effects of CpG/CFP treatment on C57BL/6 WT mice were similar to those observed for BALB/c mice, following immunization and challenge protocol outlined in Figure 1a. In keeping with results in BALB/c mice, allergic C57BL/6 WT mice treated with CpG/CFP, but not with CpG only, exhibited a significant reduction in eosinophil counts in BALF, as compared with allergic-untreated mice (Figure 5a). In addition, concentration of IL-4 and TSLP was decreased in mice treated with both CpG/CFP and CpG; eotaxin levels were decreased and IFN-γ levels were increased only in CpG/CFP-treated mice (Figure 5b). Reduction of IL-5 was observed in

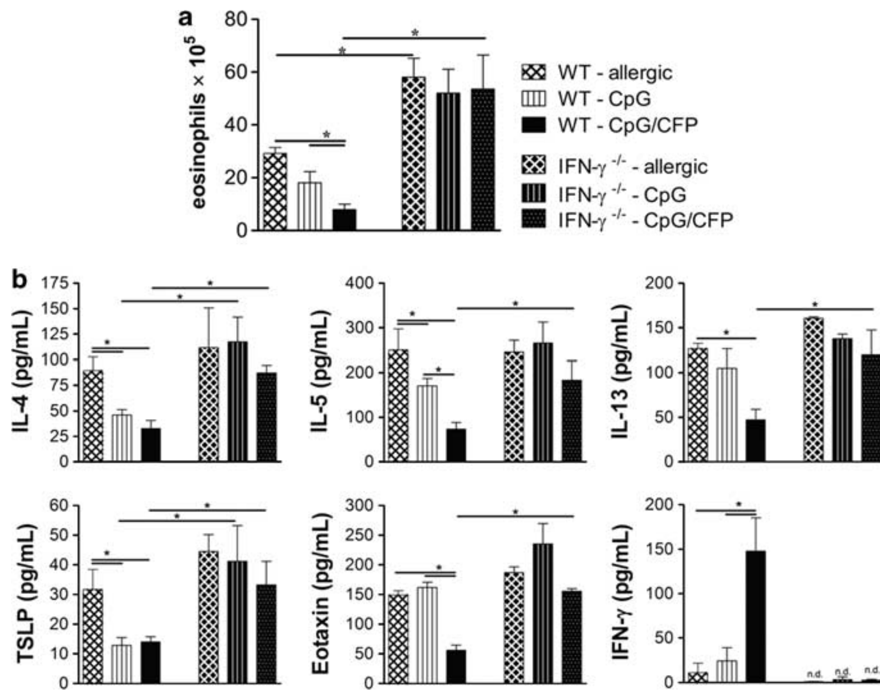

Figure 5 Role of IFN-γ production on CpG/CFP immunomodulatory effects. C57BL/6 WT or IFN-γ KO (IFN-$\gamma^{-/-}$) allergic mice were treated with CpG or CFP/CpG, according to Figure 1a. At 72 h after the second OVA-challenge cell counts (**a**) and cytokine secretion (**b**) were assessed in BALF. *$P<0.05$. (n=5)

both CpG/CFP- and CpG-treated mice, and a further reduction was found in animals treated with CpG/CFP (Figure 5b). Decrease in serum OVA-specific IgE and IgG1 and increase in IgG2a antibodies specific to OVA were observed only in the CpG/CFP-treated group (Supplementary Figure 2). In C57BL/6 IFN-γ KO mice, treatment with CpG/CFP or CpG had no effect on eosinophil recruitment, cytokine concentrations in BALF or allergen-specific antibody production (Figures 5a and b, Supplementary Figure 2).

An additional dose of CFP by intranasal route improved immunomodulatory effects of CFP/CpG treatment

Although we hypothesized that CFP-specific Th1 cells migrate to allergic sites and downmodulate allergic response, we could not explain how these cells could be activated in allergic site in the absence of CFP antigen. We performed a new schedule of treatment with CpG/CFP, using an additional dose of CFP delivered by intranasal route. Briefly, OVA-sensitized and challenged mice (allergic mice) were treated subcutaneously with three doses of CpG/CFP, and at the time of the second OVA-challenge, treated mice also received an intranasal dose of CFP, without CpG adjuvant. An additional dose of CFP given to BALB/c mice by the intranasal route, together with OVA during the second intranasal challenge, caused further reduction in eosinophil accumulation in BALF, as well as additional decrease in IL-5 and eotaxin and increase in IFN-γ concentrations in BALF (Figures 6b and c). No further decrease in IgE or IgG1, or increase in IgG2a antibodies to OVA, or changes in CFP-specific antibodies were observed in mice that received the additional CFP dose by intranasal route (Supplementary Figure 3A).

In order to evaluate if the intranasal administration of CFP modulated the pattern of cytokines secreted by lung lymphocytes, we performed lung cell cultures in which cells were stimulated with OVA or CFP. In keeping with results in BALF, further reduction of IL-5 and increase in IFN-γ production was observed in supernatants from lung cell cultures stimulated with OVA (Supplementary Figure 3B). There were no significant differences in IL-10 production by lung cells in experimental groups, and increased production of IL-17 after stimulation with CFP was found in mice treated with subcutaneous CpG/CFP, plus intranasal CFP (Supplementary Figure 3B).

These results suggest that the modulation of cytokines detected in BALF reflects the pattern of OVA- and CFP-specific T helper lymphocytes that migrate to lung tissue and airway space. Therefore, treatment with CpG/CFP induced a microenvironment characterized by the predominant production of Th1 cytokine, which modulated the pattern of cytokines secreted by OVA-specific cells and downmodulated the allergic response. All of these events were accentuated by the intranasal administration of CFP.

A similar set of experiments was carried out to assess AHR, lung inflammation and mucus production, in addition to evaluation of cell profiles in BALF (Figure 7). For these experiments, mice sensitized and challenged with OVA, treated with CpG only, served as controls. AHR as measured by Pehn values was reduced in mice treated with subcutaneous CpG/CFP that received an additional dose of CFP by the intranasal route, together with OVA during the second challenge, as compared with allergic-untreated and CpG-treated mice (Figures 7a and b). Eosinophil counts were further decreased and lymphocyte numbers were increased in BALF from mice treated with CpG/CFP that received an additional dose of CFP by the intranasal route (Figure 7c). Lung tissues from both groups of mice exhibited lower inflammatory scores and decreased mucus production, as compared with allergic-untreated mice, and no further decrease in these parameters were caused by CpG/CFP treatment with additional dose of CFP intranasally (Figures 7d–g).

DISCUSSION

In this study, we show that it is possible to accentuate the effect of an allergen-free immunotherapy with CpG-ODN by using mycobacterial

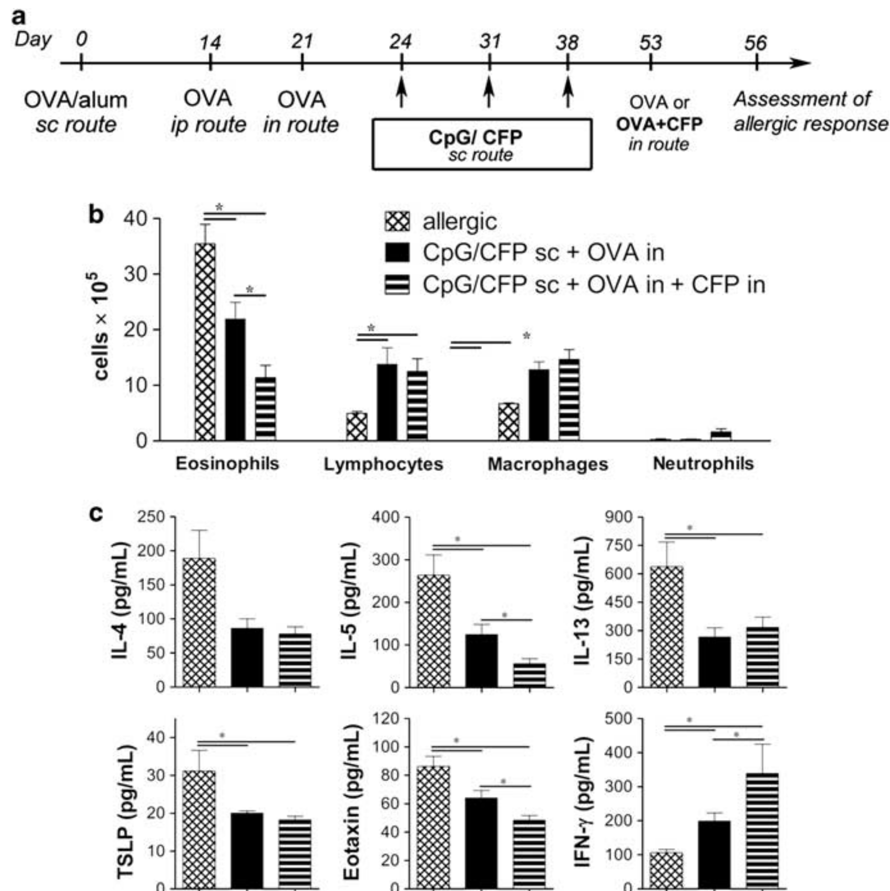

Figure 6 Effects of an additional CFP intranasal dose on immunologic response induced by CpG/CFP treatment. Allergic BALB/c mice treated with CpG/CFP sc received an intranasal CFP boost, together with OVA, during the second challenge (CpG/CFP sc + OVA in + CFP in group) (**a**). At 72 h after challenge, cells (**b**) and cytokine secretion (**c**) were quantified in BALF. $^{*}P<0.05$. Representative data of two experiments (n=10). sc, subcutaneous; ip, intraperitoneal; in, intranasal.

antigens (the CpG/CFP formulation). We observed that the administration of three subcutaneous doses of CpG/CFP to mice, with a previously established allergic response, downmodulated the allergy when these mice received a second allergen-challenge. The modulation of allergic response was significantly more pronounced in CpG/CFP-treated mice compared with CpG treatment.

The results of our study raised two main points to be explained here: (1) why and how does the CFP-specific immune response modify OVA-specific cells and (2) does an additional local CFP administration accentuate the effects of subcutaneous CpG/CFP therapy?

Considering the first point, some allergen-free therapies were designed on the basis of the use of Th1 or T regulatory-inducing infections or immunizations, such as that of BCG, *M. vaccae* or mycobacterial antigens.[6–11,25] In the present study, the modulatory effects of CpG/CFP seemed to be strictly related to IFN-γ secretion by OVA-specific cells and CFP-specific cells that migrated to allergic site. These effects were dependent on IFN-γ-producing cells because the adoptive transfer of cells from CpG/CFP-immunized mice to allergic mice also increased IFN-γ production in BALF. In parallel, the downmodulation of experimental asthma was abrogated in IFN-γ KO mice. In addition, the modulator effect of CpG/CFP therapy is dependent on the presence of CpG adjuvant, as we have previously shown that the immunization with CFP without adjuvant or with a non-stimulatory sequence of CpG did not induce activation of cellular immune response.[22]

As a Th1 cytokine, IFN-γ inhibits Th2 cell proliferation *in vitro* and *in vivo* and antagonizes pathogen-induced Th2 responses.[26] The induction or administration of IFN-γ may suppress directly Th2 cells or this cytokine could induce eosinophil apoptosis.[27–30] Recently, it has also been described that IFN-γ can act on dendritic cells and modify the functions of these antigen presenting cells, thereby downmodulating allergic responses.[31]

To address any concerns about IFN-γ production in lungs of allergic mice, one of the most important findings in our work is that CpG/CFP therapy did not exacerbate lung inflammation. CpG/CFP therapy decreased cell influx and mucus secretion, despite an increase of lung IL-17-producing CFP-specific cells.

Our hypothesis is that the Th1 immune response induced by the CpG/CFP therapy skewed the established OVA-Th2 allergic response. Th1 cells differentiated by the allergen-free therapy change the microenvironment and contribute to the generation of new OVA-specific and IFN-γ-producing cells. Possibly, Th2 cells may die or be reprogrammed as a consequence of the therapy-induced modified microenvironment. Recently, it was described that the production of type I IFNs during viral infections could reprogram Th2-specific cells *in vivo* into IFN-γ-producing Th1 cells that co-express GATA3 and Tbet.[32]

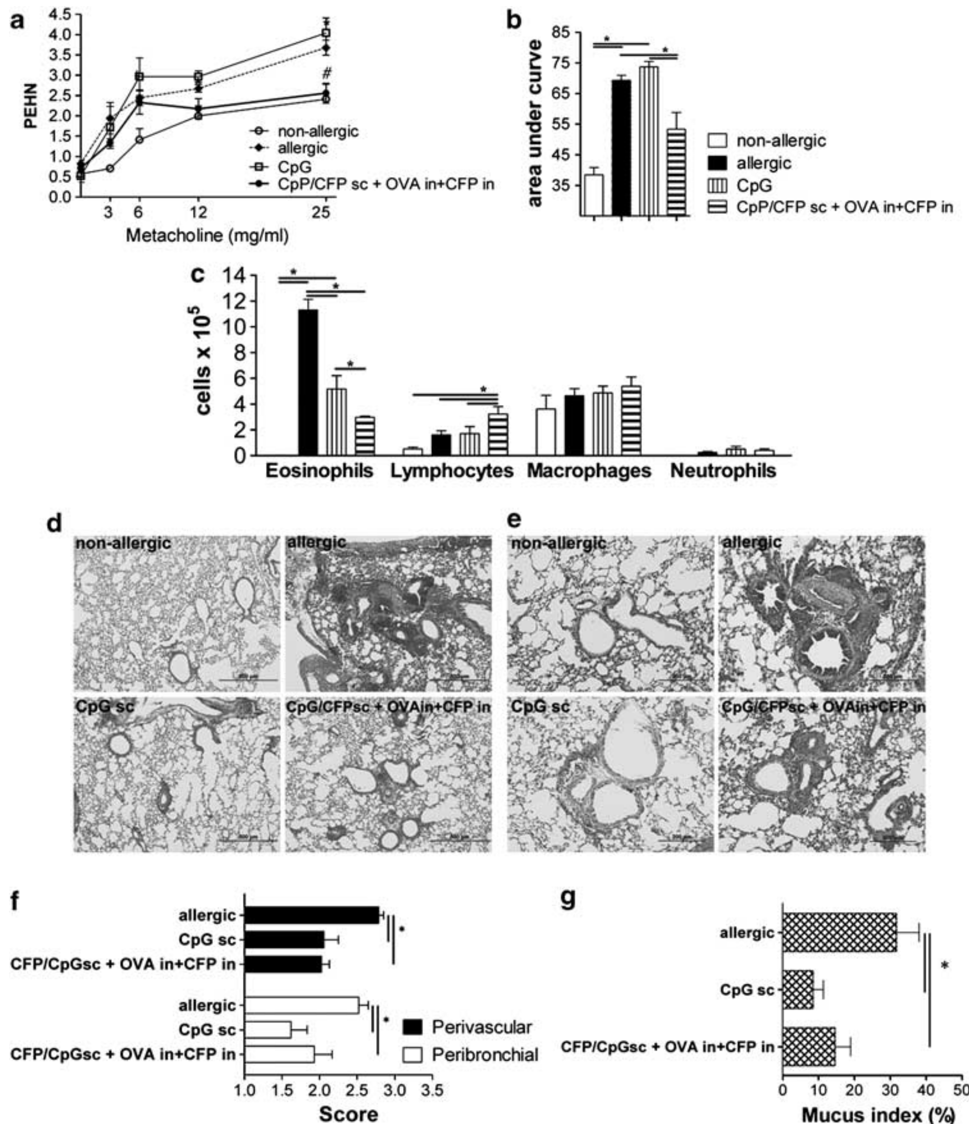

Figure 7 Effects of an additional CFP intranasal dose on AHR and lung inflammation. Allergic BALB/c mice were treated with CpG/CFP sc and boosted with CFP during the second challenge (Figure 6a). Control mice were allergic mice treated with CpG, Figure 1a. At 72 h after the second challenge, Pehn values were recorded after nebulization with increasing metacholine doses (**a**, **b**). At 48 h after challenge of BALF (**c**), lung inflammation (**d**, **f**) and mucus production (**e**, **g**) were analyzed. Magnification was ×100 (**d**) or ×200 (**e**). $^{*}P<0.05$; $^{\#}P<0.05$ versus allergic-untreated mice. Representative data of two experiments (n=5).

We showed that CpG/CFP therapy modifies the allergic response in a manner dependent on IFN-γ, and cells induced by the treatment reach the lungs and lymph nodes. Our next question was how these cells could be activated at the location of the allergic response, because it is assumed that mycobacterial antigens are not found in airways. In order to address this question, we administered an intranasal dose of CFP to CpG/CFP-treated mice and we observed a more accentuated decrease in eosinophilia, suggesting that local administration of antigen activated the CFP-specific lymphocytes and improved the effects of the immunotherapy.

Our results suggest that the modulation of allergic response by CFP-induced IFN-γ secretion might occur at two set points of allergic response: during allergen sensitization, modulating the systemic response; and after the intranasal allergen challenge, modulating the local allergic response. We believe that during allergen sensitization, the presence of the alum adjuvant promoted the formation of an antigen depot and subsequently, promoted a controlled and extended antigen release; this has been classically described for aluminum salts adjuvants.[33] Consequently, even after the OVA-alum immunization, allergen-presenting dendritic cells constantly migrate to spleens and draining lymph nodes of allergic mice and activate new allergen-specific cells, which become Th1 cells because of the microenvironment generated by the CpG/CFP treatment. This hypothesis is supported by the fact that we observed IFN-γ production by OVA-stimulated cells from CpG/CFP-treated mice and the allergen-specific isotype antibody switch from IgG1/IgE to IgG2a.

A second level of modulation could occur during the allergen challenge, when allergen-specific and CFP-specific lymphocytes migrate to the lungs and airway space. At this allergic site, the production of IFN-γ by CFP- and allergen-specific lymphocytes could downmodulate Th2 cytokine and chemokine secretion and, subsequently, eosinophil recruitment, as we observed experimentally.

It is possible that, even in the absence of the CFP in the challenge, the Th1 CFP-specific cells are activated through bystander processes. In addition, when we administered an additional dose of CFP by the intranasal route, the local response by CFP-specific cells increased and downmodulated eosinophilia, IL-5 and eotaxin. Because we identified IFN-γ as the main mediator involved in the modulation of experimental asthma, and because the additional dose of CFP by intranasal route downmodulated eosinophilia IL-5 and eotaxin, but not other Th2 cytokines, we suggest that therapy with CpG/CFP mostly affects the immune mechanisms associated with recruitment and/or number (viability) of eosinophils. It is possible that, besides the induction of IFN-γ, the intranasal CFP administration also induced eosinophil Fas/FasL-mediated apoptosis, as previously described by other IFN-γ-inducing formulations.[30] This hypothesis is under investigation by our group.

In conclusion, immunization with mycobacterial antigens, plus adjuvant CpG/CFP, may represent a promising and safe allergen-free immune therapy. This formulation was able to downmodulate allergic immune responses and AHR through an IFN-γ-dependent mechanism, without stimulating an excessive and harmful inflammatory response.

METHODS

Mice

Specific pathogen-free female BALB/c, C57BL/6 WT and C57BL/6 GFP transgenic mice, 6–8 weeks old, were obtained from local breeding facility of the School of Medicine of Ribeirão Preto, Ribeirão Preto, Brazil. IFN-γ KO were kindly provided by Dr João S Silva (School of Medicine of Ribeirão Preto, Ribeirão Preto, Brazil). Mice were housed under barrier conditions and provided with food and sterile water. Experiments were approved by the local ethical guidelines (protocol 071/2006).

M. tuberculosis culture filtrate proteins, CpG-oligodeoxynucleotides and ovalbumin

M. tuberculosis CFP antigens were obtained using an *M. tuberculosis* 14-day culture, as previously described.[22] CpG-ODN were synthesized using custom primers (Invitrogen, San Diego, CA, USA). Chicken OVA was obtained from commercial source (OVA—grade V; Sigma, St Louis, MO, USA). CFP antigens and CpG-ODN had no detectable endotoxin, whereas OVA contained 0.2 EU endotoxin/μg of protein, as determined by *Limulus* amebocyte-lysate assay (Cambrex Bio Science Walkersville, Walkersville, MD, USA).

Induction of allergic inflammation and immunotherapy

BALB/c mice were sensitized with two doses of OVA. The first dose of 100 μg was administered subcutaneously with 1.6 mg alum, and the second dose of 50 μg was injected intraperitoneally, 14 days after the first injection. At 7 days after sensitization, mice were challenged with OVA 100 μg in saline, intranasally. At 72 h after the first OVA challenge, mice were treated with three subcutaneous injections of 50 μg CpG-ODN, plus 50 μg CFP (CpG/CFP) at 7-day intervals. Control groups received saline (allergic-untreated mice) or three doses of 50 μg CpG-ODN (CpG-treated mice). At15 days after the end of treatment, mice received a second intranasal OVA challenge. AHR was evaluated 24 h following the second OVA challenge, and other parameters were assessed 72 h after challenge.

A group of CpG/CFP-treated BALB/c mice received 50 μg CFP by intranasal route, together with the second OVA challenge (CpG/CFP subcutaneouslyc—CFP intranasally). Allergic-untreated, allergic CpG-treated and allergic-untreated mice that received CFP by intranasal route, served as controls.

IFN-γ KO or C57BL/6 WT mice were OVA-sensitized, challenged and treated subcutaneously with CpG/CFP, as described above.

Cell transfer experiment

GFP transgenic mice were immunized with three doses of CpG/CFP. At 15 days after the third immunization, mice received one intravenous dose of 50 μg CFP. After 5 days, 5×10^6 spleen cells from CpG/CFP-immunized mice or from non-immunized mice were transferred intravenously to OVA-sensitized C57BL/6 WT mice. The WT mice were challenged with OVA by intranasal route, 1 day after cell transfer. At 72 h after OVA-challenge, BALF, lung, draining lymph nodes and spleen from WT-recipient mice were collected and analyzed by flow cytometry to detect GFP$^+$ cell migration. Cells from these sites were stained with anti-CD4, CD8 or CD19 mAb (BD Biosciences-PharMingen, San Diego, CA, USA) and analyzed by flow cytometry.

Determination of AHR

AHR was assessed by using a single-chamber, whole-body plethysmograph (Buxco Electronics, Wilmington, NC, USA) after nebulization with methacholine, as previously described.[34]

Spleen and lung cell cultures

At 72 h after the second allergen challenge, spleens were collected and homogenized; 5×10^6 cells ml^{-1} were cultured in complete RPMI-1640 medium (with 10% bovine serum, gentamicin, penicillin/streptomycin and polymyxin B) at 37 °C in 5% CO_2. Cells were stimulated or not with OVA (100 μg ml^{-1}) or CFP (10 μg ml^{-1}). After 48 h, supernatants were harvested for cytokine detection. Lung cells were isolated, as previously described,[35] and cultured and stimulated as described above.

Cell counts in BALF and detection of cytokines and antibodies

BALF was obtained following five consecutive 1 ml injections of RPMI-1640 medium into the lungs. Total volume of 5 ml was centrifuged (400×g, 10 min), supernatants were stored at −20 °C and cells were counted in a Neubauer chamber; 40 000 cells were centrifuged onto microscope slides and stained with panoptic (Clozarp-Hemogram, Inc., Curitiba, Brazil). Percentages of eosinophils, lymphocytes, macrophages and neutrophils were multiplied by the total number of cells from each sample.

Concentrations of IL-4, IL-5, IL-13, TSLP, eotaxin, IFN-γ, IL-17, IL-10 and TNF-α in BALF, and in spleen and lung cell-culture supernatants were determined by ELISA, according to manufacturer's instructions and as previously described.[22,35] Cytokine detection limits were as follows: 19 pg ml^{-1} for IL-5 and IL-10; 9 pg ml^{-1} for IL-4, IL-17, IFN-γ and TNF-α; and 5 pg ml^{-1} for IL-13, TSLP and eotaxin.

CFP- and OVA-specific antibodies were assayed by sandwich ELISA, according to manufacturer's instructions (BD Biosciences-PharMingen, San Diego, CA, USA), using biotin-conjugated anti-mouse IgE (R35-118), IgG1 (A85-1) and IgG2a (R19-15).

Histological analysis

Sections of 5 μm of left lung lobes were stained with hematoxylin-eosin. Perivascular and peribronchial inflammation was scored in a scale of 0–3 of all vessels and bronchi in each section, as previously described.[36]

Lung sections were also stained with periodic acid-Schiff (PAS)/hematoxylin. The mucus-occupying ratio (mucus index) was calculated using ImageJ software (NIHImage, Bethesda, MD, USA), by analyzing all bronchi micro-photographed with a magnification of ×200. Results were expressed as the ratio of total bronchi area to the periodic acid-Schiff stained area of each lung section from each mouse.

Statistical analysis

All values were expressed as means ± s.e.m. Data were compared using analysis of variance and Prisma software (Graph Pad software, Inc., San Diego, CA, USA). When the values indicated the presence of a significant difference, the Tukey test was used. Values of $P<0.05$ were considered as significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Elaine Medeiros Floriano for the general technical assistance provided. *Grant support*: This study received financial support from the 'Fundação de Amparo à Pesquisa do Estado de São Paulo' (FAPESP: Foundation for the Support of Research in the State of São Paulo; grant no. 05/59198-8

and 05/01995-0) and the 'Conselho Nacional de Desenvolvimento Científico e Tecnológico' (CNPq, Council for Scientific and Technological Development).

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The Supplementary Information that accompanies this paper is available on the Immunology and Cell Biology website (http://www.nature.com/icb)