Candidin and trichophytin stimulate the production of Th1 and regulatory cytokines by peripheral blood mononuclear cells: implication for their use as adjuvants in immunotherapy
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The prevalence of allergic asthma and other allergies is increasing worldwide [1,2]. Immunotherapy is an important alternative to the available therapies [3,4], as pharmacological therapies that are both effective and free from undesirable side effects are still unavailable for these diseases. Since its introduction for treating seasonal allergic rhinitis almost a century ago in England [5,6], allergen-specific immunotherapy has been used for many years in cases of IgE-mediated allergic diseases, including respiratory allergies (rhinosinusitis and asthma) [7,8]. The most common type of allergic diseases in humans are associated with the Gell and Coombs' type I hypersensitivity reaction, with an IgE-dependent activation of mast cells and basophils, predominance of Th2 cells and tissue eosinophilia [9].

Among the cytokines produced by Th2 cells during the allergic reactions, IL-4 and IL-13 stimulate IgE synthesis [10,11], prolong the survival of mast cells in tissues [12] and determine the selective recruitment of eosinophils by increasing vascular cellular adhesion molecule-1 expression in the vascular endothelium. IL-5 and IL-33 are the primary determinants of differentiation, recruitment, activation and survival of eosinophils [10,13]. IL-13 plays an important role in mucus secretion and in the hyperresponsiveness of airway smooth muscle [10,14].

There seems to be two distinct and perhaps sequential immunologic responses to immunotherapy, namely, immune deviation from Th2 to Th1 responses and generation of Treg cells. Inhibition of the Th2 immune response accompanied by an augmentation of a Th1 cellular immune response with IFN-[gamma] production has been reported in allergic patients following immunotherapy [15-18]. On the other hand, other authors have reported that antiallergy immunotherapy leads to activation of Treg, with production of the regulatory cytokines IL-10 and/or TGF-[beta] [18-22].

The presence of adjuvants in immunotherapeutic vaccines enhances the immune responses to allergens [23,24]. Alum is the traditional adjuvant in 'depo' extracts widely used in Europe [25]. However, similarly to what happens with preparations without adjuvants, alum-containing vaccines require several years of repeated applications, a fact that reduces patients' compliance [26,27]. Preclinical studies with adjuvants that stimulate Th1 immune responses, such as CpG oligodesoxynucleotides, have produced promising results [28]. However, a better understanding of the molecular and cellular mechanisms that lead to immune deviation and/or to the differentiation

and activation of Treg cells following an effective antiallergy immunotherapy, in addition to further clinical trials utilizing new adjuvants, is still required.

Candidin is a purified extract of the yeast *Candida albicans* cultivated in synthetic culture media [29], whereas trichophytin is a crude filtrate of *Trichophyton* spp extract [30]. There are many pieces of direct and indirect evidence in the literature indicating that these preparations may be used as adjuvants in antiallergy immunotherapy. For instance, the intradermal injections of candidin and trichophytin elicit recall Th1 cell-mediated immune reactions, and, in fact, these antigens have been widely used to induce cellular immune responses for the assessment of immune competence [31,32]. Contrary to these data, other published results support the use of candidin as an adjuvant that promotes the production of regulatory cytokines. For example, fungal polysaccharides are known to promote chronic mucocutaneous candidiasis through the production of IL-10 [33]. A major caveat, however, may hinder the use of crude fungal extracts in antiallergy immunotherapy. The exposure to *C. albicans* polarized the T-cell immune response in an arthritis mouse model toward a Th17 response, resulting in more destructive arthritis [34]. A major concern in using fungus antigens as adjuvants is, therefore, the possibility of inducing pathogenic Th17 immune responses. Th17 immune responses have indeed been associated with respiratory allergy [35,36]. As candidin and trichophytin have been injected in human beings for decades without undesirable consequences, they very likely could be used as adjuvants associated with allergens without raising untoward reactions.

The facts described above justify the carrying out of research aimed at providing support for the use of candidin and/or trichophytin as adjuvants in immunotherapeutic antiallergy preparations. In the present work, the nature of the cytokines that were produced by allergic patients' and healthy individuals' peripheral blood mononuclear cells (PBMC), when they were incubated *in vitro* with candidin or trichophytin, was investigated. This was carried out to find out whether these fungal extracts would elicit recall immune responses that could potentially inhibit a type I hypersensitivity reaction without eliciting a potentially pathogenic Th17 immune response. This was indeed observed with the PBMC from most donors.

Materials & methods

Blood donors

All blood donors, aged between 21 and 40 years, signed an informed consent to participate in the present research. They were classified into allergic (n = 9) and nonallergic (healthy individuals; n = 7) donors based on the results of skin prick tests using extracts from six common allergens (Alergolatina Produtos Alergênicos Ltd, Rio de Janeiro, Brazil), on the history of allergic symptoms and according to measurement of the levels of specific IgE to *Blomia tropicalis, Dermatophagoides pteronyssinus, Periplaneta americana, Blattella germanica* and *Ascaris lumbricoides* using the ImmunoCAP assay (Phadia Diagnostics AB, Uppsala, Sweden). The research was approved by the institutional Ethics Committee for Research in Human Subjects.

Preparation of candidin & trichophytin

Trichophyton rubrum strains 1 and 2 and *C. albicans* strains A, B and CT were from the fungus collection of the National Institute of Quality Control in Health, Oswaldo Cruz Foundation, Ministry of

Health, Rio de Janeiro, Brazil. *C. albicans* was cultivated in Sabouroud medium in a shaker at 25°C for 48 h (strains A and B) and 72 h (strain CT). The yeast was collected and washed by centrifugation in endotoxin-free 0.15 M phosphate-buffered saline, pH 7.2 (PBS), and stored at -70° C until use. *T. rubrum* was cultivated in the same conditions for 14 days, collected from the medium and washed with phosphate-buffered saline (PBS). The fungi were lyzed using an electrical triturator in the presence of silica beads (BioSpec Products, Inc, Bartlesville, OK, USA) and of PBS. Following centrifugation at 10,000 g for 20 min at 4°C, the supernatants of the *C. albicans* lysate (candidin) and of the *T. rubrum* lysate (trichophytin) were collected, filtered through a polystirene filter with 40-µm-diameter pores, aliquoted and stored at -70°C until used. Protein concentrations were measured using the Folin reagent (Bio-Rad Laboratories, Richmond, CA, USA).

Stimulation of PBMC by fungal extracts

PBMC were isolated from venous blood by centrifugation on HISTOPAQUE 1077 ® solution (Sigma-Aldrich, MO, USA). PBMC (2×10^6 cells/well) were stimulated by fungal extracts containing 5 or 50 µg of protein per ml in 96-well plates (200 µl/well) in RPMI 1640 medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (Gibco), 1% glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 20 μg/ml polymyxin B (Sigma-Aldrich), for 24 h (for quantification of IL-10, TNF-[alpha], IL-6, IL-12 and TGF-[beta]) or for 120 h (for quantification of IL-5, IL-17 and IL-13), at 37°C and 5% CO_2 . Polymixyn B was not used in wells stimulated by lipopolysaccharide (LPS). In negative control cultures, the cells were incubated with supplemented medium alone.

Cell viability assay

After cell culture for 24 h and collection of the supernatant, nine randomly chosen PBMC were subjected to a cytotoxicity assay utilizing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich).

Briefly, one million PBMC were incubated with 600 µg/ml of MTT in 100 µl of RPMI medium in wells of a 96-well microtiter plate for 4 h at 37°C and 5% CO_2 . After this period, the microculture plate was centrifuged for 5 min at 128 × g and 4°C, the supernatants were discarded by inversion, 100 µl of dimethylsulfoxide (Sigma-Aldrich) were added to each well, the microculture plate shaken on a plate shaker for 10 min and the optical densities of the reaction mixtures read with a 490-nm wavelength light. Cell viability values were expressed as the absorbance percentage in relation to those of negative control (not containing fungal extract) cultures.

Cytokine measurements

The concentrations of cytokines were assessed using commercially available ELISA duo-sets (Pharmingen, BD Biosciences, San Diego, CA, USA), according to manufacturer's instructions, and a 3,3, 5,5-tetramethylbenzidinedihydrochloride substrate system (Amresco, LLC, OH, USA). The reactions were stopped by the addition of 50 µl of 2 N sulfuric acid (Sigma-Aldrich) and the absorbances of 450-nm wavelength light were measured. The increases in cytokine concentrations in a stimulated PBMC culture were calculated by subtracting the cytokine levels in the nonstimulated culture from the levels in the stimulated culture. For most cytokines, the levels in unstimulated cultures were relatively low: a 34-179 pg range was observed for IL-10; a 19-382 pg range for TNF-[alpha], with a 663 pg outsider; a 46-243 pg range for IL-5; a 25-83 pg range for IL-

13; a 9-185 pg range for IL-17 and a 20-251 pg range for IL-12. The concentrations of TGF-[beta] were much higher than those of these other cytokines in all cultures, ranging from 2146 to 4236 pg, with an outsider of 8290 pg. As for IFN-[gamma], there was a great variation in the spontaneous production of the cytokine, whose concentrations in culture's supernatants ranged from 8 to 927 pg.

Statistical analysis

The statistical significance of differences in cytokine concentrations between stimulated and nonstimulated cultures were determined by Friedman's test, followed by Dunn's post-test. Differences of p [less than or equal to] 0.05 were considered significant.

Fisher's exact probability test was used to assess the statistical significance of differences in the proportion that fungal antigen-stimulated and unstimulated PBMC presented a given pattern of produced cytokines and in the frequencies of production of IL-17 in candidin- and trichophytin-stimulated cultures.

Results

Cell viability in fungal extract-stimulated PBMC cultures

The mean percentages of viable cells in PBMC cultures to which 50 μg/ml of candidin and trichophytin extracts were added, in relation to untreated cultures, were 98.2 and 99.0, respectively.

Cytokine production by fungal extract-stimulated PBMC

The fungal extract concentrations (5 and 50 μg/ml) that were utilized in the present work did not differ in terms of the patterns of cytokine level increases that they induced in the different PBMC cultures, although the best concentration was not always the same for the different PBMC (data not shown). The results shown in Figures 1 & 2 were those obtained with the best concentration (either 5 and 50 μg/ml) for each PBMC.

The addition of candidin significantly increased the production of IL-10, TNF-[alpha] and IL-12 by healthy individuals' PBMC, and of IL-10, TGF-[beta], IL-17, IFN-[gamma] and IL-12 by allergic patients' PBMC (Table 1), whereas trichophytin significantly increased the production of IL-12 by healthy individuals' PBMC, and of TGF-[beta] by allergic patients' PBMC (Table 1). Although statistically significant increases could be observed for some cytokines in cultures of allergic donors' PBMC and not in cultures of nonallergic donors' PBMC, or vice versa (Table 1), there were no statistically significant differences between the results obtained in cultures of allergic and nonallergic donors' PBMC.

The addition of trichophytin increased regulatory cytokine (TGF-[beta] and/or IL-10) levels in six out of seven cultures of healthy individuals' PBMC and in eight out of nine cultures of allergic patients PBMC. This also happened with the addition of candidin in all 16 individuals' (seven healthy and nine allergic) PBMC (Figure 1). In most cultures, however, these increases in regulatory cytokines were accompanied by increases in proinflammatory cytokines (Figures 1 & 2). Trichophytin increased regulatory cytokines levels exclusively, without augmenting any of the investigated proinflammatory cytokines, in two out of seven nonallergic individuals' PBMC (nonallergic donors 2 and 4; Figures 1 & 2) and in three out of nine allergic patients' PBMC (allergic donors 2, 3 and 4; Figures 1 & 2). An exclusive increase in regulatory cytokine levels was not seen in any culture of candidin-stimulated PBMC (Figures 1 & 2).

Stimulation of the production of cytokines associated with the Th1 immune response (TNF-[alpha], IL-12 and IFN-[gamma]) by either trichophytin or candidin or by both was seen in seven out of eight healthy individuals' PBMC and in all nine allergic patients' PBMC (Figures 1 & 2).

To be a feasible candidate for immunological adjuvant in antiallergy immunotherapy, a substance should stimulate the production of Treg cytokines, Th1 cytokines or both, without stimulating the production of Th2 and Th17 cytokines. Stimulation of Treg cytokine (TGF-[beta] and/or IL-10) production accompanied or not by stimulation of the production of cytokines associated with the Th1 immune response, and without stimulation of Th2 cytokines (IL-5 and IL-13) and IL-17, by either trichophytin or candidin or by both of them, was seen with four out of seven healthy individuals' PBMC (nonallergic donors 1, 2, 4 and 6; Figures 1 & 2) and with nine out of nine allergic patients' PBMC (Figures 1 & 2). This differed significantly from what was seen in unstimulated cultures ($p = 0.035$ and 0.0002, respectively; Fisher's exact probability test). Although a higher proportion of PBMC with these patterns of cytokine production was seen in the allergic patients' group than in the nonallergic group, this difference was not statistically significant ($p = 0.26$, two-tailed Fisher's exact probability test).

The levels of the allergy-associated Th2 cytokine IL-5 were increased in PBMC cultures from three out of seven healthy individuals (nonallergic donors 3, 5 and 7, Figure 2) and in none of the alergic patients' PBMC that were stimulated by candidin. Trichophytin, on the other hand, stimulated the production of IL-5 by just one of the nonallergic donors' PBMC (nonallergic donor 7) and by none of the allergic donors' PBMC.

The levels of IL-13, another allergy-associated cytokine, were not increased when candidin was added to cultures of nonallergic donors' PBMC (Figure 2). Only a small increase was seen in one of the allergic patients' PBMC cultures due to the addition of that fungal extract (allergic donor 9; Figure 2). As for trichophytin-stimulated PBMC, the level of IL-13 was increased in only one of the cultures of nonallergic individuals' PBMC (nonallergic donor 5; Figure 2) and in none of the nine allergic patients' PBMC cultures (Figure 2).

IL-17 levels were increased in cultures of trichophytin-stimulated PBMC from one out of seven healthy individuals (donor 5; Figure 2), and from none of the nine alergic patients (donors 6 and 8; Figure 2), whereas it was increased in cultures from candidin-stimulated PBMC from four out of seven healthy individuals (nonallergic donors 1, 3, 5 and 7; Figure 2), and from five out of nine allergic patients (allergic donors 2-5 and 9; Figure 2). Combining the data from allergic and nonallergic individuals for trichophytin (1 out of 16 individuals with PBMC producing IL-17) and for candidin (9 out of 16 individuals with PBMC producing IL-17), the difference observed between the frequencies of stimulation of IL-17 production by the two extracts was highly significant ($p = 0.0059$, two-tailed Fisher's exact probability test).

Discussion

The viability of the PBMC was not affected by incubation with up to 50 μg/ml of candidin or trichophytin for 24 h, as shown by the absence of additional MTT reduction. Cell death, therefore, did not affect the variation in cytokine production caused by the stimulation of the cells by the fungal extracts.

The present work, although studying a relatively small sample of individuals, clearly shows that trichophytin and/or candidin elicited the production of regulatory and/or Th1-associated cytokines without stimulating the production of the respiratory allergy-associated Th2 cytokines and IL-17 in a proportion of healthy (four out of seven in the present study) and allergic (nine out of nine in the present study) individuals' PBMC. These allergic patients, therefore, are candidates for receiving antiallergy immunotherapeutic preparations containing the allergen(s) and, as adjuvant, only candidin (allergic donor 8; Figures 1 & 2); only trichophytin (allergic donors 2, 3, 4, 5 and 9; Figure 2), or both (allergic donors 1, 6 and 7; Figure 2). These results open, therefore, the possibility of using customized immunotherapeutic preparations that would contain only the adjuvant(s) that did not induce Th2 cytokine or IL-17 production in that particular patient. For obvious reasons, adjuvants that are candidates for inclusion in antiallergy therapeutic vaccines should not induce the production of these cytokines, as they are directly associated with the pathogenesis of respiratory allergy [37]. A customized immunotherapy that would involve the previous realization of a cell-culture procedure and the assessment of cytokine production would be relatively expensive. Its price, however, should be weighed against that of carrying out a less effective immunotherapeutic procedure for years.

Candidin significantly stimulated the production of three cytokines by healthy individuals' PBMC (IL-10, TNF-[alpha] and IL-12), and of five cytokines by allergic patients' PBMC (IL-10, TGF-[beta], IL-17, IFN-[gamma] and IL-12), whereas trichophytin significantly increased the production of only IL-12 by healthy individuals' PBMC, and of only TGF-[beta] by allergic patients' PBMC (Table 1). Candidin, therefore, seems to be more stimulatory than trichophytin for human PBMC. In fact, candidin stimulated the production of IL-17 by the PBMC from a larger number of donors (9 out 16; Figure 2) than trichophytin (1 out of 16; $p = 0.0059$, two-tailed Fischer's exact probability test). However, PBMC of a larger number of individuals, and in different geographic areas, should be tested in order to determine which of the two preparations, trichophytin or candidin, would be the best antiallergy adjuvant candidate.

Although current knowledge highlights the role of T regulatory cell-mediated immune regulation, defined mechanisms that lead to successful clinical outcomes of allergen-specific immunotherapy still remains an open area of research [38].

An interesting finding, which may be relevant for the possible use of trichophytin and candidin as adjuvants in antiallergy immunotherapy, is that these fungal extracts did not stimulate the production of Th-2 cytokines by allergic donor' PBMC more intensely than by healthy donor' PBMC. Allergic patients, therefore, do not seem to have an immune system biased toward the production of Th2 cytokines when stimulated by these fungal antigens.

As trichophytin and candidin have been injected for decades in human beings for the assessment of the cellular immune response [32], a candidin- and/or trichophytin-containing immunotherapeutic preparation would be more easily approved for clinical use.

Conclusion

Trichophytin and candidin stimulated the production of regulatory cytokines on the PBMC from nearly all donors (15 out of 16 and 16 out of 16, respectively). These findings justify the realization

of studies aimed at investigating whether there are proteins in these fungus extracts that preferentially induce the production of regulatory cytokines. These proteins would be ideal Treg-inducing adjuvant candidates for inclusion in immunotherapeutic vaccines for inflammatory diseases, such as allergy and autoimmune diseases. An interesting possibility, therefore, would be the purification of molecules from candidin or trichophytin that would specifically stimulate an immune regulatory response that, in its turn, would control the allergic immune response, or alternatively, identify those molecules and have they produced by the recombinant DNA technology.

Future perspective

New tolerogenic antiallergy immunotherapies should be available in the next 5-10 years. These immunotherapies will probably utilize preparations containing Treg-inducing adjuvants. It is possible that molecules obtained from candidin or trichophytin might be used as tolerogenic adjuvants.

Table 1. Statistical significance[dagger] of variations in cytokine production by fungal extract-stimulated peripheral blood mononuclear cells.

Produced cytokine	Peripheral blood mononuclear cells from	Comparison	
Candidin-stimulated versus nonstimulated cultures	Trichophytin-stimulated versus nonstimulated cultures		
IL-10	Allergic patients	p [less than] 0.0100	NS
Healthy individuals	p [less than] 0.0001	NS	
TNF-[alpha]	Allergic patients	NS	NS
Healthy individuals	p [less than] 0.0500	NS	
IL-5	Allergic patients	NS	NS
Healthy individuals	NS	NS	
IL-13	Allergic patients	NS	NS
Healthy individuals	NS	NS	
IL-17	Allergic patients	p [less than] 0.0500	NS
Healthy individuals	NS	NS	
TGF-[beta]	Allergic patients	p [less than] 0.0001	p [less than] 0.0500
Healthy individuals	NS	NS	
IL-12	Allergic patients	p [less than] 0.0100	NS
Healthy individuals	p [less than] 0.0100	p [less than] 0.0500	
IFN-[gamma]	Allergic patients	p [less than] 0.0500	NS
Healthy individuals	NS	NS	

[dagger] As assessed by Friedman's nonparametric test followed by Dunn's post-test.
NS: No statistical significance.

Executive summary

* The identification of molecules that could be used as adjuvants in antiallergy immunotherapeutic preparations is highly desirable.
* Antiallergy immunotherapy has been associated with the induction of Treg and/or T helper 1 cells that could theoretically inhibit the development of allergy-inducing T helper 2 (Th2) immune responses.
* In this study, it was investigated whether candidin or trichophytin could elicit recall immune responses that could potentially inhibit a Th2 response, in nine allergic and seven nonallergic individuals' peripheral blood mononuclear cells (PBMC).
* PBMC were cultivated *in vitro* in the presence or absence of these extracts at 37°C and 5% CO_2 during 24 and 120 h.
* In this way it was sought to obtain indirect evidence that they could be used as adjuvants aimed at inhibiting Th2 immune responses.
* The two extracts induced cytokine production in all PBMC preparations. Stimulation of the production of Treg cytokines (TGF-[beta] and/or IL-10), accompanied or not by stimulation of production of cytokines associated with the Th1 response (TNF-[alpha], IL-12 and IFN-[gamma]), but without stimulation of Th2 cytokines (IL-5 and IL-13) and IL-17, by either trichophytin or candidin or by both of them, was seen with four out of seven healthy individuals' PBMC and with nine out of nine allergic patients' PBMC.
* As candidin and trichophytin have been injected intradermally in human beings for decades to trigger cutaneous Th1 immune reactions without adverse reactions, these results indicate that these fungal extracts could be used as adjuvants in personalized therapeutic vaccines in a fair proportion of individuals and justify the carrying out of investigations aimed at identifying molecules in these extracts that might exclusively induce Treg and/or Th1 immune responses.

CAPTION(S):

Figure 1. TGF-[beta], IL-10, TNF-[alpha] and IL-12 concentrations in cultures of healthy and allergic individuals' peripheral blood mononuclear cells stimulated with fungal extracts.
(A) Peripheral blood mononuclear cells (PBMC) from nonallergic individuals' donors. **(B)** PBMC from allergic individuals' donors. C or T were added to a final concentration of 5 or 50 µg/ml to the cells, which were incubated as described in the 'Materials & methods' section. The amounts of cytokines detected in nonstimulated cultures were subtracted from the amounts detected in the stimulated cultures. Each symbol shows the results obtained with the PBMC from an individual donor. The legends above the graphs identify the individual PBMC donors. The results obtained from the same donors' PBMC are represented by identical symbols in Figures 1 & 2.
C: Candidin; AI: Allergic donor; NAI: Nonallergic donor; T: Trichophytin.

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Authors' contributions

AL Moreno Amor and LN Santos were involved in recruiting study patients, performing the experiments, acquisition, statistical analysis and interpretation of the data, and preparing a first draft of the manuscript. A Alcântara Galvão, EM Medeiros de Andrade Belitardo and E Santos Silva performed part of the experiments. NM Alcântara-Neves helped in devising the study, and designed and supervised the experiments. L Pontes-de-Carvalho devised the study, designed and performed statistical analysis, interpreted the data and wrote the final version of the manuscript. The authors wish to thank the volunteers who participated in this study. The authors also thank the AlergoLatina Produtos Alergênicos Ltda., Rio de Janeiro, Brazil, for donation of allergen extracts.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.