The role of leukotriene B4 in early stages of experimental paracoccidioidomycosis induced in phenotypically selected mouse strains

HELANDERSON A. BALDERRAMAS*,†, ORLANDO G. RIBEIRO‡, ÂNGELA M. V. C. SOARES*,† & SILVIO L. OLIVEIRA*†
*Botucatu School of Medicine, Department of Tropical Diseases, and †Department of Microbiology and Immunology, Institute of Biosciences, Universidade Estadual Paulista (UNESP), São Paulo, and ‡Laboratory of Immunogenetics, Butantan Institute, São Paulo, Brazil

Paracoccidioidomycosis is a human systemic mycosis caused by the fungus Paracoccidioides brasiliensis. The mechanisms involved in innate immune response to this fungus are not fully elucidated. Leukotrienes are known to be critical for the clearance of various microorganisms, mainly by mediating the microbicidal function of phagocytes. We investigated the involvement of leukotriene B4 in the early stages of experimental paracoccidioidomycosis, which was induced by intratracheal inoculation of the fungus in selected mouse lines. The mouse lines utilized were produced through bi-directional phenotypic selection, endowed with maximal or minimal acute inflammatory reactivity, and designated AIRmax and AIRmin, respectively. AIRmax mice were more resistant to the infection, which was demonstrated by reduced lung fungal loads. However, the two lines produced similar amounts of leukotriene B4, and pharmacological inhibition of this mediator provoked similar fungal load increases in the two lines. The lower fungal load in the AIRmax mice was associated with a more effective inflammatory response, which was characterized by enhanced recruitment and activation of phagocytic cells and an increased production of activator cytokines. This process resulted in an increased release of fungicidal molecules and a diminution of fungal load. In both lines, leukotriene production was associated with a protective response in the lung that was consequent to the effect of this eicosanoid on the influx and activation of phagocytes.

Keywords Paracoccidioides brasiliensis, leukotrienes, acute inflammation, cytokines

Introduction
Paracoccidioidomycosis (PCM) is a human systemic mycosis caused by the dimorphic fungus Paracoccidioides brasiliensis. The natural route of infection is through the inhalation of fungal particles. This allows us to suppose that the development of an innate immune response against this pathogen, which occurs in the case of other fungi that infect the host through the same route, initiates in the lungs. This aspect is very important for consideration, because the innate immune response differs depending on the organ infected by the microorganism [1]. In general, most studies on PCM have evaluated the specific cellular and humoral immune responses to infection [2], whereas few studies have aimed to assess the innate immune response. This difficulty becomes obvious when we consider that the disease is not usually recognized until long after the establishment of infection. Thus, innate immune responses against the fungus have been studied using in vitro assays and mostly experimental models. In this context, a review by Calich et al.
[3] discussed some mechanisms involved in the innate immune response to this fungus, including complement proteins, fungicidal and modulatory activity of natural killer cells, the role of pro- and anti-inflammatory cytokines, including those with chemotactic activity, and the participation of lipid mediators, such as the eicosanoids. Among the latter, leukotrienes (LTs) have received considerable attention from researchers because they are potent mediators of the inflammatory response. LTs are derived from the metabolism of cell membrane arachidonic acid through the action of the enzyme, 5-lipoxygenase (5-LO) in concert with its helper protein, 5-LO-activating protein (FLAP) [4]. LTs act mainly on the recruitment and activation of leukocytes [5,6].

Regulation of immune cells by eicosanoid receptors is most commonly associated with the pathogenesis of diseases such as asthma [7] and atherosclerosis [8]. Recently, the role of these mediators in host defense against several microorganisms has been widely considered. Studies have shown that LT-induced host defense is mediated by the ability of LTs to increase microbial phagocytosis and killing. Alveolar macrophages from mice infected with Klebsiella pneumoniae produce LTs that increase both phagocytosis and bactericidal activity of these cells [9,10]. Further studies demonstrated that LTs enhance bactericidal activity of alveolar macrophages through the activation of NADPH oxidase [11].

Likewise, treatment of murine peritoneal macrophages with exogenous LTs results in increased production of nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α) and, consequently, in microbicidal activity against Trypanosoma cruzi [12]. Phagocytes from mice resistant to Leishmania amazonensis produce higher Leukotriene B4 (LTB4) levels and present enhanced microbicidal activity than those from susceptible mice [13]. Mycobacterium tuberculosis infection also induces LTB4 production in mouse lungs, which is important for inducing a protective immune response because the inhibition of endogenous LT synthesis abrogates mycobacterial clearance and survival [14]. LTs also play a pivotal role in inducing in vitro mycobactericidal activity of human polymorphonuclear cells (PMNs) [15]. Concerning fungal infections, important studies were reported with Histoplasma capsulatum and have shown the protective role of this eicosanoid. Accordingly, LTB4 is involved in leukocyte recruitment induced by H. capsulatum or its cell wall components [16], and a blockade of endogenous leukotrienes exacerbates murine pulmonary histoplasmosis [17]. In addition, in vivo and in vitro studies utilizing 5-LO-deficient mice to demonstrate the role of endogenous LTs in histoplasmosis demonstrated that LTs play an important role in host defense against H. capsulatum through the modulation of nitric oxide (NO) production, phagocytosis and effector cell recruitment [18]. Additionally, the recruitment and activation of memory T cells following immunization against H. capsulatum were impaired in 5-LO-deficient mice [19]. Other data identified LTB4 and LTD4 as key mediators of innate immunity against C. albicans, mainly by increasing the antimicrobial functions of alveolar macrophages [20].

Mouse lines endowed with maximal (AIRmax) or minimal (AIRmin) acute inflammatory reactivity (AIR) were produced through bi-directional phenotypic selection, starting from a highly polymorphic population (F0) obtained from the intercrossing of eight inbred mouse strains (A, DBA2, P, SWR, CBA, SJL, BALB/c and C57BL/6). The AIR phenotype was determined by the local leukocyte influx and the plasma protein exudation measured 48 h after the subcutaneous (s.c.) injection of polyacrylamide beads (Biogel), which is a non-antigenic, insoluble and chemically inert substance [21]. The progressive divergence of the AIRmax and AIRmin lines in the successive generations of selective breeding reached 20- and 2.5-fold differences in leukocyte and protein exudate infiltration, respectively. These differences resulted from the accumulation of alleles endowed with opposing effects on the inflammatory response. Genetic analysis of this selection indicated that AIR regulation involves at least 11 quantitative trait loci (QTL) [22]. Moreover, the interline difference was neither restricted to polyacrylamide beads nor to s.c. tissue because it was also detected in response to many other inflammatory agents such as bacteria [23], carrageenan and zymosan, which were injected s.c. or intraperitoneally (i.p.) (Vasquez-Bravo et al., unpublished results).

The specific immune responses of the selected lines were not markedly affected by the selective process, because AIRmax and AIRmin mice produced similar amounts of antibodies after immunization with optimal doses of such complex antigens as heterologous proteins, erythrocytes and bacterial antigens. Specific lymphocyte proliferation and delayed-type-specific reactions were also of the same intensity in both lines [23]. In contrast, AIRmax and AIRmin mice were found to differ greatly in their innate resistance/susceptibility to pathogens. This fact is well illustrated by the results of experimental infection with Salmonella typhimurium and Listeria monocytogenes, in which the respective LD50s were 1000 times and 100 times higher in AIRmax than AIRmin mice, respectively [23]. Because of these characteristics, these strains can be considered as very interesting models for studying the innate immune response to infection, besides representing extreme phenotypes found in a heterogeneous natural population. In this study, we sought to determine the role of LTB4 in
AIRmax and AIRmin mice during the early stages of 
P. brasiliensis infection.

**Materials and methods**

**Animals**

AIRmax and AIRmin mice from the 37th and 38th generation of selective breeding were obtained from the Laboratory of Immunogenetics of the Institute Butantan, São Paulo, Brazil. Each experiment was performed on male mice (2–3 months old), which were maintained under conventional conditions in our animal facilities and fed water and sterilized food ad libitum. All of the procedures involving animals were approved by the Committee for Ethics in Animal Experimentation of the Botucatu Medical School, São Paulo State University, Brazil.

**Fungal strain and culture conditions**

The P. brasiliensis strain Pb18, which is classically considered to be of high virulence [24], was maintained in the mycology culture collection of the Department of Microbiology and Immunology, Biosciences Institute, UNESP, and used throughout this study. After five days of cultivation on 2% glucose, 1% peptone, 0.5% yeast extract and 2% agar medium (GPY medium) at 35°C, yeast cells were recovered from the culture medium, washed and suspended in 0.15 M phosphate-buffered saline pH 7.2 (PBS). To obtain individual cells, the fungal suspension was homogenized with glass beads in a Vortex homogenizer (three cycles of 10 sec). Yeast viability was determined by phase contrast microscopy; while bright yeast cells were counted as viable, dark cells were considered non-viable [25]. Fungal suspensions containing more than 95% viable cells were used in the experiments.

**Infection of mice with P. brasiliensis yeast cells and treatment with MK 886**

Mice were anesthetized with a cocktail of ketamine (80 mg/kg) and xylazine (6 mg/kg) and restrained on a small board. An anterior midline incision was made to expose the trachea. A 30-gauge needle attached to a tuberculin syringe was inserted into the trachea, and intratracheal inoculation (i.t.) was utilized to introduce either 100 μl of PBS or 100 μl of a suspension of 1 × 10^7 yeast cells/ml into the lungs. The animals were divided into three groups.

Group 1: animals were infected with P. brasiliensis and treated daily with 0.5 ml of water (given by gavage) for seven days.

Group 2: animals were infected with P. brasiliensis and treated daily with MK 886 (5 mg/kg/0.5 ml; his concentration was taken from Medeiros et al. 2004 [17]) for seven days.

Group 3: animals were inoculated with PBS (i.t. route) and treated daily with water (0.5 ml given by gavage) for seven days.

**Bronchoalveolar lavage fluid (BALF)**

Mice were lavaged after cannulation of the trachea with polyethylene tubing, which was attached to a tuberculin syringe. The same procedure was applied to sham-infected mice (submitted to surgical stress and injected with 100 μl of PBS) of both mouse strains. The lungs were lavaged by repeated injections of 1.0 ml of sterile PBS (final volume, 3.0 ml). The recovered fluid was centrifuged at 250 g, and the supernatant was removed. Cells were analyzed for leukocyte subsets.

**Differential count of leukocyte population**

For differential counts, samples of BALF were cytospin onto glass slides and stained by Panótico (Laborclin, Paraná, Brazil). One hundred cells were counted from each sample. The absolute number of a leukocyte subset was calculated by multiplying the percentage of each subset in an individual sample by the total number of leukocytes in the BALF.

**Colony-forming units (CFU) determination**

Aliquots of lungs harvested from infected mice were incubated in digestion solution. Next, 0.1 ml was placed on Brain-Heart infusion (BHI) agar medium plates (Difco Laboratories) containing 0.5% gentamicin, 4% normal equine serum and 5% P. brasiliensis strain 192 culture filtrate, the latter being the source of growth-promoting factors. CFU were counted after 15 days of incubation at 37°C, and the numbers (log10) of viable P. brasiliensis colonies were expressed as CFU per gram of tissue [26].

**Quantitation of NO**

NO production was quantified by the accumulation of nitrite (as a stable end product) in the supernatants from homogenized lungs by a standard Griess reaction. Briefly, 50 μl of supernatant was incubated with an equal volume of Griess reagent (1% sulfanilamide: 0.1% naphthylene diamine dihydrochloride: 2.5% H₃PO₄) at room temperature for 10 min. The absorbance at 550 nm was determined with a microplate reader. The conversion of absorbance to
micromoles of NO was deduced from a standard curve using a known concentration of NaNO₂ diluted in RPMI medium. All of the determinations were performed in triplicate and expressed as micromoles of NO₂.

Measurement of cytokines and LTB4

For cytokine and LT measurements, the lungs were removed on days 1, 3 and 7 post-infection and homogenized in 2 ml of RPMI 1640. The suspension was centrifuged at 1500 g, filtered, sterilized and stored at −80°C until it was assayed. Levels of LTB4, TNF-α, IL-10, and IFN-γ were measured using commercial ELISA kits that were obtained from Cayman Chemical Co. (LTB4) and BD Systems (cytokines).

Statistical analysis

The statistical procedures were developed using the software package Graph Pad Instat (San Diego, CA, USA). Significant differences between the groups were determined by Analysis of Variance followed by the Tukey-Kramer test for Multiple Comparisons. The statistics were considered to be significant when \( P < 0.05 \).

Results

LTB4 production in the lungs of AIRmax and AIRmin mice infected with P. brasiliensis

In the first set of experiments, we compared LTB4 production in the lungs of AIRmin- and AIRmax-infected mice and determined whether this production could be inhibited by MK 886 (Fig. 1), a mammalian 5-LO-activating protein inhibitor. LTB4 was chosen to be evaluated because it is the best known lipid mediator for its role in the immune response, particularly in initiating the inflammatory response [27]. Uninfected mice produced very low LTB4 levels, which were similar between AIRmax and AIRmin lines. However, infected mice showed an increase in lung LTB4 levels as early as the first day of infection, and LTB4 levels were also not significantly different between the two lines. These levels were not altered at three and seven days post-infection. It is remarkable that the lung LTB4 levels for all animal groups were reduced after MK 886 treatment.

Effect of LTB4 in the degree of infection in lung

Given that MK 886 significantly inhibited LTB4 production, we have used this pharmacological approach to study this eicosanoid effect on the responses of both lines to P. brasiliensis infection. First, we analyzed the degree of infection in the lung by evaluating the recovery of viable fungi (Fig. 2). A significant recovery was obtained from the lungs as early as day one post-infection in both AIRmax and AIRmin mice. However, the CFU number was significantly higher in the AIRmin lungs in comparison to the AIRmax lungs. At three days post-infection, fungus recovery was proportionally increased for both lines followed by a decrease at seven days post-infection. Notably, a significant decrease in the recovery of viable fungi was detected in the lungs of animals from each of the groups that were treated with MK 886. Overall, the results indicated that the AIRmax mice presented a lower degree of infection than that of the AIRmin mice. However, inhibition of LTB4 production results in a proportional increase in the recovery of viable fungi in both lines, showing a similar role of this eicosanoid in controlling fungal viability in the two strains. Thus, to evaluate other mechanisms involved in the different infectivity detected in the AIRmax and AIRmin lungs and to address the involvement of LT in these same mechanisms, the following parameters were analyzed in the lungs of all of the animal groups: influx of...
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PMNs and mononuclear cells and NO, IFN-γ, TNF-α and IL-10 production.

Polymorphonuclear cells (PMN) influx

The PMNs influx at day one of infection was significantly higher among AIRmax mice. A proportional but slight increase in PMNs was detected at day three post-infection, followed by a significant reduction at seven days. Notably, an inhibition in PMN influx was detected in each group treated with MK 886 in comparison to the respective untreated group, demonstrating the probable role of LTB4 in attracting these cells to the lungs. In uninfected (control) animals, PMN influx remained very low or undetectable (Fig. 3A).

Mononuclear influx

Mononuclear cell influx in non-infected animals was consistently very low or undetectable. However, as early as day one of infection, both lines were presenting an increased influx of mononuclear cells but with no significant interline differences. Cell influx increased at three days post-infection and peaked at seven days, when AIRmax mice presented a significantly higher infiltrate. Remarkably, the influx in each line of MK 886-treated animals was unchanged compared to untreated animals (Fig. 3B).

NO production

At day one of infection, an increase in NO levels was detected in infected AIRmax and AIRmin mice in comparison to uninfected groups. However, higher levels of the metabolite were detected in the lungs of AIRmax in relation to AIRmin. The levels were maintained at three days post-infection. At seven days, a slight diminution in NO production was detected in all of the groups, but the difference between AIRmax and AIRmin mice was still maintained. In all of the groups, MK 886 treatment resulted in lower metabolite levels in treated mice than those in untreated animals (Fig. 4).

TNF-α and IFN-γ levels

As illustrated in Fig. 5A and 5B, a similar response profile was detected in TNF-α and IFN-γ expression, respectively. At day one of infection, cytokine levels of AIRmax and AIRmin mice were greater than those of their uninfected counterparts. However, higher levels of both cytokines were detected in the lungs of AIRmax mice. At three and seven days post-infection, the levels were not changed, except for a slight reduction in TNF-α at three days post-infection. A significant reduction in TNF-α production was detected in each group of mice treated with MK 886 in relation to their respective untreated counterparts. Regarding IFN-γ expression, the treatment significantly reduced cytokine production in the lungs of AIRmax mice. However, the differences between treated and untreated AIRmin mice were not significant.

IL-10 levels

As shown in Fig. 5C, on the first day of infection, infected mice of both lines presented higher IL-10 levels than their respective controls. However, it is noteworthy that higher

Fig. 2 Colony-forming units (CFU) recovered from the lungs of AIRmax and AIRmin mice intratracheally infected with Pb18 (Pb), treated or not with MK 886 (MK) and evaluated at 1, 3 and 7 days after infection. Data are expressed as means ± standard errors from the results of 11–15 animals from both lines in each group. *P < 0.05 vs. AIRmin Pb, **P < 0.05, ***P < 0.01, ****P < 0.001 AIRmin or AIRmax vs. the respective MK-treated mice.

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levels of IL-10 were produced by the AIRmin animals. At three and seven days post-infection, cytokine production was not significantly changed in any group. Interestingly, no significant differences were detected between the mice treated with MK 886 and untreated animals.

**Discussion**

This study examined the role of LTB4 in the evolution of lung PCM in mouse lines endowed with maximal or minimal acute inflammatory reactivity. Pharmacological inhibition of LTs with MK 886 was used as an experimental approach to establish an eicosanoid effect. Throughout infection, AIRmax mice presented a lower degree of lung infection than the AIRmin mice. Despite these interline differences, infected AIRmin and AIRmax mice produced high but similar levels of LTs. In addition, LT inhibition provoked similar increases in fungal load among both AIRmin and AIRmax mice. Thus, it is clear that LTs are associated with fungus destruction. However, differences in inflammatory processes detected between the two lines cannot be attributed to a differential capacity to produce this eicosanoid.

In this context, to determine the mechanisms involved in the differences in infectivity between the two lines, we first evaluated the kinetics of PMN and mononuclear cell influx in the organ. Throughout infection, there was a marked increase in the influx of PMNs in AIRmax animals in relation to AIRmin mice. AIRmax animals showed an increase in mononuclear cells only at day seven. NO is an important molecule used by murine phagocytes to kill *P. brasiliensis* [28,29]. In this study, higher NO levels detected as early as day one of infection were only slightly reduced on the seventh day. Interestingly, higher levels of the metabolite were produced by AIRmax mice. In light of these results we may propose that at days one and three of infection, the fungal load was controlled mainly by the fungicidal activity of PMN cells because the recruitment of mononuclear cells was very low. As the PMN influx was significantly increased in AIRmax mice, fungus killing was...
also higher in the lungs of this strain. This notion may be corroborated by an association between this process and the high NO levels released in the lung of this same line. Some reports have demonstrated that neutrophils may be important effector cells in PCM mainly during the initial stages of infection [30]. In vitro studies have shown that neutrophils possess efficient fungicidal or fungistatic activity, especially after activation by certain cytokines [31–33]. At seven days post-infection, both lines demonstrated a proportional decrease in the recruitment of PMNs. On the contrary, a significant change was observed in the mononuclear influx, which was higher in AIRmax mice. Interestingly, P. brasiliensis killing was also significantly higher at this timepoint compared to days one and three, mainly among AIRmin mice. Thus, we can propose that at day seven, the fungus was killed mainly by mononuclear cells. Unexpectedly, the NO levels were not increased during this period. Alternatively, one possibility is that the mononuclear cells in this period killed P. brasiliensis using another metabolite such as H₂O₂. This molecule has been shown to mediate the killing of P. brasiliensis by human and murine phagocytes [29,34,35].

We also asked whether the differences in the response of both lines to infection could be associated with alterations in the levels of cytokines that modulate phagocyte functions such as TNF-α, IFN-γ, and IL-10. TNF-α and IFN-γ presented similar response kinetics during the infection. For example, as early as day one, infection induced high levels of both cytokines, which were unchanged in the subsequent infection periods and were higher among AIRmax mice. IL-10 levels were also enhanced during the three infection periods in both lines, but in contrast to TNF-α and IFN-γ, IL-10 levels remained higher in AIRmin mice. IFN-γ and TNF-α were shown to effectively activate both neutrophils and monocytes/macrophages to kill P. brasiliensis [29,34,35]. On the contrary, IL-10 deactivates these cells [38,39].

Thus, we can propose that in both lines, PMNs especially at one and three days and macrophages at seven days are activated by IFN-γ and TNF-α to release NO and/or H₂O₂ and to consequently kill the fungus. This process was most likely associated with the prevalent production of IFN-γ and TNF-α despite the concomitant production of IL-10, which was not able to eliminate the effect of activator cytokines. In addition, the lower IL-10-to-IFN-γ and TNF-α ratio in the AIRmax mice results in higher activation than in the AIRmin line.

Of particular interest in this study are the results on the role of LTB4 in P. brasiliensis infection. As already discussed, we cannot attribute the differences in inflammatory processes detected between the two lines to a differential production of cytokines. However, it should be noted that independent of the lines, the results support a significant role of LTs in modulating an inflammatory response that results in the killing of P. brasiliensis. In this context, our results showed that LTB4 is involved in PMN but not in mononuclear influx. These results are in agreement with other studies that found LTB4 to be a potent chemoattractant for neutrophils and, to a lesser extent, for eosinophils and mononuclear cells and showed that it is released after cell activation by pathogens or soluble mediators [40–42]. LTB4 is also involved in P. brasiliensis killing in the lungs because fungus recovery was significantly higher after MK 886 treatment. This effect was detected in other infections within which LTs have potent effects on...
phagocytosis and the killing of microorganisms by phagocytic cells [9–17]. We are proposing that this process may be attributed to the augmented production of fungicidal substances by this eicosanoid. Indeed, NO production was significantly reduced by MK 886 treatment in our study. Alternatively, the effect of LT on fungicide molecules may

Fig. 5  Levels of TNF-α (A), IFN-γ (B) and IL-10 (C) in the supernatants of lung homogenates from AIRmax and AIRmin mice that were uninfected (control) or intratracheally infected with Pb18 (Pb), treated or not with MK 886 (MK) and evaluated at 1, 3 or 7 days after infection. Data are expressed as means ± standard errors from the results of 11–15 animals from both lines in each group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. the respective control, #P < 0.05, ##P < 0.01 vs. AIRmin Pb, *P < 0.05, **P < 0.01 vs. the respective MK-treated mice.
reflect its role in increasing activator cytokines, which were also detected in our study, as IFN-γ and TNF-α production was reduced by MK 886 treatment. Other studies in the literature corroborate this notion because LTB4 is involved in T cell activation and increasing in IL-2, IL-4, IFN-γ, IL-1 and TNF-α [17,43,44].

In summary, we have demonstrated that mouse lines that respond to a pulmonary *P. brasiliensis* infection with a more robust inflammatory response in early phases are able to better control the infection during this period. This more effective inflammatory response is characterized by a higher recruitment of both PMN and mononuclear phagocytes, accompanied by their enhanced activation resulting from a higher production of activator cytokines. This process results in a greater release of fungicidal molecules and a consequential diminution in fungal load. Interestingly, this study demonstrated that intratracheal infection with *P. brasiliensis* induces LTB4 production in mouse lung. Animals treated with a leukotriene inhibitor demonstrated an increase in viable fungus recovery, showing that endogenous production of leukotrienes is associated with a protective immune response. These results reinforce the mechanism already proposed for other infections, including fungal ones, whereby leukotrienes are protective during the innate immune response of the host because of their ability to actuate on phagocytic cells, increasing their recruitment and ability to kill the microorganism. Our results are in agreement with this idea because we found that leukotrienes are involved in cell recruitment and NO production, the metabolite involved in fungus killing. This eicosanoid is also involved in TNF-α increases in the lung. We propose that LTs released in the lung could directly kill the fungus by activating macrophages and neutrophils or could indirectly kill it by increasing TNF production, one of the main cytokines that activates phagocytic cells. In addition to modulating innate immune responses, LTS can play a role in protective adaptive immune responses in PCM by promoting the release of TH1 cytokines. Identification of the role of leukotrienes on protective immune responses to *P. brasiliensis* can have important clinical implications because we can hypothesize that leukotriene production is inhibited in patients with PCM. Some studies have demonstrated that this eicosanoid is downregulated in situations such as malnutrition [45] and smoking [46]. However, further studies are needed to consider leukotrienes as a potential target for immunotherapy in PCM.

**Acknowledgements**

Helanderson A. Balderramas has a Doctoral fellowship from CAPES.

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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This paper was first published online on Early Online on 6 March 2013.