Cytokine production in lungs and adrenal glands of high and low antibody producing mice infected with Paracoccidioides brasiliensis


Department of Microbiology and Immunology, Biosciences Institute, UNESP-São Paulo State University, Botucatu, State of São Paulo, Brazil

Mice genetically selected for high (H) and low (L) antibody production (HIV-A and LIV-A) were used in an experimental model of paracoccidioidomycosis. In a previous work, it was observed that male HIV-A animals were more susceptible to the infection due to adrenal gland damage. Male HIV-A and LIV-A animals were intravenously inoculated with Paracoccidioides brasiliensis (strain 18) and sacrificed 2, 4, 6, 8 and 10 weeks after inoculation. At each time interval, lungs and adrenals were removed to estimate recoverability of the fungus, as well as to determine Th1 (IFN-γ, TNF-α) and Th2 (IL-4 and IL-10) cytokine profiles. While viable fungi recoverability from the lungs of HIV-A mice was higher after 4 and 8 weeks, there was less fungal recovery from the adrenals of LIV-A animals after the 2nd week, with total fungal elimination after the 8th week. With regard to Th2 cytokines, there was an inhibition in IL-4 production in the organs from infected animals, the extent of which varied according to the organ and the time period after initiation of infection. IL-10 production was found to be lower in both organs. Determination of Th1 cytokines revealed that IFN-γ production increased in both organs, mainly in the adrenal of LIV-A after 8 and 10 weeks, when these animals showed a total fungal elimination. A significant difference was observed between HIV-A and LIV-A concerning TNF-α production in both organs and at all recovery times, in that LIV-A produced a higher level of this cytokine, mainly in the adrenal. These results may explain the high susceptibility of HIV-A to P. brasiliensis infection, is due, at least in part, to adrenal involvement. The higher production of Th1 cytokines by LIV-A in comparison to HIV-A mice may account for LIV-A resistance to P. brasiliensis infection. Our data reveal the importance of this experimental model in the study of the adrenal involvement in paracoccidioidomycosis, since this gland may be highly compromised in the patients, leading to the development of Addison’s Disease.

Keywords Biozzi mice, experimental paracoccidioidomycosis, cytokines, adrenal gland

Introduction

Paracoccidioidomycosis (PCM) is a human systemic mycosis caused by the dimorphic fungus Paracoccidioides brasiliensis (Pb). This disease shows several clinical and pathological manifestations, varying from benign and localized forms to a chronic granulomatous
disease with involvement of the mucocutaneous areas, the lungs and other organs [1].

In PCM, the main host defense is mediated by cellular immune responses. Although high levels of specific antibodies are produced in association with the most severe forms of the disease, they have no protective role [2].

Animal models have been very useful in the study of host-parasite interaction in PCM [3]. Variables such as the experimental animal, sex and age of host, as well as strain, routes of inoculation and virulence of the fungus strains affect the susceptibility of the same animal species to *P. brasiliensis* [4,5].

Biozzi mice, high (H) and low (L) antibody producers of IV-A, showed modifications in macrophage activity in an inverse correlation to that of antibody production [6,7]. L mice macrophages presented a high catabolism of the immunogens with consequent deficiency in their antigen presentation which in turn lead to low antibody production. These animals, however, presented a greater resistance to intracellular pathogens compared to H mice, whose macrophages are less catabolic, leading to high antibody production and greater susceptibility to these pathogens [6,8,9].

In a previous work using H and L strains of IV-A mice infected with Pb, Soares et al. [10], observed that male H IV-A mice were more susceptible to the infection due to adrenal gland damage. An appropriate model for pathogen study of PCM that leads to compromise in adrenal function would provide a better understanding of the immunological mechanisms involved in *Paracoccidioides brasiliensis* infection.

The purpose of this work was to evaluate Th1 (IFN-γ and TNF-α) and Th2 (IL-4 and IL-10) production of cytokines in lungs and adrenal glands of H IV-A and L IV-A mice infected with *Paracoccidioides brasiliensis*.

**Materials and methods**

**Animals**

Male IV-A mice that show high (H) and low (L) antibody response (H IV-A and L IV-A), aged 8–10 weeks, were bred in the animal facilities of the Department of Microbiology and Immunology, Institute of Biosciences, São Paulo State University, Campus of Botucatu, and were fed water and sterilized food *ad libitum*.

**Fungus and infection**

*P. brasiliensis* yeast cells (strain 18) were maintained by subcultivation at 36°C in semisolid Fava Netto medium [16]. After 6 days of culture, samples were taken from each tube, washed and suspended in phosphate-buffered saline (PBS, pH 7.2). In order to avoid clumps and to facilitate the yeast count, fungal suspensions were homogenized with glass beads in a Vortex homogenizer (three cycles of 10 s). After counting, the fungus concentration was adjusted to $5 \times 10^6$ viable fungal cells mL$^{-1}$ and each mouse was infected by the intravenous (i.v.) route with 0.2 ml of this suspension. Fungal viability was determined by vital staining [17] and was always higher than 90%.

**Sacrifice**

Groups of 25 H IV-A and 25 L IV-A male mice were infected by i.v. route with 0.2 ml of the *P. brasiliensis* suspension as described above and five animals from each lineage were sacrificed after the 2nd, 4th, 6th, 8th and 10th week of infection. Each mouse was anesthetized with ether and killed by exsanguination. After sacrifice, lungs and adrenal glands were aseptically removed to analyze the degree of infection (through the recovery of viable fungi) and determination of Th1 (IFN-γ, TNF-α) and Th2 (IL-4, IL-10) cytokines by ELISA.

As control group, 25 H IV-A and 25 L IV-A male mice received an i.v. inoculum of 0.2 ml phosphate-buffered saline (PBS), and five animals from each lineage were sacrificed and analyzed at the same periods as infected
mice comparing each control period group with each experimental period group.

Evaluation of infection degree

Yeast-cell determination was performed by counting the number of CFUs after plating [18]. Briefly, lung fragments and adrenal glands were aseptically removed, weighed and homogenized in 2.0 ml of PBS pH 7.2 using a tissue grinder. Aliquots of 100 μl of each homogenate were plated in triplicate on brain-heart infusion (BHI) agar (Merck) supplemented with 4% (v/v) normal equine serum and 5% \( P. \) brasiliensis strain 192 culture filtrate, the latter acting as the source of the growth-promoting factor. The plates were incubated at 36°C in plastic bags to prevent drying. The number of \( P. \) brasiliensis colonies was determined daily, until no increase in counts was observed. The results were expressed as CFUs per milligram of tissue and represented the mean of three replicate experiments.

Determination of cytokines

After plating, portions of the homogenates were centrifuged at 2500 \( \times \) g for 10 min, the supernatants were recovered and stored at -70°C. IFN-\( \gamma \), TNF-\( \alpha \), IL-4 and IL-10 concentrations were measured by ELISA (R&D Systems, Inc), according to manufacturer’s instructions. In brief, microplates (Nunc) were sensitized overnight at 4°C with anti-IFN-\( \gamma \), anti-TNF-\( \alpha \), anti-IL-4 and anti-IL-10 monoclonal antibody (mAb). Plates were washed four times with PBS containing 0.05% Tween (PBS-T) and were then incubated for 1 h, at room temperature, with 200 μl per well of 3% bovine serum albumin (BSA-Sigma) in PBS. Plates were washed four times with PBS-T and incubated for 2 h at 37°C in 5% CO\(_2\) with 100 μl of each homogenate in triplicates, besides the respective cytokine standard curve. Then, plates were washed four times with PBS-T, and incubated for 2 hours at 37°C in 5% CO\(_2\) with 100 μl per well with biotinylated anti-IFN-\( \gamma \), anti-TNF-\( \alpha \), anti-IL-4 and anti-IL-10 (detection antibodies). Afterwards, plates were washed four times with PBS-T and incubated 1 h at 37°C in 5% CO\(_2\) with 100 μl per well of alkaline phosphatase-conjugated streptavidin. Plates were washed four times with PBS-T and enzymatic activity was revealed by incubation with 100 μl per well of o-phenylenediamine (Merck). Absorbance was read at 492 nm in microtiter plate-reading equipment. Results were expressed in pg/ml.

Statistical analysis

The data were statistically analyzed using the INSTAT software (Graph Pad, San Diego, CA, USA) [19]. The Student’s \( t \)-test was used for the assay of the recovery of viable fungi in the lungs and adrenal glands and also for the analysis of cytokines. A \( P \)-value <0.05 was considered significant.

Results

Infection degree

Fig. 1 shows the recovery of viable fungal elements from the lungs and adrenal glands. HIV-A mice presented significantly higher numbers of viable fungi from the lungs than LIV-A mice at the 4th and 8th weeks after infection. The number of viable fungi in adrenal glands was significantly higher in HIV-A lines than LIV-A, and this difference was observed at the 2nd, 4th, 8th and 10th weeks.

Determination of cytokines

The profile of IL-4 production in lungs and adrenal glands is presented in Fig. 2. In lungs, its production in both animal lines varied as a consequence of infection when compared to control animals, showing a significant decrease after 2, 6 and 8 weeks. Comparing HIV-A and LIV-A mice at each time interval during infection, a significant difference was observed only at the 2nd and 8th weeks when LIV-A mice produced lower levels of IL-4 than control animals, but produced higher levels than HIV-A. Adrenal glands of both strains demonstrated lower IL-4 production during infection when compared to the respective controls animals at the 2nd, 4th, 6th and 8th weeks. Between HIV-A and LIV-A lines, a statistical difference was observed at the 6th week of infection, when HIV-A mice produced more IL-4 than LIV-A.

Concerning the IL-10 levels shown in Fig. 3, lungs of the two lines demonstrated a statistical difference when compared to controls animals at the 2nd, 4th, 6th and 8th week. Between HIV-A and LIV-A lines, a statistical difference was observed at the 6th week of infection, when HIV-A mice produced more IL-10 than LIV-A. Adrenal glands presented suppression of IL-10 production in both lines after 2 weeks of infection in relation to respective controls and an increase at the 4th and 6th weeks, but these differences were not statistical significant. At the two final weeks of infection (8th and 10th), suppression of IL-10 was again noted in HIV-A and LIV-A mice as compared to control animals, with LIV-A mice presenting a
A statistically significant difference was observed in the lungs at the 10th week. Data from IFN-γ production in lungs and adrenal glands are shown in Fig. 4. No differences in lungs were found between H IV-A and L IV-A mice in each period post inoculation. However, H IV-A did show significant differences with respect to their controls at the 2nd and 8th weeks of infection, while L IV-A presented similar difference at the 2nd, 4th, 8th and 10th week of infection.

Comparing H IV-A and L IV-A with regard to IFN-γ production in adrenal glands, a significant difference was detected only at the 2nd week. H IV-A mice compared with their control group presented a significant difference in IFN-γ production at the 2nd, 8th and 10th weeks, while L IV-A showed a statistically significant difference with its control group at the 2nd, 4th, 8th and 10th weeks post-inoculation.

Fig. 5 shows that lung levels of TNF-α in L IV-A mice were statistically higher than H IV-A at the 2nd, 4th, 6th and 8th weeks of infection. Analyzing the TNF-α production in relation to control, H IV-A mice showed a significant inhibition at the 2nd week, while L IV-A presented an increase at week 6.

In agreement with TNF-α levels in adrenal glands, a comparison between H IV-A and L IV-A mice in each period showed a significant difference at the 2nd, 4th and 6th weeks (L IV-A > H IV-A). This difference was also observed among control animals. Comparing H IV-A mice with their respective controls, a decrease was observed only at the 2nd and 6th weeks of infection, while L IV-A mice did not differ from the control.

**Discussion**

This work investigated the production of Th1 (IFN-γ and TNF-α) and Th2 (IL-4 and IL-10) cytokines in lungs and adrenal glands of H IV-A and L IV-A mice experimentally infected with *Paracoccidioides brasiliensis* in order to obtain a better understanding of the high involvement of these organs, especially the adrenal glands, in an experimental model of PCM.
In a previous study using this same experimental model, Soares et al. [10] showed that H IV-A mice were more susceptible to Paracoccidioides brasiliensis infection due to the extensive involvement of the adrenal glands, as seen by the numerous lesions caused by the fungus. The authors also detected increased levels of corticosterone in this line of mice. Other work with experimental models have shown adrenal-gland involvement in infections caused by mycobacteria, parasites and fungi were related to the impairment of the local cellular immune response [20].

Our results indicated that the H IV-A mice strain was more susceptible to infection than L IV-A mice probably due to greater involvement of the adrenal gland, with high levels of recoverable fungus and lower Th1 cytokines.

With respect to Th2 cytokines, IL-4 induces antibody production, inhibits Th1 cytokine and stimulates the
expression of histocompatibility complex molecules [21]. It has been associated with the response to parasitic infections and both allergic and chronic inflammations. However, its involvement in the host defense against bacterial and fungal infections has not been explained [22].

Calich et al., analyzing the in vivo depletion of IL-4, did not observe an increased recovery of *P. brasiliensis* in studied organs after intratracheal (i.t.) inoculation. The IL-4 depletion caused an increase of colonization in lungs [23], confirming our findings relative to low IL-4 production.

Among the biological properties of IL-10 is the ability to block cytokine synthesis, mainly IFN-γ, by Th1 cells. This suppression occurs indirectly, since it inhibits the activity of antigen-presenting cells [24].
Another indirect form of suppression is through cytokine inhibition caused by activated macrophages. Some of the cytokines secreted by these cells and inhibited by IL-10 are: TNF-α, IL-1, IL-6, IL-8, GM-CSF, IL-12 and IL-10 [25].

As shown in Figs. 3 and 4, we were able in our animal model to reproduce the biological effect of IL-10 in suppressing IFN-γ production, mainly in lungs at the 6th week after inoculation.

The association of high IFN-γ production with resistance to the fungus was demonstrated in the two different mice lines during the course of experimental infections [26]. The importance of IFN-γ in the control of PCM was shown in sensitive and resistant (B10/A and A/Sn respectively) lines i.t. inoculated with *Paracoccidioides brasiliensis*. Evaluating the pulmonary infection by the fungus at 4 and 8 weeks, the authors demonstrated a mixed pattern of Th1 and Th2 cytokine secretion in
both lineages, and still detected higher levels of IFN-γ, IL-4, IL-5 and IL-10 in susceptible mice [26]. A/Sn animals presented a restricted pulmonary infection, low mortality, persistent delayed-type hypersensitivity (DTH) reaction and bronchoalveolar macrophage activation [27], indicating that the lower levels of Th2 cytokines at the infection site resulted in a prevalent effect of IFN-γ. On the other hand, the susceptible mice presented higher levels of pulmonary IFN-γ conferring local protection to control fungal growth, but the concomitant presence of Th2 cytokines suppressed macrophage activation and DTH responses, resulting in dissemination of yeast cells to extra pulmonary sites. So, the authors concluded that, independent of the lineage, IFN-γ plays a fundamental role in resistance to *P. brasiliensis* infection [26].

Other works demonstrated that the treatment of murine pulmonary or peritoneal macrophages with
IFN-γ potentiated the anti-fungal activity of these cells [28,29], with a clear relationship between activation of macrophages, verified by hydrogen peroxide release, and the resistance to fungal challenge [27]. These data suggest that IFN-γ has a fundamental role in macrophage activation and inhibition of P. brasiliensis multiplication.

The continuous macrophage activation process by these cytokines and fungal components [30], leads to TNF-α production that autocrinally increases the fungicidal activity of these cells. TNF-α induces accumulation and differentiation of macrophages in epithelioid cells, with persistence and well-defined granulomas and consequent inhibition of fungal replication [31]. However, TNF-α has other roles besides macrophage activation, which are considered to be important in the resistance of the animals to the fungus, so that one cannot assure that the high concentration of this cytokine means protection against the disease. Thus, the prevalence of this in vivo protective mechanism depends on all activities exerted by TNF-α [32].

Therefore, we can suggest, on the basis of histopathological analysis of Soares et al. [10] that the resistance of the LIV-A line occurred due to greater TNF-α production. Thus, the low-level compromise of the adrenal gland in this lineage with the medullar region conserved, and well-defined granulomas indicating control of the infectious process, would be due to the larger presence of this cytokine in these structures as confirmed in the present work.

Important differences were detected in both lineages, mainly in relation to adrenal gland fungal recovery, where the LIV-A lineage presented total elimination of the fungus from the 8th week of infection when compared to the HIV-A lineage, indicating greatest compromise of this system in this experimental model.

The production of type 2 cytokines such as IL-4 and IL-10 are not involved in control of the infectious process, and the data confirm the important role of IFN-γ in the control of fungal multiplication in the analyzed organs in both lineages.

Other studies report the importance of TNF-α in the resistance to P. brasiliensis, in experimental studies involving knockout mice for IFN-γ and for p55 TNF-α receptor.

The animals without IFN-γ infected with virulent fungus strain presented 100% mortality after 16 days of infection with dissemination of yeast cells in the liver, spleen and lungs. These data suggest that mice without the functional gene for IFN-γ were unable to contain and control the infection, in spite of the intense inflammatory reaction and formation of incipient granulomas associated with a large amount of yeast. Besides, these data showed that TNF-α, acting through p55 receptor is also important in containing the growth and dissemination of the fungus and contribute to inflammatory response. The infection of knockout p55 TNF-α receptor mice also resulted in propagation of fungus and absence of granuloma formation [33]. These findings agree with recent studies, which show that TNF-α activities by p55 receptor are important for granuloma formation in response to bacteria and protozoa [34,35].

Concerning TNF-α involvement, our results indicate its role in the PCM resistance mechanism. The higher TNF-α amount presented by the LIV-A mice may be associated with the higher resistance of this lineage to infection, and the greater involvement of the adrenal gland HIV-A mice may be related to the lower production of this cytokine.

Again, this experimental model was shown to be important and original to the study of the involvement of adrenal glands in experimental PCM, since these glands are frequently attacked in human disease.

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