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Cytokines Expressed in the Granulomatous Lesions in Experimental Paracoccidioidomycosis: Role in Host Protective Immunity and as Fungal Virulence Factor

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Abstract

Paracoccidioidomycosis (PCM) is a systemic granulomatous disease caused by the fungus *Paracoccidioides brasiliensis* (Pb). In the murine model of PCM, susceptible (S) mice develop disseminated disease with loose granulomas containing several viable fungi whereas resistant (R) mice show low fungal dissemination and encapsulated granulomas with few numbers of degenerated fungal cells. Here, we report the results of the expression of mRNA of these cytokines, as well as their distribution in the paracoccidioidomycotic granulomatous lesions and a semi quantitative score, that was correlated with the histological and biological data. Overall, our data show that the total area of granulomatous lesions and the relative areas of lesions containing Pb were, respectively, 1.2x and 1.9x more extensive in S than in the R mice. Also, the expression of IFN-γ and TNF-α mRNA was, respectively, 8x and 11x higher R mice and immunohistochemistry showed that the number of IFN-γ cells was 2.5x higher in R than in S mice. However, TNF-positivity was similar in the granulomas from S and R mice. In contrast, TGF-β mRNAs was 1.2x more expressed in S mice and this inhibitory cytokine was detected in higher concentration in the omental tissue from S mice.

We hypothesize that the infection of R mice by Pb leads to the preferential synthesis of TNF-α and IFN-γ that promote macrophage activation, probably enhancing Pb killing and control of fungal dissemination, in parallel with the development of compact granulomatous lesions containing few fungi. On the other hand, the infection of S mice elicits preferential synthesis of TGF-β that deactivates macrophages and may inhibit Pb killing by macrophages, favoring fungal dissemination and formation of loose granulomatous lesions. The positivity to TGF-β in Pb yeast cells may consist in a virulence factor of Pb, inducing the suppressive milieu that favors fungal dissemination.

Keywords: Pro- and anti-inflammatory cytokines; Paracoccidioidomycosis; Granulomatous lesions; *P. brasiliensis;* Virulence factor

Introduction

Paracoccidioidomycosis (PCM) is a systemic granulomatous disease, prevalent in Latin American countries, and whose causative agent is the thermally dimorphic fungus *Paracoccidioides brasiliensis* (Pb). PCM manifests in different clinical forms and may present as acute or chronic disease [1]. In the experimental murine model of PCM, previously described by our group [2], we demonstrated differences in susceptibility among inbred strains. Two strains of mice were shown to develop polar forms of the disease, A/Sn mice were resistant and B10.A mice susceptible to *Paracoccidioides brasiliensis* (Pb) infection [3].

The susceptible B10.A strain mice presented high fungal dissemination, as measured by the number of viable fungi found in the organs leading to high mortality. There was the formation of loose and disseminated granulomas, presenting innumerous multinucleated giant cells (MGCs), intense deposits of extracellular matrix (ECM) and numerous viable yeast cells, indicating progression and ineffective control of the disease. There was also observed low activation state of phagocytes, high levels of specific antibody, suppressed delayed type hypersensitivity (DTH) and lymphoproliferative responses and a preferential Th2 cytokine production patterns, characterizing the anergic pole of the experimental disease [4].

On the other hand, resistant mice of the A/J and A/Sn strains showed low fungal dissemination (CFU) and low mortality. The lesions were constituted by active compact and encapsulated granulomas with intense deposits of ECM, intense cellular infiltrate, few number of mostly degenerated yeast cells that evolved in the course of the infection to residual lesions with pseudoxantomatous macrophages containing fungal debris, indicating effective control of the disease. These mice also showed high activation state of phagocytes, synthesized low levels of specific anti-Pb antibodies, with preferential production of IgG2a and IgG3 isotypes and had preserved DTH and lymphoproliferative responses and produced preferentially Th1 cytokine, constituting the hiperergic pole of experimental paracoccidiodomycosis [5,6].

The role of cytokines in human and experimental paracoccidioidomycosis has been extensively studied; but the participation of cytokines at the lesions, which are the actual site of interaction between the *P. brasiliensis* and the immune system, has been less studied. Some cytokines including tumor necrosis factor-

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alpha (TNF-α), interferon-gamma (IFN-γ) and transforming growth factor- beta (TGF-β) play an important *in situ* role in the granulomas, and the participation of these cytokines in granuloma formation has been found to be involved in several infectious diseases [7].

IFN-γ, the main Th1 cytokine, has a major role in protective immunity in fungal infections; the main mechanisms are activation of macrophage activation leading to fungicidal activity, associated with the presence of TNF-α. The important role of IFN-γ in host resistance to infection with *P. brasiliensis* is well established, except for its participation in fungal granulomas [4,8]. At the site of lesions, IFN-γ is anti-fibrotic and was found in paracoccidioidal granulomas in patients [9] and in experimental models [10].

TNF-α is a typically pro-inflammatory cytokine with important protective function in infectious diseases (e.g., tuberculosis, PCM), and also with an important role as a granulomatogenic mediator at the site of lesions. TNF-α has an important role in the onset of granuloma formation [11], exerting an inhibitory effect on the gene and protein synthesis of collagen [12]. Furthermore, TNF-α deficient mice had uncontrolled granuloma formation [13].

TGF-β is a potent immunosuppressive cytokine, produced by lymphocytes, macrophages, and dendritic cells, which modulates excessive immune responses and immunopathology in infectious diseases, as well as in allergy and autoimmunity. TGF-β has a central role in fibrosis, acting in concert with chemokines, cytokines, reactive oxygen species and other factors. Its production has been linked with the development of fibrosis in several diseases [14]. Although various cell types produce and respond to TGF-β, circulating monocytes and tissue macrophages are the main cellular source [15].

The *in situ* expression of cytokines in paracoccidioidal granulomas has been poorly investigated. In view of these facts, we studied the numerous granulomatous lesions developed by susceptible and resistant mice to *P. brasiliensis* infection. Resistant mice inoculated with a highly virulent *P. brasiliensis* isolate present efficient macrophage activation, presence of delayed type hypersensibility (DTH) response, low levels of specific antibodies and a tendency to resolution of the infectious process, suggesting that a T helper-1 mode of immune response is mounted. Susceptible mice, on the contrary, seem to mount a predominantly T helper-2 type of immune response activation in which an inefficient macrophage activation, depressed DTH reactions (anergy) and high levels of antibodies result in progressive disease [16].

We compared the granulomas architecture, areas of lesions comprising *P. brasiliensis* with preserved or altered morphology, the presence and distribution of different cell populations and relevant cytokines to granuloma formation equilibrium maintenance. In this way, we proposed to study the participation of IFN-γ, TNF-α and TGF-β in the host-fungal interactions at the intimate site of the lesions by determining the expression of their mRNAs and their localization, as well as their concentration in culture supernatant of primary tissues, comparing this data with the architecture of the lesions developed in the omentum of resistant (R) and susceptible (S) mice to detect eventual differences due to the genetic background of the mice.

Materials and Methods

Animals

Specific pathogen-free 8 week-old female A/J and B10.A (resistant and susceptible mouse strain to PCM, respectively) mice were purchased from the Department of Immunology; Biomedical Sciences Institute of University of São Paulo animal facility and housed in micro isolator cages with free access to water and food. All protocols were carried out in accordance with the guidelines of the University of São Paulo Ethic Committee on the Use and Care of Animals.

Infection of mice

The highly virulent isolate Pb18 of *P. brasiliensis* was used throughout the experiments. Fungal cells were grown at 36°C in Fava Netto's medium and used on day 7 of growth. Briefly, *Paracoccidioides brasiliensis* cells were collected and the fungal mass was suspended in phosphate-buffered saline (PBS), mixed twice for 10s on a Vortex-mixer, centrifuged and double-washed in PBS. Concentration was adjusted to 107 cells/ml and viability of fungal suspensions, determined by staining with Janus Green B (SIGMA, St. Louis, MO, USA), was always higher than 90%. Mice were inoculated intraperitoneally with 5×10^6 yeasts of *P. brasiliensis* in 500 µl of PBS or with PBS alone (control group) and killed at 120 days after the inoculation.

Collection of tissue samples

At 120 days after Pb18 infection, mice were sacrificed and the peripancreatic omentum was removed and collected to RT-PCR analysis and also to fixation in Methacarn solution and later embedded in paraffin for histopathological and immunohistochemical analysis.

Preparation of organs for histological procedures

The omentum (which was described as the target organ in the murine model of *P. brasiliensis* infection [6]) of the animals were stretched on cardboard, to ensure a more homogeneous tissue and fixed in Methacarn solution (60% methanol, 30% chloroform and 10% acetic acid) at 4°C for approximately 4 hours under stirring. The fixed materials were placed in absolute alcohol for 24 hours, subjected to three passages in alcohol for 30 minutes each, followed by passage through xylene (three times 15 minutes each), embedded in paraffin (three times 15 minutes each) and finally, mounted on blocks for subsequent completion of sections of 5 µm thickness for histological evaluation.

Histopathological analysis

The omentum of each mouse were analyzed using the following histological staining: hematoxylin and eosin (HE) to observe the overall architecture and also the cellular components present in the granulomatous lesions, and methenamine silver impregnation to identify the presence and morphology of fungal cells considering its morphology and presence of budding [17]. Both staining were visualized by under a light microscope (NIKON, Japan) coupled to a digital camera (DS-FI1, NIKON Co., Japan) and by 8 image capture and analysis program NIS-Br version 2.34 (NIKON, Japan) with total magnification of x40, x100, x250 and x400.

Area of granulomatous lesions

A quantitative analysis was made to calculate the total area of the lesions and also the area of lesions containing *P. brasiliensis* yeast cells. We employed a reticulated ocular lens (total 100 grids) with total magnification of x125, resulting in the counting of fields that corresponded to an area of 1120 mm^2. All the results were expressed as mean of semi quantitative analysis of 10 fields (μm^2).

Colony-forming units assay

The counting of colony forming units (CFU) was performed to quantify the number of viable *P. brasiliensis* yeast cells in the organs

of infected mice. Spleen, liver, omentum and lungs were collected and homogenized in 5 mL of sterile PBS using a tissue grinder. The homogenate was centrifuged at 3000 rpm for 5 minutes. The supernatants were collected and stored at -80°C. The pellet was resuspended in 1 ml of PBS and 0.1 ml was seeded in brain-heart infusion (BHI) agar plates according to the protocol described previously [18]. Plates were incubated at 37°C and colony growth was accompanied by a period of between 13 and 15 days, with results expressed as the number of log CFU per gram of tissue.

Cytokine gene expression

Omentum RNA was extracted from tissues using the Illustra RNAspin Mini (GE Health care, Amersham, UK). After RNA quantification and analysis of RNA integrity on a 1.5% agarose gel, reverse transcription was performed with approximately 2 µg of RNA using the High Capacity cDNA Reverse Transcription Kit (APPLIED BIOSYSTEMS, Foster City, CA, USA) according to the manufacturer's instructions. The cDNA was quantified in Nanodrop by determining the absorbance at 260 nm, and the 260/280 nm absorbance ratio was calculated. Quantitative (q)RT-PCR was performed with an ABI Prism 7300 device (APPLIED BIOSYSTEMS, Foster City, CA, USA) and the reactions were carried out in 25 µL volume and in the presence of the TaqMan PCR Master Mix™ (APPLIED BIOSYSTEMS, Foster City, CA, USA), using different sets of specific oligonucleotides and probes for the amplification of the RNA 18S (Hs99999901_s1), IL-10 (Mm00439614_m1), TGF-β (Mm01178820_m1), TNF-α (Mm00443260_g1), and IFN-γ (Mm01168134_m1) genes. The relative quantification of target genes was determined by using the ΔΔCT method [19]. Expression levels of cytokine genes are represented as a ratio to RNA 18S gene controls.

Immunohistochemical analysis for cytokines

Tissue sections with five- m thickness were used for immunohistochemical analysis of TNF-α, TGF-β and IFN-γ expression. Briefly, slides sections were deparaffinized and rehydrated, then incubated with hydrogen peroxide (30%) for 30 min at room temperature in a shaker, for blocking endogenous peroxidase. After that, blocking of nonspecific sites with normal serum/bovine serum albumin 10% (1:1 dilution) was carried out for 30 min at room temperature and followed by application of Tris-buffered saline (TBS) for blocking endogenous biotin in tissues for 2 h. Slides were incubated with adequate volume of rabbit pan-specific polyclonal antibody anti-TNF-α or anti-TGF-β (1/30) (R&D SYSTEMS, Inc., Minneapolis, MN, USA) and monoclonal antibody anti-IFN-γ (according to the protocol described previously by [20-22], overnight at 4°C, diluted in PBS/Tween 20 0.3%. After incubation with primary biotinylated anti-IgG antibody (1/1000) (Rockland, Gilbertsville, PA, USA) was applied on tissues for 1 h at room temperature, followed by incubation with streptavidin-peroxidase (1/50) (Vector Laboratories, Burlingame, CA, USA). Color development was achieved using a 0.05% H2O2 and 3,3' diaminobenzidine tetrahydrocloride (DAB, SIGMA-ALDRICH, St. Louis, MO, USA) solution and reaction was stopped after visualization of brown color on tissue sections. Sections were then counterstained with Mayer's Hematoxylin and examined using a light microscope (HUND WETZLAR H500, Germany). Image capture was carried out by using a microscope coupled to a video camera (KODO, Tokyo, Japan) and Microsoft Video Capture software for Windows. Control slides were made with specimens of uninfected mice and without primary antibody replaced by diluent (PBS - 0.3% Tween 20) and were negative for any reactivity.

Quantitative analysis of immunohistochemical reaction

The quantitation of TNF-α , IFN-γ and TGF- β in the lesions was done by using a reticulated eyepiece (X12.5) with square grid and a x40 objective (total magnification: x500, total area=280 μm^2). The number of positive cells was counted in 10 fields randomly chosen for each tissue slides (3 mice/group) and the results were expressed as mean ± SEM of IFN-γ-positive cells / μm^2. We also determined the percentage of weakly and strongly IFN-γ-positive cells. The quantitation of TNF-α and TGF-β in the lesions was done by the sum of the positive areas at the lesions observed at 3-5 selected fields, captured from microscopic images using a video camera (KODO, Tokyo, Japan) and the Video Capture for Windows program. The quantitation was expressed by the mean of the positive areas in square pixels (2-3 mice per group). The images selected for quantitation were always observed at 400x.

Statistical analysis

Results are presented as the means ± SE. Statistical evaluation of the results comparing resistant (A/J) and susceptible (B10.A) strains was performed with Student´s *t* -test and values of $p<0.05$ were defined as significant.

Results

Analysis of granulomatous lesions

Figure 1 shows that resistant mice (A/J) had higher capacity to control the infection, in contrast to progressive increase in the severity of the lesions in susceptible mice (B10.A), associated to fungal dissemination. Also, the total areas of lesions as well as the percentage of these lesions occupied by Pb were significantly higher in susceptible than in resistant mice, in parallel with the higher viable fungal loads in the former strain. This was in parallel with higher numbers of viable

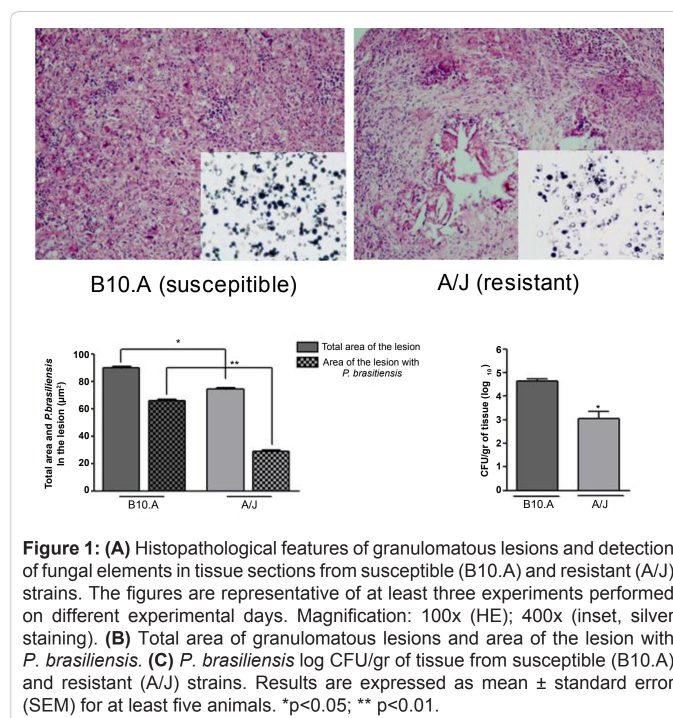

Figure 1: (A) Histopathological features of granulomatous lesions and detection of fungal elements in tissue sections from susceptible (B10.A) and resistant (A/J) strains. The figures are representative of at least three experiments performed on different experimental days. Magnification: 100x (HE); 400x (inset, silver staining). **(B)** Total area of granulomatous lesions and area of the lesion with *P. brasiliensis.* **(C)** *P. brasiliensis* log CFU/gr of tissue from susceptible (B10.A) and resistant (A/J) strains. Results are expressed as mean ± standard error (SEM) for at least five animals. *p<0.05; ** p<0.01.

P. brasiliensis recovered from the omentum of the susceptible mice, as compared to the resistant A/J mice.

Differential gene expression in susceptible and resistant strain

Comparing resistant mice (A/J) and susceptible mice (B10.A), we found that resistant mice showed significantly enhanced gene expression of the inflammatory cytokines IFN-γ and TNF-α (~34-fold and ~9.5-fold, respectively). In contrast to this enhanced expression of inflammatory genes in resistant mice, susceptible mice showed no up regulation of these genes (Figures 2a and 2b). However, the gene expression of the anti-inflammatory cytokine TGF-β was significantly increased in susceptible mice as compared to resistant mice (~28-fold) (Figure 2c).

Immunohistochemistry

At 120 days post infection, susceptible (B10.A) mice showed disseminated loose lesions with IFN-γ stained cells circumscribing granulomatous foci. In resistant (A/J) mice, intense positivity was detected in mononuclear cells forming several aggregates surrounding central necrosis and compact granulomatous lesions (Figure 3a). At this later phase of infection, the lesions developed by both mouse strains showed marked extracellular matrix deposition, but with weak immunostaining for IFN-γ (data not shown). IFN-γ expression increases in the lesions developed in both susceptible and resistant mice throughout the infection, but more markedly in the lesions of resistant mice, which show a 4-fold increase of positive cells (data not shown). IFN- γ positive cells found in the lesions of resistant mice were 2.6 fold that of positive cells for this cytokine in the susceptible mice (Figure 3b). This increased number of IFN-γ positive cells, mostly in the resistant mice points towards an intense tissue degradation and remodeling, and the development of a highly activated state (marked inflammatory response), leading to the control of the fungal dissemination.

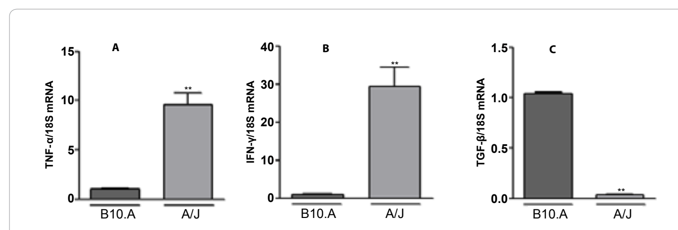

Figure 2: Gene expression, quantified by real-time quantitative RT-PCR, of TNF-α (A) TNF-α (B) and TGF-β (C) in susceptible (B10.A) and resistant (A/J) strains. Results are expressed as mean ± standard error (SEM) for at least five animals. ** p<0.01.

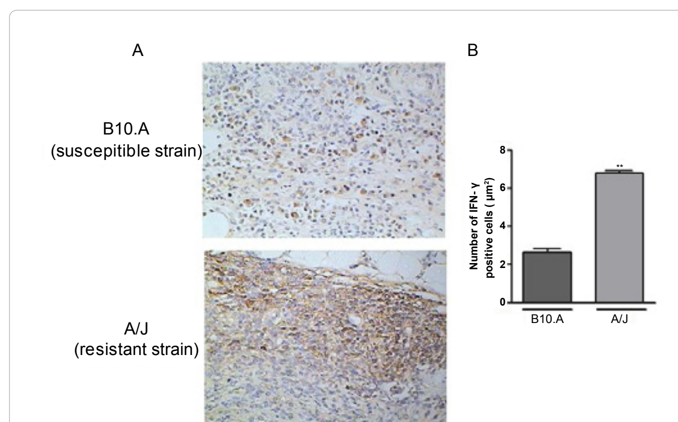

Figure 3: (A) Expression of IFN-γ, detected by immunohistochemichal analysis, in the granulomatous lesions of omentum of susceptible (B10.A) and resistant (A/J) mice, 120 days after ip infection with the virulent Pb18 *P. brasiliensis* isolate. The figures are representative of at least three experiments performed on different experimental days. Magnification: 400x. **(B)** The graphic corresponds to the quantitative analysis of selected microscopic fields of lesions from susceptible (B10.A) and resistant (A/J) mice in terms of IFN-γ positive cells and represents the mean ± SEM for at least five animals. ** p<0.01.

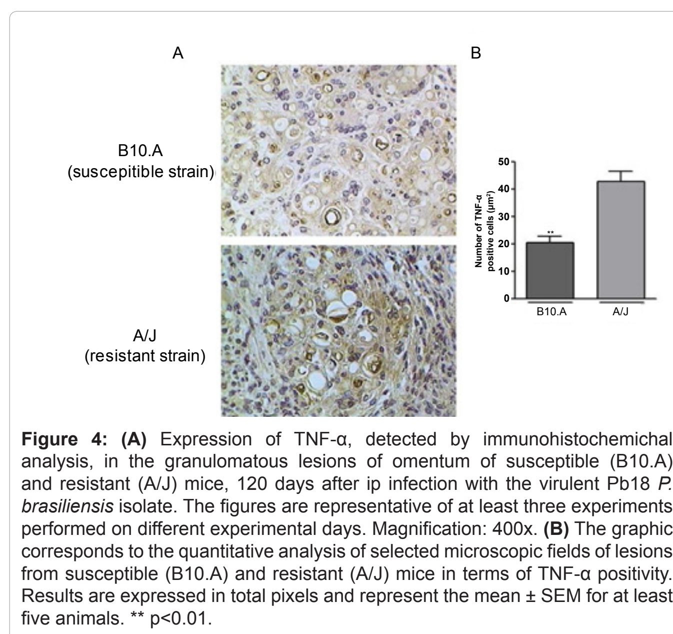

Figure 4: (A) Expression of TNF-α, detected by immunohistochemichal analysis, in the granulomatous lesions of omentum of susceptible (B10.A) and resistant (A/J) mice, 120 days after ip infection with the virulent Pb18 *P. brasiliensis* isolate. The figures are representative of at least three experiments performed on different experimental days. Magnification: 400x. **(B)** The graphic corresponds to the quantitative analysis of selected microscopic fields of lesions from susceptible (B10.A) and resistant (A/J) mice in terms of TNF-α positivity. Results are expressed in total pixels and represent the mean ± SEM for at least five animals. ** p<0.01.

TNF-α was found to be expressed in the extracellular matrix, with intense localization in mononuclear cells and in multinucleated giant cells present in granulomas of both susceptible and resistant strains (Figure 4a).

Figure 4b shows that TNF-α positive cells in resistant mice (A/J) were 2.1 fold that of positive cells for this cytokine in the susceptible mice (B10.A). This higher number of TNF-α positive cells in the resistant mice suggests that the cells present at the site of lesions are in a highly activated state, which could be responsible for the control of Pb yeast cells dissemination.

TGF-β was present in the omentum on cellular components, such as macrophages, multinucleated giant cells, lymphocytes and fibroblasts, however, absent on neutrophils in granulomatous lesions presented in both resistant and susceptible mice. It was also detected in areas of fibrosis and necrosis, as well as scattered on amorphous extracellular matrix, mostly in resistant mice that developed residual lesions containing TGF-β-positive pseudoxantomatous macrophages.

Interestingly, TGF-β positivity was observed in many Pb yeast present at the lesions in the susceptible mice whereas no positivity was observed in Pb yeast cells present at the lesions in the resistant mice (Figure 5a). Also, the number of TGF-β positive cells in susceptible mice was about 2.5 times that of positive cells for this cytokine in the resistant mice (Figure 5b). These results suggest that TGF-β is critical in increasing the fungal growth and dissemination in experimental murine paracoccidioidomycosis.

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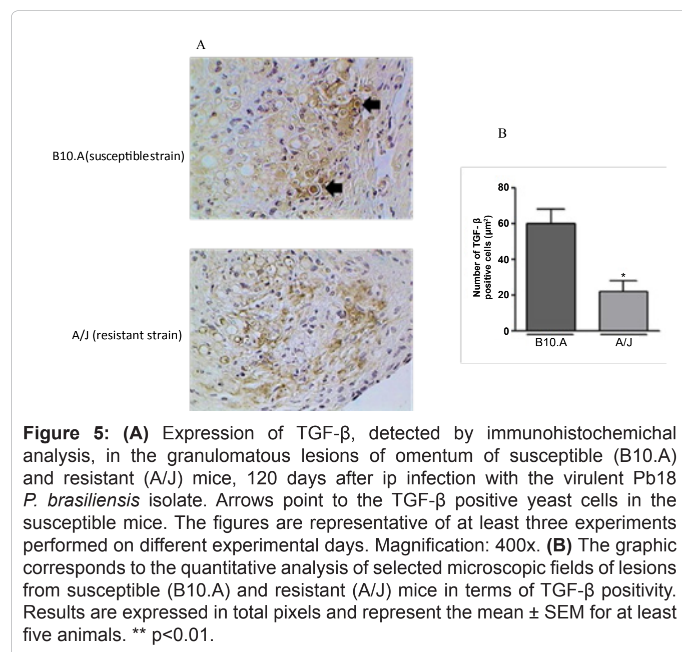

Figure 5: (A) Expression of TGF-β, detected by immunohistochemichal analysis, in the granulomatous lesions of omentum of susceptible (B10.A) and resistant (A/J) mice, 120 days after ip infection with the virulent Pb18 *P. brasiliensis* isolate. Arrows point to the TGF-β positive yeast cells in the susceptible mice. The figures are representative of at least three experiments performed on different experimental days. Magnification: 400x. **(B)** The graphic corresponds to the quantitative analysis of selected microscopic fields of lesions from susceptible (B10.A) and resistant (A/J) mice in terms of TGF-β positivity. Results are expressed in total pixels and represent the mean ± SEM for at least five animals. ** p<0.01.

Discussion

The present work aimed to analyze the overall *in situ* participation of three cytokines (TGF-β, TNF-α and IFN-γ), in attempt to infer the consequences of their cooperative and antagonistic effects on the outcome of experimental paracoccidioidomycosis, using as a measure of control or progression of the disease the morphology of the granulomatous lesions, and the presence of *P. brasiliensis* with preserved or altered morphology. We also correlate these findings with the expression of mRNA for these cytokines in granulomatous lesions, trying to assess parallelism between mRNA and protein synthesis. This is the first study showing the *in situ* detection of TGF-β, TNF-α and IFN-γ in experimental paracoccidioidomycosis. The concomitant presence of these cytokines and the possibility of their interactions and their effects over both *P. brasiliensis* and the cell populations present at the site of lesions open many possibilities for their local importance.

The frequent involvement of the omentum/pancreas in murine PCM has been reported and the omentum has been characterized as a main target organ in intraperitoneal infection [6]. As shown before, the overall morphology of the lesions was different in the B10.A and in the A/J mice, which developed, respectively, loose and compact granulomas [6]. In the granulomatous lesions observed in experimental murine model of paracoccidioidomycotic lesions, cytokines could influence these different morphological aspects of the granulomas, as well as the evolution or the control of PCM infection, by activating or deactivating the cells present or even acting directly on the fungi.

In cutaneous leishmaniasis, TGF-β expression is more marked in long lasting lesions compared with acute lesions, suggesting that this cytokine plays an important role in the development and chronicity of that disease [23,24]. Mouse and human macrophages infected with different *Leishmania* species actively produce TGF-β, with the amounts correlating with both the strain virulence and the multiplication of parasites within the macrophages, so TGF-β secretion is considered as a virulence factor for *Leishmania* parasite [25].

Lopera et al. [26] studied the lungs of control and Pb-infected mice at different time post-infection with *P. brasiliensis* in order to define histopathological patterns of pulmonary lesions, multiplex-cytokine profiles and their dynamics during the course of this mycosis and observed different patterns of pulmonary lesions according to time post-infection, as well as different cytokine patterns: an early increase in some cytokines including IFN-γ and TNF-α, followed by down regulation of various lung cytokines, again including IFN-γ and TNF-α.

In previous studies on the murine model of PCM developed by our group, IFN-γ-positive cells with lymphocyte morphology were localized mainly at the periphery of granulomas in both mouse strains; a significant increase in positive cells was found in compact granulomas of A/J mice at 120 days compared to 15 days post infection and a significantly higher number of positive cells was detected in compact granulomas of resistant mice than in loose and multifocal granulomas of susceptible at 120 days post infection [22]. Also, TNF-α (unpublished data) and TGF-β [21] immunostaining was detected in macrophages and multinucleated giant cells, as well as in extracellular matrix components. Our present results confirm that all three cytokines (TNF-α, IFN-γ and TGF-β) were found *in situ* in the granulomatous lesions of both, resistant (A/J) and susceptible mice (B10.A).

Mononuclear cells and multinucleated giant cells were positive for both, TNF-α and TGF-β, but the only IFN-γ-positive cells were lymphocytes, localized at the periphery of the lesions.

This pattern may suggest strongly that during *P. brasiliensis* infection, TNF-α, TGF-β and IFN-γ are simultaneously synthesized by different cell populations (mononuclear cells and multinucleated giant cells secreting TNF-α and TGF-β, and lymphocytes, localized at the periphery of the lesions producing IFN-γ) both in susceptible and in resistant mice. However, the presence of IFN-γ-secreting lymphocytes and TNF-α- secreting cells was significantly higher in the resistant mice than in the susceptible ones, and, on the other hand, TGF-β positivity was significantly higher in the susceptible mice than in the resistant ones, both at the cells and diffuse in the extracellular matrix. The expression of mRNA of all three cytokines was totally parallel with the actual production of these cytokines.

TNF-α and IFN-γ act synergistically on NOS2 (inducible nitric oxide synthase) expression and macrophage activation, and consequently on the subsequent induction of fungal killing. Taking this in account, it is interesting to note that the mRNA of these two cytokines are much more expressed in resistant mice than in the susceptible ones, as is the number of IFN- γ -positive cells at the lesions, suggesting that the local synthesis of IFN-γ exerts a protective role at granulomatous lesions.

Some cytokines play important *in situ* roles in the granuloma. The tumor necrosis factor (TNF-α) has an important role in the onset of granuloma formation, exerting an inhibitory effect on the gene and protein synthesis of collagen; furthermore, another study showed that TNF-α deficient mice had uncontrolled granuloma formation. On the other hand, IFN-γ, much more than its important role in the defense mechanisms against various pathogens, has anti-fibrotic action through the inhibition of collagen synthesis and increased degradation of collagen type I and III. TGF-β exerts pro-fibrotic effects through induction of ECM components and is an inducer of fibrogenesis in various granulomatous infections, by activating fibroblasts, leading to collagen production and promoting the granuloma formation.

In our model, the presence of TGF-β at the granulomatous lesions of susceptible mice may provide a suppressive milieu, favoring fungal persistence and multiplication but also preventing immunopathologic responses. This is suggested by the preferential finding of both parameters, expression of TGF-β mRNA, and TGF-β producing cells

in the granulomas of these mice. On the other hand, as TGF-β is also somewhat expressed in the granulomas of resistant mice, it is suggested that a complex balance involving various cell populations and the cytokines they secrete occurs in this mycosis, depending on the genetic background of the host.

It is well known that beyond the classical Th1 or Th2 cells effector T helper cells, characterized largely by the production of IFN-γ or IL-4, respectively, there is the participation in protection against fungal pathogens of Th17, a third subset of effector cells, characterized by the production of IL-17, and whose development and differentiation involves cytokines including TGF-β and thought to be important for mobilization of immune responses against fungal pathogens [27].

Another cell population that may be present in the paracoccidioidomycotic lesions is Mucosal Associated Invariant T (MAIT) cells that reside preferentially in mucosal tissues and could be present at the pancreatic omentum of these P. brasiliensis-infected mice. It has been reported that these cells appear to function as innate T lymphocytes and may control intracellular bacterial growth at the early stage of infection, when they are a potent source of innate IFN-γ to provide direct control of bacterial growth, whereas their IL-17 production may help to stimulate the adaptive immune response [28]. Although not the scope of our present investigation, we cannot rule out these cell populations as the sources of some of the cytokines present at the lesions adding to the complex interactions herein described.

The unexpected finding of positivity for TGF-β in Pb yeast cells may be due to the synthesis of this cytokine by the fungus. This phenomenon will be investigated in the future, and if confirmed, points towards a very effective virulence factor of Pb, inducing the suppressive milieu earlier which would favor fungal dissemination.

The mRNA expression and the local production of TNF-α, IFN-γ and TGF-β seems to be functional, since the number of viable *P. brasiliensis* is lower in the resistant mice, in which the pro-inflammatory TNF-α, IFN-γ prevail and the CFU counts are higher in the susceptible mice, in which the regulatory anti-inflammatory TGF-β predominates.

In view of our previous and present findings, we can postulate some hypothesis on the participation of the cytokines in experimental paracoccidioidomycosis: the infection of resistant mice by a virulent *P. brasiliensis* isolate leads to the preferential synthesis of mRNAs and of proteins TNF-α and IFN-γ that promote macrophage activation, probably enhancing *P. brasiliensis* killing and control of fungal dissemination, in parallel with development of few compact granulomatous lesions containing few fungi; whereas infection of susceptible mice elicits preferential synthesis of mRNAs and of protein TGF-β which deactivate macrophages and may inhibit *P. brasiliensis* killing by macrophages, favoring fungal dissemination and formation of numerous disseminate granulomatous lesions.

Some discrepancies have been reported comparing the data of IL-10 mRNA and immunohistochemistry. IL-10 mRNA was found in only 70% of biopsies by means of quantitative PCR [28], but IL-10 secreting cells were detected in 100% of the sections studied by immunohistochemistry [29].

Our results of mRNA expression and immunohistochemistry were in accordance when IFN-γ and TGF-β were analyzed; but with TNF-α, the differences observed with the former technique were not found with the latter. It is known that the cytokine TGF-β is synthesized and secreted as a latent form that needs activation to be functional.

Therefore, in our present study if the results of mRNA and immunohistochemistry were different, the protein analysis experiments would be more reliable than gene expression studies [30]. However, as they furnished similar results, we can infer that TGF-β mRNA expression is followed by both, synthesis and activation of this cytokine.

It is well known that T_H1 responses (mediated by IFN-γ and TNF-α) are proinflammatory and drive the immune response to cell-mediated responses, whereas T_H2 responses (mediated by IL-13/TGF-β) skew the response to humoral immune responses and are anti-inflammatory. T_H1/T_H2 responses also have opposite roles in fibrosis and tissue repair: T_H2 responses activate collagen deposition, whereas T_H1 responses inhibit this process. In a persistent infection, the initial response is often a T_H1-dominant, response, mediated by IFN-γ, leading to almost completely attenuated development of tissue fibrosis, followed by a T_H2 response mediated by IL-4, IL-5, IL-13 and TGF-β cytokines which promote repair of host tissues [31].

On the other hand, these phenomena may be associated with the presence of MAIT cells that reside preferentially in mucosal tissues and could be present at the pancreatic omentum of these *P. brasiliensis*-infected mice. It has been reported that these cells appear to function as innate T lymphocytes and may control intracellular bacterial growth at the early stage of infection, when they are a potent source of innate IFN-γ to provide direct control of bacterial growth, whereas their IL-17 production may help to stimulate the adaptive immune response [32].

Our present results confirm this hypothesis, because in resistant mice, which develop preferential T_H1 type responses, IFN-γ and TNF-α are prevalent, whereas in the susceptible mice, which respond with a T_H2 pattern, TGF-β is preferentially produced.

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Disclosure and Conflict of Interests

The authors state they have no conflict of interest.

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