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Kinetics of IFN-γ, TNF-α, IL-10 and IL-4 production by mononuclear cells stimulated with gp43 peptides, in patients cured of paracoccidioidomycosis

Cinética da produção de IFN-γ, TNF-α, IL-10 e IL-4 por células mononucleares, de pacientes curados de paracoccidoidomicose, estimuladas com peptídeos da gp43

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ABSTRACT

We analyzed the kinetics of cytokine production by mononuclear cells from 17 patients who had been treated for paracoccidioidomycosis, using the stimulus of gp43 peptide groups (43kDa glycoprotein of Paracoccidioides brasiliensis*) at 0.1 and 1μM, gp43 (1μg/ml) and crude* Paracoccidioides brasiliensis *antigen (PbAg; 75μg/ml). IFN-γ production was a maximum at 144 hours in relation to the G2 and G8 peptide groups at 1μM and was greatest at 144 hours when stimulated by gp43 and by PbAg. The maximum TNF-α production was at 144 hours for the G2 group (0.1μM) and for gp43. IL-10 production was highest after 48 and 72 hours for G7 and G6 at 1μM, respectively. We also suggest the best time for analysis of IL4 production. These results may contribute towards future studies with gp43 peptides and encourage further investigations with the aim of understanding the influence of these peptides on the production of inflammatory and regulatory cytokines.*

Key-words: *Paracoccidioidomycosis.* Paracoccidioides brasiliensis*. Gp43. Peptides. Cytokines.*

RESUMO

Analisamos a cinética da produção de citocinas de células mononucleares de 17 pacientes com paracoccidioidomicose tratada, usando como estímulo: grupos de peptídeos da gp43 (glicoproteina de 43kDa de Paracoccidioides brasiliensis*) a 0,1 e 1μM, gp43 (1μg/mL) e antígeno bruto de* Paracoccidioides brasiliensis *- AgPb (75μg/mL). A produção de IFN-γ foi máxima em 144 horas frente aos grupos de peptídeos G2 e G8 a 1μM e maior em 144 horas quando estimuladas por gp43 e por AgPb. A produção de TNF-α foi máxima em 144 horas para G2 (0,1μM) e para gp43. A produção de IL-10 foi maior após 48 e 72 horas para G7 e G6 a 1μM, respectivamente. Sugerimos também o melhor período para a análise da produção de IL4. Tais resultados podem contribuir para estudos com peptídeos da gp43, estimulando investigações posteriores visando entender a influência de tais peptídeos na produção de citocinas inflamatórias e regulatórias.*

Palavras-chaves: *Paracoccidioidomicose.* Paracoccidioides brasiliensis*. Gp43. Peptídeos. Citocinas.*

Paracoccidioidomycosis is a type of systemic mycosis caused by a dimorphic fungus, *Paracoccidioides brasiliensis* (*P. brasiliensis*). The host-fungus interaction without disease expression (i.e. in the absence of signs and symptoms) is known as paracoccidioidomycosis infection. Imbalance in this interaction leads to further fungal multiplication and lymphohematogenous dissemination and expression of the disease in children and young adults as the acute form of paracoccidioidomycosis. The chronic form reflects the reactivation of fungal lesions in the lungs or any other organ or tissue many years later in adult life[22].

Evolution from infection to disease in its different clinical forms depends on many factors, such as environmental factors, host immune response and parasite virulence. As cell immunity plays an important role in resistance to this fungal infection, cytokine production has been widely investigated, and valuable

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data to help understand the pathogenesis of the disease has become available[3 12 14 17 21].

In experimental models, the presence of IFN-γ and IL-2 has been related to protection. Furthermore, preferential type 1 (Th1) response has been associated with a resistance phenotype. On the other hand, susceptibility is linked to a type 2 (Th2) response, with production of IL-4, IL-5, IL-10 and TGF-β or production of the regulatory cytokines that are responsible for progressive paracoccidioidomycosis[6].

Individuals with paracoccidioidomycosis infection without symptoms or signs of the disease present high levels of lymphocyte proliferation when stimulated by *P. brasiliensis* antigens, and also high levels of IFN-γ, but have low levels of IL-4, IL-5 and IL-10. Presence of the paracoccidioidomycosis infection is represented by the Th1 pole, and the acute form by the opposite pole, while patients with the chronic form have an intermediate immune response pattern between the Th2 and Th1 responses[17].

Recently, the kinetics of cytokines were analyzed by mRNA expression in individuals with paracoccidioidomycosis infection and in patients with the chronic and acute forms of paracoccidioidomycosis. Early high expression of Th1 cytokines was observed in individuals with paracoccidioidomycosis infection, while in acute-form patients a predominance of Th2 cytokine mRNA was observed. In patients with the chronic form, a mixed pattern of Th1 and Th2 cytokine mRNA was detected. These findings suggest that the differential kinetic patterns and mRNA expression may significantly influence the outcome of paracoccidioidomycosis infection[14].

The aim of this study was, for the first time, to evaluate the kinetics of cytokine production by mononuclear cells from patients who had been treated for paracoccidioidomycosis, using groups of peptides that constitute the gp43 glycoprotein of *P. brasiliensis* as a stimulus.

POPULATION AND METHODS

Sample. Seventeen patients who had been successfully treated for paracoccidioidomycosis were selected at the Systemic Mycosis Outpatient Clinic of the Infectious and Parasitic Diseases Division, Hospital das Clínicas, University of São Paulo School of Medicine. Thirteen patients had chronic multifocal paracoccidioidomycosis, and four had the acute form of the disease.

At the time the patients were included in the study, they fulfilled the following criteria: a) they were clinically cured, with low levels or absence of antibodies, as shown by counterimmunoelectrophoresis (CIE); b) they had a positive paracoccidioidin test result or exhibited a lymphoproliferation response to gp43 or crude *P. brasiliensis* antigen.

The clinical forms were defined according to the classification recommended in the International Colloquium on Paracoccidioidomycosis that was held in Medellín, Colombia, in 1986[10].

The Ethics Committee of Hospital das Clínicas approved the study design, and written informed consent was obtained from all patients. Brazilian Ministry of Health guidelines for human experimentation were followed strictly.

Gp43 peptides, gp43 and *Paracoccidioides brasiliensis* crude antigen. Forty-one gp43 peptides obtained from *P. brasiliensis* isolate B339[16] (Genbank access number AY005437) were synthesized in the Biophysics Department Laboratory, Federal University of São Paulo (UNIFESP). Forty of them contained 15 amino acids, and one contained 16 (Table 1). These peptides were synthesized with a 10-amino acid overlap.

Table 1 - Gp43 peptide sequences.

Peptide	Position	Amino Acid Sequence
P1	(01-15)	MNFSSLNLALASCVL-NH_2
P2	(11-25)	ASCVLAWVCLASASS-NH_2
P3	(21-35)	ASASSHVASHIVPRQ-NH_2
P4	(31-45)	IVPRQAGSAIYGVNI-NH_2
P5	(41-55)	YGVNIGGWLLLEPWI-NH_2
P6	(51-65)	LEPWISPSVFEAGGS-NH_2
P7	(61-75)	EAGGSSSVDEYTLSK-NH_2
P8	(71-85)	YTLSKNLGRDAKRHL-NH_2
P9	(81-95)	AKRHLSKHWDTFITE-NH_2
P10	(91-105)	TFITEDDFKNIAAAG-NH_2
P11	(101-115)	IAAAGLNHVRIPIGY-NH_2
P12	(111-125)	IPIGYWAVNPIEGEP-NH_2
P13	(121-135)	IEGEPYVQGQLDYLD-NH_2
P14	(131-145)	LDYLDKALVWAKNSN-NH_2
P15	(141-155)	AKNSNLRVVIDLHGV-NH_2
P16	(151-165)	DLHGVPGSQNGFDNS-NH_2
P17	(161-175)	GFDNSGHRGAINWQK-NH_2
P18	(171-185)	INWQKGDTIKQTLIA-NH_2
P19	(181-195)	QTLIAIHTLAIRYAN-NH_2
P20	(191-205)	IRYANRTDVVDSIEL-NH_2
P21	(201-215)	DSIELVNKPSIPGGV-NH_2
P22	(211-225)	IPGGVQVSLLKEYYE-NH_2
P23	(221-235)	KEYYEDGYHIVRDID-NH_2
P24	(231-245)	VRDIDSTVGVAISDA-NH_2
P25	(241-255)	AISDASLPPRTWNGF-NH_2
P26	(251-265)	TWNGFLAPKTYKNVY-NH_2
P27	(261-275)	YKNVYLDTYHNQVFD-NH_2
P28	(271-285)	NQVFDDIFRTFTIDQ-NH_2
P29	(281-295)	FTIDQHVKLACSLPH-NH_2
P30	(291-305)	CSLPHDRLRGADKPL-NH_2
P31	(301-315)	ADKPLIVKEWSGAMT-NH_2
P32	(311-325)	SGAMTDCAMYLNGRG-NH_2
P33	(321-335)	LNGRGIGSRFDGSFP-NH_2
P34	(331-345)	DGSFPSGKPSGACGA-NH_2
P35	(341-355)	GACGARSKGSSSELS-NH_2
P36	(351-365)	SSELSAQQKKDTLRY-NH_2
P37	(361-375)	DTLRYIEAQLDAFEV-NH_2
P38	(371-385)	DAFEVAAGWYFWTWK-NH_2
P39	(381-395)	FWTWKTEGAPGWDMQ-NH_2
P40	(391-405)	GWDMQDLLNQKLFPQ-NH_2
P41	(401-416)	KLFPQPIWARKYGGCR-NH_2

Gp43 was kindly provided by Professor Luiz R. Travassos of UNIFESP and was produced in accordance with Puccia et al[20]. *P. brasiliensis* crude antigen (PbAg) was provided by Professor Gil Benard of the University of São Paulo (USP) and was prepared in accordance with Benard et al[1].

Measurement of cytokines from cell-culture supernatants. Peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation (δ = 1,077). Cells were cultivated in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum and 5mM L-glutamine, in triplicate 96-well flat-bottom culture plates (2.5 x 10^5 cells/well, final volume 0.2ml) at 37°C and 5% CO_2 for 48, 72 and 144 hours. The cells were stimulated with 41 gp43 peptides distributed into seven groups containing five peptides each and one group containing six peptides: **G1** (P1, P2, P3, P4 and P5); **G2** (P6, P7, P8, P9 and P10); **G3** (P11, P12, P13, P14 and P15); **G4** (P16, P17, P18, P19 and P20); **G5** (P21, P22, P23, P24 and P25); **G6** (P26, P27, P28, P29 and P30); **G7** (P31, P32, P33, P34 and P35) and **G8** (P36, P37, P38, P39, P40 and P41). The peptides were tested at concentrations of 0.1 and 1μM. *P. brasiliensis* crude antigen (PbAg) (75μg/ml) and purified gp43 (1μg/ml) were also used for stimulating cytokine production.

Culture supernatants were harvested after 48, 72 and 144 hours and stored at -80°C before the assay. The cytokine levels were measured by ELISA. The assay was performed in 96-well plates (Nunc) coated with mice anti-human IFN-γ, TNF-α, IL-10 or IL-4 antibodies (R&D) and blocked with bovine serum albumin (BSA) fraction V (Sigma) in PBS buffer or reagent diluent at pH 7.2. Biotinylated anti-human IFN-γ, TNF-α, IL-10 or IL-4 antibodies were used. The ELISA procedure was performed according to the manufacturer's protocol. The lowest detection limits for IFN-γ, TNF-α, IL-10 and IL-4 were 15,625pg/ml, 7,813pg/ml, 62,500pg/ml and 15,625pg/ml, respectively.

Statistical analysis. Analysis of cytokine secretion after different stimulus durations was performed by analysis of variance for non-parametric data with repeated measurements (Friedman's test) and multiple comparisons with Dunn's test[7]. *P* values under 0.05 were considered significant.

RESULTS

Determination of IFN-γ levels. The production of IFN-γ from the 17 paracoccidioidomycosis patients was higher at 144 hours than at 48 hours for the G2 (p = 0,0213) and G8 (p = 0,0033) gp43-peptide groups at 1μM concentration. Figures 1A and 1B show the IFN-γ levels in the supernatants from the patients' cell cultures stimulated by G2 and G8 peptides, respectively. The G4 group of peptides induced IFN-γ production in four patients, with the concentration of some supernatants exceeding 300pg/ml when stimulated for 144 hours, although there were no statistically significant differences in IFN-γ production for any of these peptides (data not shown).

For gp43 and *P. brasiliensis* crude antigen (PbAg), statistically significant differences were found between IFN-γ levels at 144 hours and those at 48 and 72 hours (p=0,0001 for both antigens). Figures 1C and 1D show high levels of IFN-γ secretion after stimulation by both gp43 and PbAg. In some patients with the acute

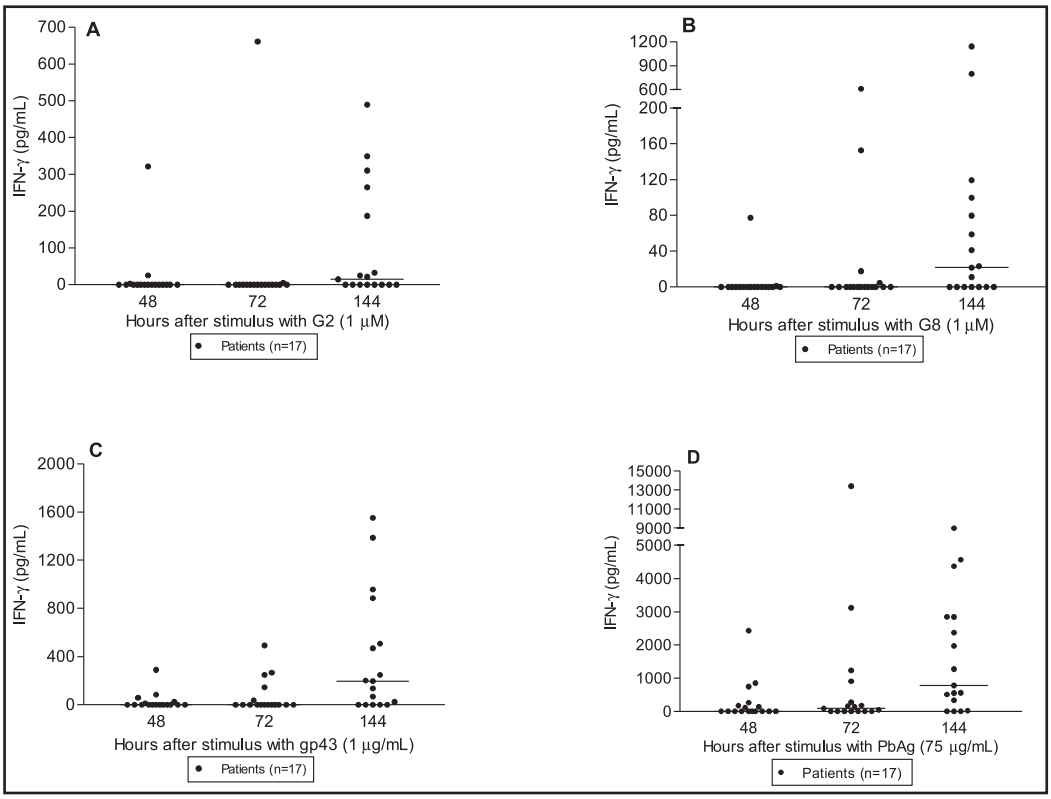

Figure 1 - IFN-γ levels in supernatants from mononuclear cell cultures from cured paracoccidioidomycosis patients, with stimulation using G2 peptides (A) (48h vs. 144h, p=0,0213) and G8 peptides (B) (48h vs. 144h, p=0,0033), gp43 (C) (48 and 72h vs. 144h, p=0,0001) and PbAg (D) (48 and 72h vs. 144h, p=0,0001) for 48, 72 and 144 hours. The horizontal bars represent medians.

form of paracoccidioidomycosis, cells that were reactive to gp43 produced high levels of IFN-γ (data not shown).

Determination of TNF-α levels. The levels of TNF-α production in the 17 patients treated for paracoccidioidomycosis were higher at 144 hours than at 48 hours in mononuclear cells stimulated with G2 peptides (p = 0,0020) at 0.1μM and gp43 (p = 0,0008). Figures 2A and 2B show the TNF-α levels in supernatants from the patients' cell cultures stimulated by G2 and gp43, respectively. Four patients with the acute form of paracoccidioidomycosis produced TNF-α after stimulus with gp43. Of these, two had TNF-α levels greater than 1,000pg/ml (Figure 2B). Other peptides induced TNF-α production in the majority of patients, but no significant differences were observed between the durations of stimulation (Figures 3A and 3B).

Determination of IL-10 levels. IL-10 levels were higher in supernatants from 17 patient mononuclear-cell cultures stimulated with the G7 group of peptides at 1μM (p = 0,0438) after 48 hours than after 144 hours. Figure 4A shows IL-10 levels in supernatants of patients cell cultures stimulated by G7. However, when the cells were stimulated with the G6 group of peptides at 1μM (p = 0,0260), IL-10 levels were higher at 72 hours than at 144 hours (data not shown).

G3 group of peptides at 0.1μM induced IL-10 production in patients cured of the chronic multifocal form of the disease although no statistically significant differences were found between the different periods (Figure 4B).

Determination of IL-4 levels. IL-4 was detected at low levels in most of the cell-culture supernatants. However, this cytokine level tended to be higher at 72 hours when stimulated by G2 peptides at 0.1μM (p = 0,0595) and at 48 hours when stimulated by G6 peptides at 1μM (p = 0,0581). However, the results were not statistically significant for the time periods studied (data not shown).

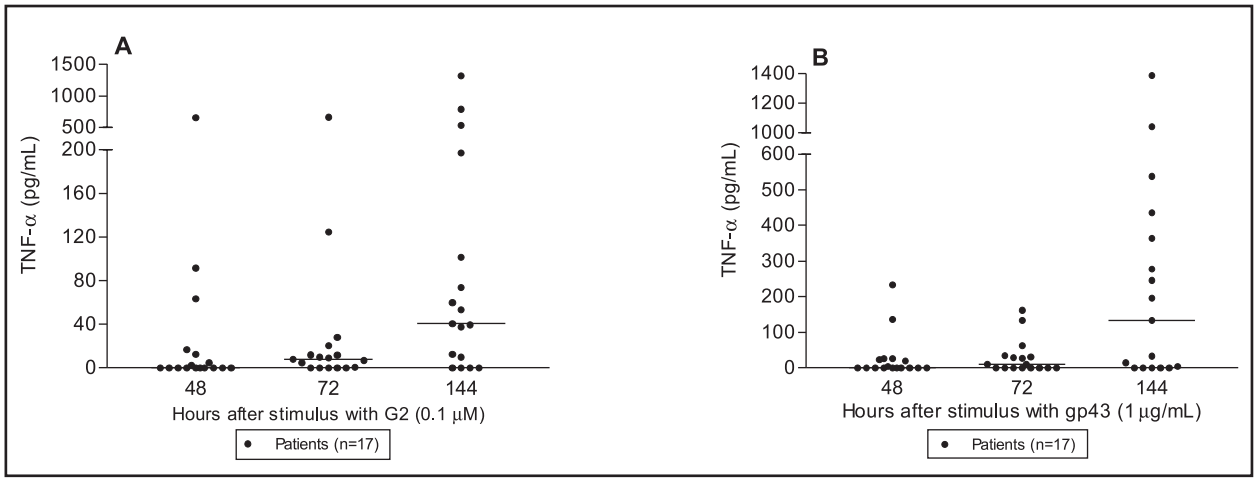

Figure 2 - TNF-α levels in supernatants from mononuclear cell cultures from cured paracoccidioidomycosis patients, with stimulation using G2-group peptides (A) (48h vs. 144h, p=0,0020) and gp43 (B) (48 and 72h vs. 144h, p=0,0008) for 48, 72 and 144 hours. The horizontal bars represent medians.

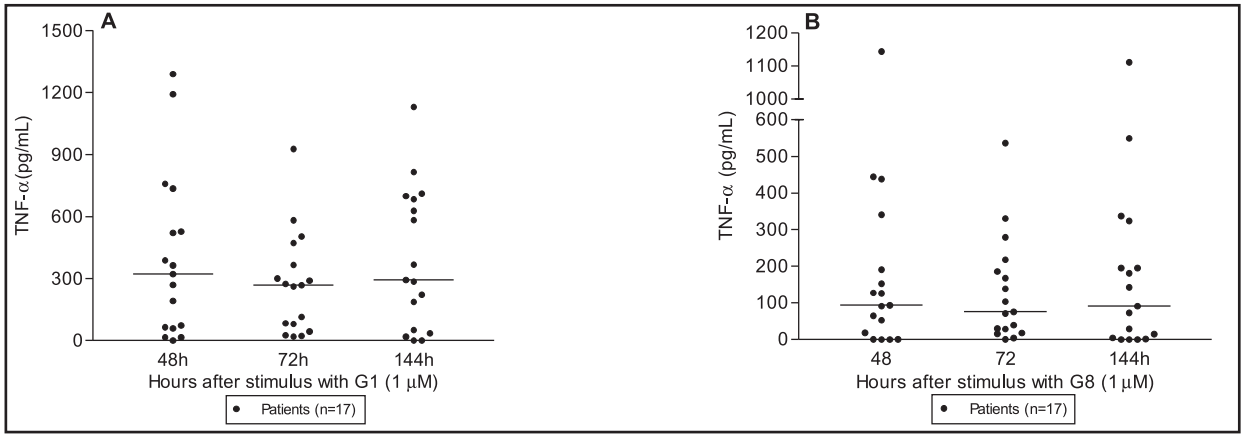

Figure 3 - TNF-α levels in supernatants from mononuclear cell cultures from cured paracoccidioidomycosis patients, with stimulation using G1 (A) and G8 (B), for 48, 72 and 144 hours. The horizontal bars represent medians.

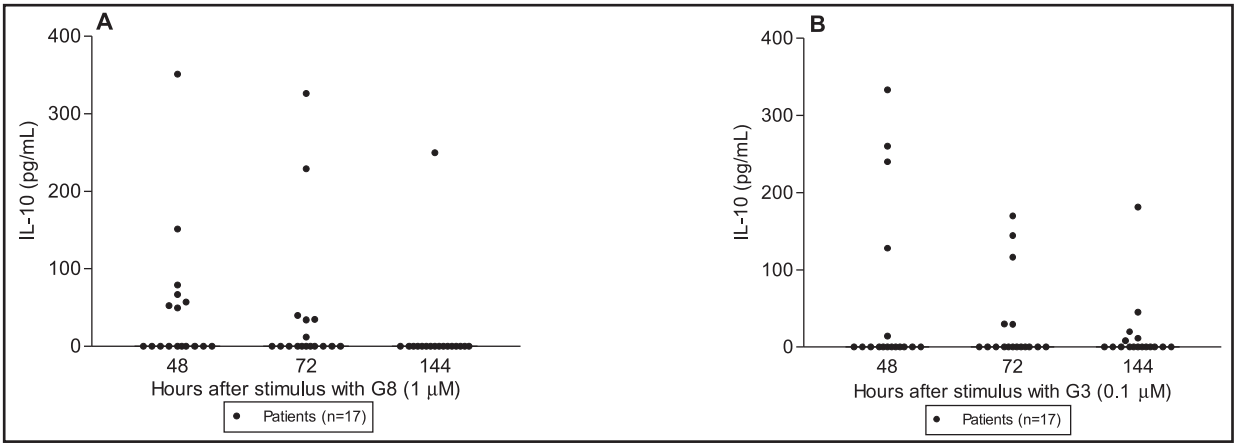

Figure 4 - IL-10 levels in supernatants from mononuclear cell cultures from cured paracoccidioidomycosis patients, with stimulation using G7-group peptides (A), G3-group peptides (B), for 48, 72 and 144 hours.

DISCUSSION

The present study was performed using cells from clinically cured patients, because patients with active paracoccidioidomycosis present transitory cell immunosuppression to antigens from *P. brasiliensis*, whereas this response is restored following clinical cure[2]. The best stimulus duration observed for IFN-γ production in mononuclear cell cultures from treated paracoccidioidomycosis patients was 144 hours for two groups of gp43 peptides (G2 and G8), for *P. brasiliensis* crude antigen (PbAg) and for *P. brasiliensis* gp43 glycoprotein.

A number of authors who studied cytokines in human paracoccidioidomycosis, using similar lymphoproliferation assays with mononuclear cells, used stimulus periods of 48 to 96 hours for IFN-γ analyses[3 12 17]. The differences between the stimulus duration for IFN-γ production in those studies and the stimulus duration in our study may be due to the different techniques used for obtaining and purifying *P. brasiliensis* antigens. In experimental work with BALB/c mice using the gp43 and P10 (gp43[181-195]) peptides, the best length of time found for IFN-γ detection was the same as in the present study, namely 144 hours[24], which confirms this result.

Analysis of IFN-γ mRNA expression stimulated with PHA suggests that low levels of IFN-γ in the acute form indicate that the initial phase of host interaction with *P. brasiliensis* is followed by the Th2 cytokine pattern and increased levels of non-protective fungal antibodies[14]. Only after specific therapy and control of fungus multiplication was recovery of cellular response and a drop in antibody levels observed.

It is important to note that several studies have shown that IFN-γ plays an essential role in resistance to paracoccidioidomycosis and that it is associated with cell-mediated immunity, which is critical for host defense against *P. brasiliensis*[13 17]. Furthermore, studies in animal models have been described in which IFN-γ-activated macrophages represent one of the most important mechanisms against this pathogen and induce a fungicidal effect[4 8]. This process is nitric-oxide dependent[11].

For TNF-α, the peak production with G2 peptides (0.1μM) and gp43 was found to be at 144 hours (Figures 2A and 2B). This result was interesting because most other studies analyzed the production or expression of these cytokines between six and 72 hours after stimulus with different types of antigens[9 12 18 19]. TNF-α mRNA expression in cells from individuals with paracoccidioidomycosis infection peaked three hours after stimulus with PHA[14]. In our study, this cytokine was detected in the majority of the peptide groups tested for almost all the durations of stimulation (Figures 3A and 3B) although TNF-α production was higher after 144 hours for G2 peptides and *P. brasiliensis* gp43 glycoprotein (Figures 2A and 2B).

The high levels of IFN-γ and TNF-α production found in cell cultures from the cured patients in our study after 144 hours of stimulation with gp43 suggest that in some cases the antigen-specific immunosuppression was transitory and immune reactivity to gp43 was recovered after the treatment, as reported by Benard et al[2]. Moreover, the present finding of reactivity to gp43 peptides, in particular to the G2 and G8 peptides, is helpful in understanding the immunopathogenesis of the disease and has possible applications in immunotherapy and immunoprophylaxis. As reported in the literature, P10 peptide stimulated IFN-γ production in a murine model and provided protection against virulent *P. brasiliensis*[23 24], thus corroborating the findings of our study in human paracoccidioidomycosis cells stimulated by the G4 peptide group, which contains the same peptide (P19 in our study) as the one involved in the protection of mice against *P. brasiliensis* (P10).

In the present study, IL-10 was detected for two groups of peptides - G7 (1μM) and G6 (1μM) - and the best stimulus durations observed were 48 and 72 hours, respectively. Studies carried out with *P. brasiliensis* antigens have described detection of IL-10 after stimulation of between 18 and 72 hours[3 5 15 17 18]. In human paracoccidioidomycosis, IL-10 appears to perform a regulatory function in the apoptosis process, thereby avoiding the loss of reactive cells during infection by *P. brasiliensis*[5]. However, so far, only gp43 has been tested, and none of its epitopes are known to stimulate the production of this regulatory cytokine. High

levels of this cytokine observed in supernatants of cell cultures from patients with active disease[3 17] may be important in limiting the loss of reactive cells. IL-10 was also detected at high levels in treated paracoccidioidomycosis patients when the cells were stimulated *in vitro* with PHA and *P. brasiliensis* antigens[3 12]. In our study, the presence of IL-10 in the supernatants of the patients' cell cultures suggests that this regulatory response persists, even if treatment had been interrupted many months or years before.

The levels at which the IL-4 cytokine was detected in cultured supernatants were low in most of the samples analyzed, and no statistically significant difference was observed between the results from the different durations of stimulation. However, IL-4 was detected at 48 and 72 hours when stimulated with G6 (1μM) and G2 (0.1μM) respectively. These low levels of detection may be explained because these patients had already been cured. IL-4 was detected in cell culture supernatants from patients with active paracoccidioidomycosis, following stimulation with *P. brasiliensis* antigen, and was strongly detected in patients with the acute form of the disease[17]. Furthermore, high levels of IL-4 mRNA expression were observed in patients with the acute and chronic forms up to 24 hours after stimulus with PHA[14], thus suggesting that IL-4 has a role in the Th2 response at this phase of the disease. Low levels of IL-4 or failure to detect this cytokine in cell-culture supernatants from treated patients could be related to the absence or low levels of antibodies, since the presence of this cytokine has been associated with the presence of IgG4 antibodies in the active phase of the illness. The presence of this cytokine in the supernatants of some cured patients' cells (data not shown) suggests that, even after a long period of treatment, the immune response is not restored in some patients.

Our results indicate the best time for detection of IFN-γ, TNF-α and IL-10 in supernatants from cultures stimulated by gp43, crude *P. brasiliensis* antigens and groups of gp43 peptides and also suggest the best time for detection of IL-4. These data may contribute towards future studies on the role of gp43 peptides and gp43 in the host immune response relating to paracoccidioidomycosis. In addition, increased levels of IFN-γ and TNF-α were observed in cells from paracoccidioidomycosis patients in response to stimulation by gp43 and gp43 peptides, thus encouraging further research towards understanding the influence of gp43 peptides on the production of inflammatory and regulatory cytokines.

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