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Interferon-gamma and interleukin-4 single nucleotide gene polymorphisms in Paracoccidioidomycosis

A. Bozzi [a], B.S. Reis [a], P.P. Pereira [b], E.P. Pedroso [b], A.M. Goes [a,*]

[a] Departamento de Bioquímica e Imunologia do Instituto de Ciências Biológicas, UFMG, Caixa Postal 486, CEP 31.270-901, Belo Horizonte, MG, Brazil
[b] Faculdade de Medicina, Universidade Federal de Minas Gerais (UFMG), Brazil

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ABSTRACT

The gene polymorphisms interferon-gamma (IFN-γ) +874 T/A and interleukin (IL)-4 −590 C/T have been associated with the altered production of cytokines. Therefore, they might be indicative of the occurrence of Paracoccidioidomycosis (PCM) caused by *Paracoccidioides brasiliensis*. The analysis of single nucleotide polymorphism (SNP) at position +874 IFN-γ showed an increase occurrence of A/T genotype in both PCM patients and healthy individuals as control (HIC) (56% and 45%, respectively), while the allelic distribution showed 82% of A allele in the patients and 80% in the controls. The SNP of −590 IL-4 showed that C/T genotype was significantly ($p < 0.05$) more prevalent (39%) in PCM group compared to the HIC group (19%), while IL-4 C/C genotype was significantly less frequent (59%) in the patient group compared to the control group (81%). Otherwise, 41% of PCM patients and 19% of HIC individuals carried the IL-4 T allele. Stimulation of peripheral blood mononuclear cells (PBMC) from PCM patients with cell extract antigenic preparations (PbAg) as well as secreted and surface antigens (MEXO) of *P. brasiliensis* evidenced that there is no difference in the IFN-γ production related to A and T alleles between PCM and HIC individuals. However, with IL-4 production, PCM patients classified as C phenotype showed two times more IL-4 production than PCM patients classified as T phenotype and HIC controls. In conclusion, our results suggest that functional genetic variants in the IL-4 promoter could influence the production of IL-4 in PCM.

1. Introduction

PCM is a granulomatous mycosis caused by the dimorphic fungus *P. brasiliensis* [1]. This disease presents a broad spectrum of clinical and pathological manifestations, ranging from localized lesions to severely disseminated infection [2]. Host resistance to *Paracoccidioides brasiliensis* is related to the ability to develop a cell-mediated immune response characterized by Thl type immune responses and by the ability of T-cells to proliferate in response to fungal antigens [3–6].

The development of the PCM is highly influenced by cytokine levels. The resistance to PCM is related to IFN- production and the susceptibility has been linked to the preferential production of the IL-4, IL-5, IL-10, and IL-13 [7–9]. A limited number of studies are available regarding IL-4 and PCM. 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 [10].

There are several reports suggesting that host genetic factors play significant roles in susceptibility or resistance to infectious diseases, including PCM. Thus, many studies were already conducted in order to investigate whether a genetic basis for these diseases may be found in polymorphisms of cytokine genes [11,12]. This would explain why there are differences between individuals in their ability to produce cytokines after *in vitro* stimulation of peripheral blood leukocytes.

Various polymorphisms, mostly SNPs, or microsatellites, were described to affect gene transcription, causing individual variations in cytokine production. These polymorphisms are involved in the susceptibility, severity and clinical outcome of several diseases, including infectious ones [13,14].

Various SNPs have been detected within IFN-γ and IL-4 gene sequences. Several of these polymorphisms may be associated with differential levels of gene transcription. IFN-γ production seems to be influenced by a SNP at position +874—being the most extensively studied SNP of this cytokine—that corresponds to a transition of T/A. A SNP +874 (T/A) is located at the 5′-end of a CA repeat at the first intron of human IFN-γ [15]. The +874 T allele is linked to the 12 CA repeats, whereas the A allele is linked to the non-12 CA repeats [16]. The specific sequence of the T allele

* Corresponding author. Tel.: +55 31 3409 2639; fax: +55 31 3409 2614.
E-mail address: goes@icb.ufmg.br (A.M. Goes).

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provides a binding site for the transcription factor nuclear factor-κB [17,18]. Because transcription factor nuclear factor-κB induces IFN-γ expression, this T allele correlates with high IFN-γ expression, whereas the A allele correlates with low expression.

IL-4 is an important cytokine for the development of Th2-mediated immune response and B-cell differentiation and proliferation [19]. Several polymorphisms of the IL-4 gene have been studied [20]. A single nucleotide polymorphism (−590 C/T) in the promoter region of IL-4 gene has been identified with −590 T variant associated to enhanced transcriptional activity *in vitro* and with increased total serum IgE *in vivo*. This polymorphism has been implicated in the immune-mediated diseases asthma and atopic dermatitis [21,22]. Rosenwasser et al. [21] found a polymorphism with a C to T exchange at position 590 upstream of the open reading frame of the IL-4 gene that is associated with greater luciferase activity [23].

To our knowledge, little is known about the association between cytokine genetic polymorphisms and PCM. Considering the potential role of IFN-γ and IL-4 cytokines in PCM and limited studies on the relationship between these cytokine gene polymorphisms and resistance or susceptibility to PCM, we aimed to investigate the occurrence of polymorphisms in IFN-γ +874 T/A and IL-4 −590 C/T in PCM patients in this study.

2. Materials and methods

2.1. Study population

Adult patients (50 males) with active PCM, age range 18–75 years, were enrolled in this study after admission to the Centro de Treinamento e Referência em Doenças Infecto-Parasitárias Orestes Diniz (CTR-DIP), Hospital das Clínicas da Universidade Federal de Minas Gerais (UFMG), Brazil. The patients were selected based on clinical examinations and the diagnosis of the disease was confirmed by histopathological examination and microscopic demonstration of *P. brasiliensis* in clinical specimens. These patients were classified as non-treated patients with a positive diagnosis of PCM.

HIC individuals (31 males) were blood donor volunteers in public hospitals with no evidence of any clinical history of PCM or other serious illness. Samples from HIC controls and patients had been collected consecutively. Neither of individual group was submitted to any therapeutic schedule or immune-modulator medication (e.g., corticosteroids). All PCM patients and controls used did not suffer from diseases with marked influence on immune function such as HIV infection or other severe diseases. Both patients and controls were informed, gave their written consent to participate in the study and to allow their biological samples to be genetically analyzed. Approval for the study was given by the ethic committee of the Hospital das Clínicas da Faculdade de Medicina da UFMG (COEP).

2.2. Antigens

Paracoccidioides brasiliensis (Pb 18 isolate) cell extracts were used as antigenic preparation (PbAg), which was obtained by mechanical disintegration of yeast cells with glass beads as previously detailed [8]. Other antigen used was secreted and surface *P. brasiliensis* antigen (MEXO). Briefly, yeast cells were cultured in YPD agar (0.5% yeast extract, 0.5% peptone, 1.5% D-glucose, 1.5% agar, pH 7.0) medium (Sigma, St. Louis, MO, USA) at 35 °C and collected on the 7th day of culture. The yeast cells were carefully removed from the culture medium and submitted to agitation in a vortex in 0.05 M PBS, pH 7.4, for 30 s. The solution was centrifuged (14,000*g*) for 10 min at 4 °C [24]. Both antigen preparations were sterilized by filtration, and the protein concentration was measured according to the Bradford micro-assay protocol [25].

2.3. Enzyme-linked immunosorbent assays (ELISA)

To evaluate the reactivity of sera from PCM patients and HIC controls against *P. brasiliensis* antigens, flat-bottomed microtiter plates (Immulon II, Dynatec Corp., Alexandria, VA, USA) were coated overnight with 100 μL of a 10 μg/mL solution of MEXO or PbAg in 0.5 M carbonate buffer, pH 9.6. The plates were washed five times with washing buffer (0.05 M PBS containing 0.05% Tween 20 (PBS-T)), and blocked with 150 μL of blocking solution (10% bovine serum albumin in 0.15 M PBS, pH 7.4) at room temperature. After 1 h of incubation, plates were filled with 100 μL of 1:400 dilution sera of patients or HIC controls in 0.15 M PBS, pH 7.4, and re-incubated for 1 h. The plates were washed 10 times with PBS-T and incubated for an additional hour with 100 μL of a 1:10,000 dilution of goat anti-human IgG peroxidase-conjugated antibody (Sigma) in 0.15 M PBS, pH 7.4. The plates were then washed 10 times with PBS-T and the peroxidase activity was assayed with 100 μL of *o*-phenylenediamine dihydrochloride (OPD) solution (3.4 mg of OPD and 20 μL of hydrogen peroxide to 10 mL of citrate/phosphate buffer, pH 5.0) from Sigma. Color development was stopped with 50 μL of 2 N H_2SO_4. An optical density of 492 nm was registered in an automated ELISA reader (Bio-Rad 2550 Reader EIA).

2.4. Cell preparations

Human PBMC were isolated from heparinized venous blood of PCM patients or HIC controls by Ficoll-diatrizoate density gradient centrifugation (Sigma Chemical Co., St. Louis, MO, USA). PBMC were suspended in RPMI 1640 (Sigma) culture medium containing 1.6% L-glutamine, 300 U/mL of penicillin, 0.3 mg/mL of streptomycin, gentamycin and 10% heat-inactivated human AB + serum (RPMI 10% AB+) at a final concentration of 3.0×10^5 cells per well for cell proliferation assays.

2.5. Cellular proliferation assay

PBMC from PCM patients and HIC controls (3.0×10^5 cells per well) were cultured in 96-well flat-bottomed plates in a final volume of 200 μL of RPMI containing 10% AB + human serum at 37 °C and 5% CO_2. Cells were stimulated with MEXO (25 μg/mL) or PbAg (25 μg/mL) or 50 μL of medium (control) or in the presence of phytohemaglutinin (PHA) (10 μg/mL). Cells were set up in triplicate and incubated for 5 days, pulsed for an additional 18 h with 0.5 μCi/well of methyl-[^{3}H]thymidine (specific activity, 37 Ci/Mm; Amersham, Life Sciences) and harvested. Cell bound radioactivity was measured using a β-plate scintillation counter (Wallac Oi, Turku, Finland). Results were expressed as mean counts per minute (cpm) of triplicates.

2.6. Cytokine detection

Cytokines were measured in the supernatant of antigen stimulated cell cultures with commercial capture ELISA kit (Pharmingen) according to the manufacturer's protocol. For cytokine titration, microplates (Nunc) were sensitized overnight at 4 °C with anti-IL-4 (Pharmingen) or anti-IFN-γ (Pharmingen) monoclonal antibodies (mAb). Plates were washed three times with phosphate buffered saline (PBS) containing 0.05% Tween (PBS-T), and non-specific binding was prevented by incubating plates for 1 h at room temperature with 200 μL per well of 10% fetal bovine serum (FBS, Sigma) in PBS 0.15 M. Plates were washed three times with PBS-T and incubated for 2 h at room temperature with 100 μL per well of

each culture supernatant, next to the respective cytokine standard-curve (Pharmingen) in a properly serial dilution in PBS with 10% FBS. Plates were washed five times with PBS-T and incubated for 1 h at room temperature with anti-IL-4 mAb (Pharmingen) or anti-IFN-γ (Pharmingen) plus enzyme reagent. Plates were washed seven times with PBS-T and incubated for 30 min at room temperature with 100 μL per well of substrate solution (tetrametilbenzidina and hydrogen peroxide). Absorbance was read at 450 nm within 30 min of stopping reaction with 50 μL of stop solution (2 N H_2SO_4).

2.7. DNA extraction

Genomic DNA was obtained from granulocytes isolated from heparinized venous blood of patients or non-infected controls by Ficoll-diatrizoate density gradient centrifugation (Sigma Chemical Co., St. Louis, MO). After the rupture of erythrocytes with lyses buffer (1.68 mol/L NH4Cl; 0.09 mol/L KHCO3; 0,001 mol/L EDTA), the genomic DNA was extracted from granulocytes pellet with DNAzol reagent (Life Technologies), according to the manufacture's protocol.

2.8. Single nucleotide polymorphisms (SNP) detection

To determine single nucleotide polymorphism, the allele-specific polymerase chain reaction (ASPCR) were used to detect the T → A SNP at position +874 of IFN- gene [16] and the C → T SNP at position −590 of IL-4 gene. Three primers were constructed for each ASPCR: For +874 IFN- SNP analyses, the downstream primer (5′-CATCTACTGTGCCTTCCTGT-3′) as combined with either the upstream primer (5′-TTCTTACAACACAAAATCAAATCT-3′) complementary to IFN- +874 T allele, or upstream primer (5′ TTCTTA CAACACAAAATCAAATCA 3′) complementary to INF- +874 A allele. The upstream primers (+874 T and +874 A) differ only in their 3′ terminal nucleotide.

For −590 IL-4 SNP analysis, the downstream primer (5′-AGTA-CAGGTGGCATCTTGGAAA-3′) was combined with either the upstream primer (5′ CTAAACTTGGGAGAACATTGTT3′), complementary to IL-4 −590 T allele, or upstream primer (5′-CTAAACTTGGGA GAACATTGTC-3′) complementary to IL-4 −590 C allele. The upstream primers (−590 T and −590 C) differ only in their 3′ terminal nucleotide.

All PCRs were performed with final reagent concentrations of reaction buffer (1×), $MgCl_2$ (2.5 mM), dNTP (4 M), each primer (0.5 pmol/l), thermoprimer DNA polymerase (0.25 U) and DNA (25–100 ng). PCRs were performed using a 9600 Thermal Cycler (Applied Biosystems, Foster City, CA, USA), in the conditions: 95 °C 3′; 35 cycles of 93 °C 30″, 57 °C 40″, 72 °C 40″; then 72 °C 7′. The dDNA amplified fragments were digested by 0.1 M NaOH for 5 min at 95 °C and loaded with "loading buffer" onto 2% agarose gels (containing 0.5 mg/mL ethidium bromide) and separated by electrophoresis. The SNPs were detected according to the presence or absence of the PCR band visualized by IFN-γ +874 T → A SNP 116 pb and IL-4 −590 C → T SNP 131 pb. The polymorphic alleles were identified by differential fragments of sDNA migration in the gel. All reactions were normalized to the known DNA polymorphism. A non template control with non genetic material was included to eliminate contamination or nonspecific reactions. Each sample was tested in duplicate.

2.9. Statistical analysis

Statistical analysis was performed by Epi Info Software Version 3.3.2 for Windows. The distribution of cytokine genes polymorphisms were compared between PCM patients and healthy infected individuals by the X^2 or Fisher's exact test. P values smaller than 0.05 were considered significant. The other data were analyzed statistically by ANOVA or Student's t test with the level of significance set at $p < 0.05$. Bonferroni was used to compare subgroups.

3. Results

3.1. IFN-γ +874 T/A SNP analysis

The genotype and allele frequencies analysis of SNP at position +874 IFN-γ among PCM patients and HIC individuals are shown in Table 1. There was no significant difference ($p = 0.6186$) in the genotype frequencies of the IFN-γ +874 T/A gene polymorphism in patients with PCM when compared with HIC individuals (PCM:HIC 26%:36%, 56%:45%, 18%:19%, for A/A, T/A, T/T genotypes, respectively). The T/A genotype is the most frequent in both groups, followed by the A/A and T/T genotypes. The allelic frequency at position +874 was not different between patients and controls. The frequency of the A allele was greater ($p < 0.05$) in both patients (82%) and controls (80%) followed by T allele in patients (18%) and controls (20%).

3.2. IL-4 −590 C/T SNP analysis

The genotype and allele frequencies in the IL-4 −590 polymorphism are shown in Table 2. The detective rates of T/T, C/T and C/C genotypes were 0%, 19% and 81% in healthy controls, and 2%, 39% and 59% in patients with PCM. It was detected that IL-4 C/T genotype frequency was significantly ($p < 0.05$) more prevalent (39%) in PCM group compared to the HIC group (19%), while IL-4 C/C genotype was significantly less frequent (59%) in the patient group compared to the control group (81%). Otherwise, 41% of PCM patients and 19% of HIC individuals carried the IL-4 T allele. On the other hand, the C allele was lower ($p < 0.05$) in PCM (59%) than in HIC (81%).

Table 1
Genotype and allele distributions for the +874 polymorphism of the IFN-γ gene in PCM patients and healthy individuals.

	PCM patients ($n = 50$)*	Healthy individuals ($n = 31$)
Genotype (%)		
A/A	13 (26%)	11 (36%)
T/A	28 (56%)	14 (45%)
T/T	9 (18%)	6 (19%)
Allele (%)		
A	41 (82%)	25 (80%)
T	9 (18%)	6 (20%)

n = number of PCM patients or healthy individuals.
* $p = 0.6186$ when compared PCM with Healthy individuals.

Table 2
Genotype and allele distributions for the −590 C/T polymorphism of the IL-4 gene in PCM patients and healthy individuals.

	PCM patients ($n = 50$)*	Healthy individuals ($n = 31$)
Genotype (%)		
T/T	1 (2%)	0 (0%)
C/T	20 (39%)**	6 (19%)
C/C	29 (59%)	25 (81%)
Allele (%)		
T	21 (41%)**	6 (19%)
C	29 (59%)	25 (81%)

n = number of PCM patients or healthy individuals.
* $p = 0.1041$; chi-square test, 3 × 2 contingency table.
** Statistically significant $p < 0.05$ in relation to C/T or T distribution when compared PCM to healthy individuals.

3.3. Proliferation of PBMC stimulated with soluble P. brasiliensis antigens

The PBMC from PCM patients and HIC individuals were cultivated with *P. brasiliensis* antigens for 5 days and their proliferation responses were analyzed by the measuring of [^{3}H]-thymidine incorporated. The results in Fig. 1 show that MEXO and PbAg antigens significantly ($p < 0.05$) induced proliferation of PBMC from PCM patients when compared to HIC. On the other hand, significant increase ($p < 0.05$) in cell proliferation was observed when PBMC from HIC individuals were stimulated with PHA as compared with PCM patients. It was observed that PHA was not capable of promotting strong proliferative response in cells from PCM patients, and this proliferation was not statistically significant when compared with the responses induced *by P. brasiliensis* antigens.

3.4. Profiles of cytokine production by PBMC from PCM patients stimulated with P. brasiliensis antigens

To analyze whether PBMC from PCM patients differed in the cytokine profile secretion in response to stimulation with *P. brasiliensis* antigens MEXO or PbAg, the IFN-γ or IL-4 production was determined in culture supernatant by ELISA. PBMC from PCM patients did not produce significant IFN-γ production when compared to HIC individuals after stimulation with MEXO or PbAg. However, the PBMC stimulation with PHA promoted significant ($p < 0.05$) IFN-γ production in both PCM patients and HIC individuals (Fig. 2A) when compared to *P. brasiliensis* antigen stimulations. The IL-4 production by PBMC from PCM patients was significant higher ($p < 0.05$) than in the HIC individuals after stimulation with MEXO or PbAg. Moreover, IL-4 production of PBMC after stimulation with PHA was not different between PCM and HIC groups (Fig. 2B).

3.5. Production of IFN-γ, IL-4 and relationship with polymorphism

To further determine whether the SNP interfered in the production of IFN-γ and IL-4 cytokines, the phenotypic frequency results were associated to cytokine production. There is no difference in the IFN-γ production related to A and T phenotypes compared with PCM and HIC individuals. When the IL-4 production was analyzed, it was found that PCM patients classified as C phenotype showed two times more IL-4 production than PCM patients classified as T phenotype (Fig. 3B). On the other hand, both PCM patients classified as T and C phenotypes presented significant ($p < 0.05$) IL-4 production compared to HIC individuals with the same phenotypes.

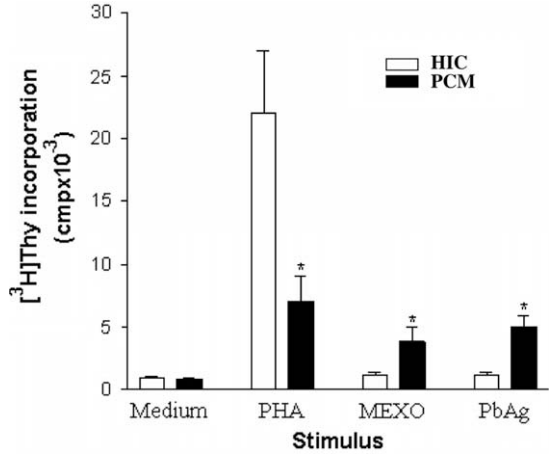

Fig. 1. Proliferation of PBMC from PCM patients and HIC individuals induced by *P. brasiliensis* antigens MEXO or PbAg. A total of 3×10^5 cells per well, isolated from PCM patients ($n = 50$) or from HIC individuals ($n = 31$) were cultured with Medium, PHA (10 μg/mL), MEXO (25 μg/mL) or PbAg (25 μg/mL) for 5 days, and the last 18 h in the presence of 0.5 μCi [^{3}H]-thymidine. Data were reported as mean ± SD of triplicates in counts per minute (cpm). Statistically significant ($p < 0.05$) data are indicated by an asterisk (*) in relation to controls and antigen stimulation.

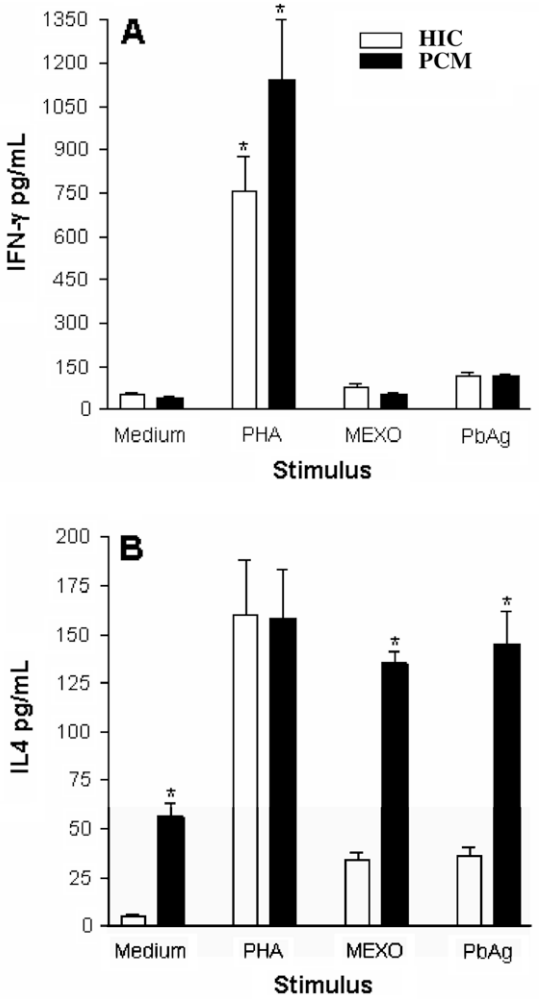

Fig. 2. Production of IFN-γ (A) and IL-4 (B) by PBMC from HIC individuals ($n = 31$) and PCM patients ($n = 50$) after cells stimulation *in vitro* with Medium or with *P. brasiliensis* antigens MEXO (25 μg/mL) or PbAg (25 μg/mL). The data are reported as mean ± SD of triplicates concentration of each cytokine. Statistically significant ($p < 0.05$) data are indicated by an asterisk (*) in relation to PHA stimulation.

4. Discussion

In this study, we investigated whether or not genetic variation in IFN-γ and IL-4 (as Th1 and Th2 cytokines) genes influences susceptibility to PCM. Our experimental results demonstrated that a higher frequency of T/A genotype was observed in the PCM patients in respect to A/A and T/T genotypes of IFN-γ +874 polymorphism and a significantly higher frequency of A allele. However, no significant difference was found in relation to HIC group. It has been shown that T to A polymorphism at +874 position of IFN-γ gene can directly influence the level of IFN-γ production. In our experiments, analysis of cytokine gene expression in PBMC of PCM patients after *in vitro* stimulation with MEXO or PbAg antigens showed low levels of IFN-γ production and higher IL-4 production. The general analysis of our previously results demonstrated that the use of MEXO or PbAg is an important tool for identification of specific components of *P. brasiliensis* that elicit

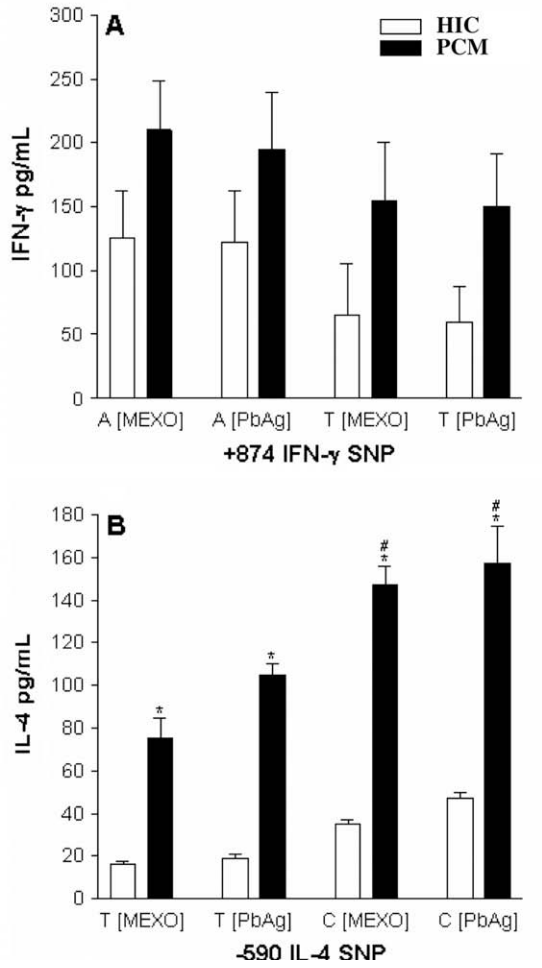

Fig. 3. Association between single nucleotide polymorphism and cytokine production by PCM patient cells after MEXO and PbAg *in vitro* stimulation. The production IFN-γ (panel A) or IL-4 (panel B) was available and correlated to phenotypic frequency, subjects A or subjects T for IFN-γ SNP studied and T or C subjects for IL-4 SNP. Data are reported as mean ± SD for PCM patients ($n = 50$) or HIC individuals ($n = 31$) of triplicates concentration of each cytokine. Statistically significant ($p < 0.05$) data are indicated by an asterisk (∗) in relation to controls. $^{\#}P < 0.05$ significantly different between groups.

T-cell activation, cytokine release and antibody response. The identification of antigenic components of *P. brasiliensis* that elicit antibody production during the course of infection may contribute to a better understanding of the disease and the host protective mechanisms involved. In addition, identification and isolation of specific antigens may lead to a better definition of the course of the infection, to more specific diagnostic tests, and also to the development of precisely characterized vaccines.

Considering the importance that IFN-γ plays in protective immunity against *P. brasiliensis* infection, it is possible that different amounts of IFN-γ production may influence the susceptibility to PCM.

The A allele with low IFN-γ production has been previously reported to be associated with tuberculosis and SARS [26,27]. It is possible that a low IFN-γ production may impair the antiviral response against these infectious diseases. Polymorphisms within promoter sequence, e.g., IFN-γ +874 (T/A), could affect transcription by altering the structure of transcription factor binding sites. In fact, our group previously detected an association between PCM presence and cytokine gene polymorphisms, including IL10 and tumor necrosis factor alpha (TNF-α) [28]. Therefore, the data discussed above led us to hypothesize that the propensity of individuals to develop resistance in PCM is associated in part due to their ability to produce IFN-γ and the possibility of being associated with their genotype at the IFN-γ +874 T/A polymorphism.

To our knowledge, this is the first report of IL-4 genotype association to PCM, although the association of IL-4 gene polymorphism with different diseases has been investigated [29,30]. On the other hand, our results showed that PCM patients presented high IL-4 production after PBMC stimulation with *P. brasiliensis* antigens. These data were similar to those previously detected by Bozzi et al. [31]. It is well recognized that the balance between different cytokines may be a more important indicator of the outcome of infection than the absolute concentrations of particular cytokines. The different IL4 to IFN-γ levels may alter the control of infection in each individual. Thus, the balance between IL4 and IFN-γ could then be linked to protection or to disease severity. Another explanation for this finding is that the IL-4 −590 C/T polymorphism is just a marker of disease and not the cause of disease. Indeed, other functional single nucleotide polymorphisms with linkage disequilibrium to IL-4 −590 C/T polymorphism may be associated with susceptibility to develop PCM. Therefore, individuals with low genetic ability for IL-4 production (T/T genotype) may have a good Th1 response and will be more resistant to *P. brasiliensis* infection than individuals with intermediate (C/T) or high (C/C) IL-4 producing genotypes. It seems that the two observations on allele distribution and IL-4 production would lead to the conclusion that the increased IL-4 production promotes susceptibility in PCM.

Acknowledgments

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