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Candidate gene case-control and functional study shows macrophage inhibitory factor (MIF) polymorphism is associated with cutaneous leishmaniasis

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ABSTRACT

American tegumentary leishmaniasis (ATL) is an infectious disease caused mostly by *Leishmania* (*Viannia*) *braziliensis* in Southeast Brazil. The clinical manifestations are vast, ranging from asymptomatic to severe mucosal leishmaniasis (ML). It has been suggested that variation of the pathogen does not fully explain the response spectrum and the variability of clinical manifestations. Previous data have shown that host genetics also play a role in disease outcome. Herein, we have tested the association of *TNF*, *IL10*, *IL12* and *MIF* single nucleotide polymorphisms (SNPs) using a case-control study design including 110 cutaneous leishmaniasis (CL) patients and 682 healthy subjects. The genotype–phenotype correlation was also assessed using leishmania antigens to stimulate peripheral blood mononuclear cells obtained from cured CL patients. Results demonstrated that the *MIF* −173C allele is associated with leishmaniasis outcome and also with lower levels of MIF in culture supernatants. Also, the *TNF* −308AA genotype was statistically increased among leishmaniasis patients. The results showed here suggest that the lower levels of MIF produced by *MIF* −173C carriers could influence the host–*Leishmania* interaction, favoring infection and disease progression. On the other hand, high TNF levels can contribute to tissue damage, consequently leading to skin lesions.

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1. Introduction

American tegumentary leishmaniasis (ATL) is an infectious disease caused by parasites of the *Leishmania* genus, which are transmitted by several species of sand flies to either humans or different species of wild or domestic animals [1,2]. In Brazil, the incidence of ATL has increased over the past 20 years in virtually every state [3,4]. *Leishmania* spp. is an obligatory intracellular parasite that infects mainly macrophages [5]. This infection is characterized by a broad clinical and immunopathological spectrum, ranging from an asymptomatic infection with spontaneous healing to severe disease. The cutaneous leishmaniasis (CL) is the most frequent clinical form and often tends to heal spontaneously, while mucosal leishmaniasis (ML), observed in 3–5% of infected subjects, is the most severe clinical manifestation [6].

Regarding the immunological response in the ATL, has been described that the type 1 immune response prevail in CL and ML, with an exacerbated production of IFNγ and TNF that fails to be modulated either by IL-10, TGFβ or by neutralizing antibodies to IL-12 and IL-15 [7,8]. Patients with ML produce higher levels of IFNγ and TNF than CL, so this exaggerated type 1 immune response is unable to control the parasite and also contributes to pathological tissue damage [8,9]. Curiously, ML patients present low IL-10 production which in turn can contribute to the exacerbated inflammatory immune response [8,10]. In this context, macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine produced by the pituitary gland and multiple cell types, that exhibits a broad range of immune and inflammatory activities, including the induction of cytokines such as TNF, IFN-γ, IL-1β, IL-12, IL-6 among others, and also can increase the survival of macrophage [11–13], which might affect the immune response to intracellular pathogens.

The exacerbated cytokine response in some individuals might be genetic controlled. In the past few years, population-based case-control studies used single nucleotide polymorphisms (SNPs) in candidate genes as markers of susceptibility and severity of ATL

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have suggested that the different outcomes of *Leishmania* infections, may be, at least in part, influenced by the host immunogenetic background [14–19].

The aim of this study was to replicate previous findings regarding the association between the *IL10* gene and ATL and also investigate whether additional cytokine genes involved in the control of intracellular parasite infections (MIF, TNF and IL-12) as players of susceptibility to cutaneous leishmaniasis in an endemic Brazilian region. Here, the genetic association of these genes was evaluated in a case-control study and the functional role of each gene was assessed by determining the cytokine levels in of peripheral blood cells cultures stimulated *in vitro* with leishmanial antigens.

2. Material and methods

2.1. Subjects and study design

To assess the genetic susceptibility towards ATL, we used a case-control design comparing CL cases with healthy controls (HC). A total of 110 CL patients selected from areas endemic for *L. braziliensis* infections were enrolled in this study (CL, 74 males and 36 females; mean age = 38 ± 17 years old). The diagnostic criteria were based on clinical, parasitological and immunological parameters as described elsewhere [20]. Patients were reexamined one year after the end of the therapy and were considered clinically cured at the moment of this study. Of these, functional analysis was carried out with 52 clinically cured CL patients for genotype–phenotype correlation. The HC group included a total of 682 subjects (359 males and 322 females; mean age = 33 ± 9.6 - years old) recruited in the same geographic area as the cases (Rio de Janeiro, Brazil). A second control group including 22 healthy subjects (17 males and 5 females; mean age = 33 ± 10 years old) was also selected for functional analyses. Written informed consent was obtained from all individuals included in the study. The present study was approved by the Ethical Committee for Human Research of the Oswaldo Cruz Foundation, Brazilian Ministry of Health (protocol number 577/09).

2.2. SNP genotyping

Genomic DNA was extracted from whole blood samples using the salting out method. The polymorphism at *IL12B* +1188 (rs17875322) was genotyped by polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis, as described elsewhere [21]. The *IL10* −819 (rs1800871), *TNF* −308 (rs1800629) and *MIF* −173 (rs755622) polymorphisms were genotyped by real-time PCR using "assay-by-design" TaqMan probes for allelic discrimination according to manufacturer's instructions (Life Technologies, CA, USA). All reactions were performed in the StepOne Plus instrument (Life Technologies).

2.3. Cell culture and IL-12p70, IL-10, MIF and TNF measurements

Peripheral blood mononuclear cells (PBMC) were obtained from a total of 52 clinically cured CL patients and 22 HC using Ficoll-Hypaque density centrifugation. Cell cultures (3×10^6 per well) were stimulated *in vitro* with crude extract of *L. braziliensis* promastigote antigens (Lb-Ag, 50 μg/well) for 24 h for detection of IL-12p70 and TNF and 72 h and 120 h for detection of IL-10 and MIF, respectively, as previous described [20]. The levels of IL-12p70, IL-10, MIF and TNF were measured in culture supernatants by ELISA according to manufacturer's instructions (R&D, Emeryville, CA, USA). Samples were tested in duplicate and concentrations were measured using the SOFTmax®PRO 4.0 program (Life Sciences Edition, Molecular Devices Corporation, USA). Assay sensitivity ranged from 15.62 to 1000 pg/mL for IL-12p70 and TNF, and 31.25 to 2000 pg/mL for IL-10 and MIF.

2.4. Statistical analyses

The genotype and allele frequencies of the *IL12B* +1188A>C, *IL10* −819C>T, *MIF* −173G>C and *TNF* −308G>A SNPs were determined by direct counting and a χ^2-test was applied to determine if the observed frequencies conformed to the expected under Hardy–Weinberg Equilibrium (HWE). The frequencies of each genotype, allele and minor allele carriers in cases and controls were compared by logistic regression models with adjustment for the non-genetic covariate sex. A Cochran–Armitage trend test was applied to assess allele dose effects. Odds ratios (ORs) were used as the point estimates of risk and were calculated along with their 95% confidence intervals (CIs). All analyses were performed using the statistical software R for Windows version 2.9.2 (R Development Core Team, 2008) [22] with the packages "genetics" and "coin". Results obtained from quantitative variables were analyzed by One-way Analysis of Variance (ANOVA) with Tukey-Kramer post-test, using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

In total, 110 CL patients and 682 HC were genotyped. The genotypic, allelic and minor allele carrier frequencies are shown in Table 1 along with the results of the logistic regression models. None of the SNPs deviated from HWE in both populations (cases and controls) tested.

As observed in Table 1, the *MIF* −173C carriers (CG + CC genotypes) had a higher risk of developing CL (adjusted OR = 1.79; $p = 0.008$). Furthermore, the risk estimates of GC and CC genotypes (adjusted OR = 1.54 and 3.43, respectively) suggested an allele dose effect, which was supported by the Cochran–Armitage trend test ($p = 0.001$). For the TNF −308G/A SNP, the AA genotype was significantly increased among CL patients (6%) as compared to controls (2%). The *IL10* and *IL12B* SNPs were not associated to CL outcome (Table 1).

Subsequently, the functional relevance of these SNPs was investigated by comparing the *in vitro* production of cytokines (IL-12p70, IL-10, MIF and TNF) in PBMCs cultures of CL patients and HC grouped according to their genotypes. The patient group produced higher levels of all cytokines tested when compared to controls (Fig. 1). The secretion levels of IL-12p70 in the control group were not quantified because it was below of the minimum levels of assay detection (31.25 pg/mL). After stratification of patients according to their genotypes no differences were observed for IL-10, IL-12p70, TNF and MIF secretion in leishmania stimulated PBMC cultures. However, among controls the −173C carriers produced lower levels of MIF when compared to non-carriers (4.3 ± 4.4 ng/mL vs. 14.5 ± 7.7 ng/mL; $p = 0.01$).

4. Discussion

This study set out to investigate the effects of cytokine polymorphisms on leishmaniasis outcome. We first selected the *IL10* gene, which had been previously found to be associated with leishmaniasis and other infections [23,17] and also tested other candidate genes such as *MIF*, *IL12* and *TNF* due to their association with other infectious diseases caused by intracellular pathogens [24–32].

The present study using a sample from Southern Brazil did not replicate the association of the *IL10* −819C>T SNP which had been previously described in the Northeast [17]. The present data indicates that IL-10 is neither associated with cutaneous leishmaniasis

Table 1
Genotypic and allelic frequencies for the *IL10* −819C>T, *IL12*+1188 A>C, *MIF* −173G>C and *TNF* −308G>A SNPs in CL patients and HC.

SNP	Genotype/allele	Patients*	Healthy controls*	OR (95%CI; *p*-value)	OR(95%CI; *p*-value)**
IL-10	CC	40 (0.38)	237 (0.42)	Reference	Reference
−819C>T	CT	54 (0.51)	248 (0.43)	1.29 (0.82–2.01; 0.26)	1.25 (0.80–1.97; 0.31)
	TT	12 (0.11)	86 (0.15)	0.82 (0.41–1.64; 0.58)	0.77 (0.38–1.55; 0.47)
	Total	106	571		
	C*	134 (0.63)	722 (0.63)	Reference	Reference
	T	78 (0.37)	420 (0.37)	1.00 (0.65–1.53; 0.99)	0.97 (0.62–1.49; 0.89)
	T carriers	66 (0.62)	334 (0.58)	1.17 (0.76–1.79; 0.46)	1.13 (0.73–1.73; 0.56)
IL-12	AA	58 (0.57)	381 (0.56)	Reference	Reference
+1188A>C	AC	34 (0.34)	252 (0.37)	0.88 (0.56–1.39; 0.60)	0.87 (0.55–1.37; 0.56)
	CC	9 (0.09)	49 (0.07)	1.20 (0.56–2.58; 0.62)	1.20 (0.55–2.58; 0.63)
	Total	101	682		
	A*	150 (0.74)	1014 (0.74)	Reference	Reference
	C	52 (0.26)	350 (0.26)	1.00 (0.62–1.61; 0.98)	0.99 (0.61–1.60; 0.98)
	C carriers	43 (0.43)	602 (0.44)	0.93 (0.61–1.43; 0.76)	0.92 (0.60–1.41; 0.72)
MIF	GG	47 (0.50)	389 (0.64)	Reference	Reference
−173G>C	GC	35 (0.37)	186 (0.31)	1.55 (0.97–2.49; 0.06)	1.54 (0.96–2.47; 0.07)
	CC	12 (0.13)	30 (0.05)	3.31 (1.58–6.90; 0.001)	3.43 (1.64–7.20; 0.001)
	Total	94	605	($p = 0.005$)***	($p = 0.005$)***
	G*	129 (0.69)	964 (0.80)	Reference	Reference
	C	59 (0.31)	246 (0.20)	1.79 (1.11–2.89; 0.01)	1.80 (1.11–2.91; 0.01)
	C carriers	47 (0.50)	216 (0.36)	1.80 (1.16–2.78; 0.008)	1.79 (1.15–2.78; 0.008)
TNF	GG	80 (0.77)	501 (0.76)	Reference	Reference
−308G>A	GA	18 (0.17)	148 (0.22)	0.76 (0.44–1.31; 0.32)	0.75 (0.44–1.30; 0.32)
	AA	6 (0.06)	12 (0.02)	3.13 (1.14–8.57; 0.02)	3.10 (1.12–8.53; 0.02)
	Total	104	661		
	G*	178 (0.86)	1150 (0.87)	Reference	Reference
	A	30 (0.14)	172 (0.13)	1.12 (0.62–2.03; 0.69)	1.12 (0.61–2.03; 0.70)
	A carriers	24 (0.23)	160 (0.24)	0.93 (0.57–1.53; 0.80)	0.93 (0.57–1.52; 0.79)

* Results are shown as total counts (frequency).
** Results adjusted for the covariate sex.
*** Overall *p*-values for genotype comparisons (2 degrees of freedom). CI: confidence interval, SNP: single nucleotide polymorphism.

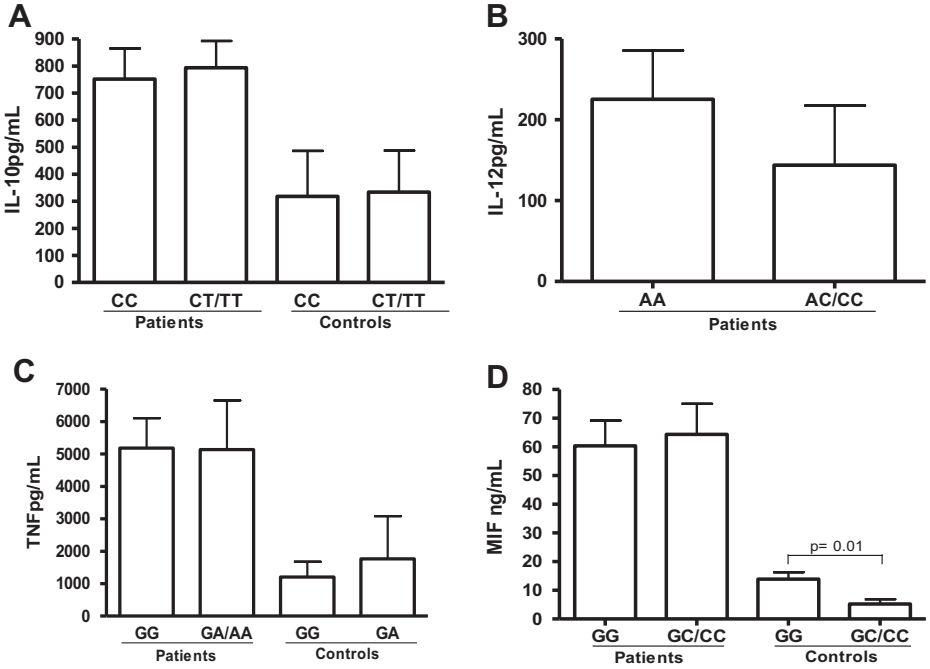

Fig. 1. Leishmania induced-cytokine production according to genotypes. Peripheral blood mononuclear cells (PBMCs) of cutaneous leishmaniasis patients and healthy controls according to different genotypes were cultured with *Leishmania* antigens and supernatants were collected after 72-h for IL-10 quantification (A), after 24-h for IL-12p70 quantification (B) and after 120-h for TNF (C) and MIF (D) quantification by ELISA. Results are shown for each sample (pg/mL or ng/mL). Each bar represent mean and SEM of each group.

nor regulates IL-10 secretion. It can be suggested that interaction between different *L. braziliensis* strains and different host genetic backgrounds may lead to distinct final outcomes.

When candidate genes were tested, the genetic epidemiological and functional analysis of the *IL12* +1188A>C SNP did not show any association with leishmaniasis, although patients presented a

heterogeneous production of IL-12 with high and low producers that did not cluster according to +1188A>C genotypes. A very interesting result was the association of the TNF −308AA genotype with cutaneous leishmaniasis. Functional analysis suggests that TNF −308A carriers secrete subtly higher amounts of TNF, although this data did not show statistical significance. Due to the low frequency of the AA genotype, it is still unclear as to how −308AA individuals respond to *Leishmania* antigens. It has been shown that TNF has a broad range of functions, both in the resistance or immunopathology of several infectious diseases, such as Chagas disease, leprosy, malaria and tuberculosis [33,34,31,35–37]. In ATL, exacerbated levels of TNF were correlated with extensive tissue damage among mucosal leishmaniasis (ML) patients, the most severe form of ATL [38,8,39,40]. Additionally, it was documented that carriers of this allele produce higher levels of TNF protein, although results are still controversial [41,42]. TNF −308A have been systematically associated with autoimmune, metabolic, infectious and neurodegenerative diseases [43,44]. We can, therefore, hypothesize that TNF −308AA carriers are high producers of TNF in leishmaniasis. In this situation, strong localized secretion of TNF in −308AA individuals could be deleterious, since high TNF levels would be mediating immunopathology, tissue damage and, consequently, disease outcome.

The macrophage migration inhibitory factor (MIF) has been described as an important component of the host antimicrobial response and promotes the activation and secretion of pro-inflammatory cytokines, including IFNγ and TNF, by immune cells [45,12]. MIF also increases macrophage survival through inhibition of P53 activity, thus reducing activation-induced apoptosis [13], which might affect the immune response to intracellular pathogens.

For leishmaniasis, a study by Satoskar et al. [46] showed that MIF gene-deficient mice ($MIF^{-/-}$) were highly susceptible to cutaneous *Leishmania major* infection, developing severe skin lesions late after infection exhibiting greater parasite burdens than wild-type (WT) mice. Curiously, similar proliferative responses and production of IL-4 and IFNγ cytokines among T-cell from mice ($MIF^{-/-}$) and WT were observed. However, IFNγ-stimulated macrophages harvested from $MIF^{-/-}$ mice had a defective parasite clearance which was associated with low superoxide and nitric oxide levels. This finding suggests that susceptibility to *L. major* in $MIF^{-/-}$ mice is due to an impaired macrophage leishmanicidal activity rather than deregulation of Th1 and Th2 responses. Thus, it is also possible that MIF has a similar biological function in humans enhancing the activation of macrophages and contributing for parasite clearance.

Genetic studies have associated the *MIF* −173C allele with increased susceptibility to infections, [47] such as Chagas' disease [28], tuberculosis [48,26,30] and severe malaria [27,31]. Our data corroborate these previous findings by showing that the *MIF* −173C allele is also associated with CL susceptibility. Thus, it is appealing that MIF play an important role during the innate immune response against intracellular pathogens. Nevertheless, data regarding the functional influence of this SNP are still controversial, with studies reporting lower levels of MIF mRNA or protein among −173C carriers [27,49,47], while other authors showed otherwise [50,51]. Here, our results showed that the MIF −173C allele was able to induce less MIF secretion in cell culture supernatants of healthy controls stimulated with *Leishmania* antigens. However, this effect was not observed when cells from CL clinical cured subjects were investigated. Temple and coworkers [49] found increased basal levels of MIF mRNA among *MIF* −173C carriers, although cells stimulated *in vitro* did not induce different levels of MIF irrespective the donor's genotype. We hypothesize that the patients already have a steady programmed cellular response because of the previous development of leishmaniasis and, thus, the subtle effect of the −173C allele would not be able to influence MIF secretion at this stage. In fact, previous reports have showed that even years after cure, patients may continuously produce high levels of cytokines such as IFNγ [20], indicating that epigenetic factors may alter the expression of cytokines for a lifetime. Moreover, we cannot exclude the possibility of an interaction between additional polymorphisms, such as the MIF −794CATT5-8 microsatellite polymorphism, which might play a role in the regulation of MIF gene expression. Nevertheless, moderate linkage disequilibrium ($r^2 = 0.5–0.7$) has been demonstrated between MIF −794 and −173 polymorphisms in different populations, and it is likely that, at least in part, −173G>C is a good genetic tag of this region for the present study [48,52,53,27,47,51].

In summary, the genetic and biological data suggest that MIF plays a role in the very early events of the *L. braziliensis* infection by regulating its replication and controlling disease outcome. Our data suggest that the *MIF* −173C allele is associated with lower levels of MIF, and this decreased production may be associated with leishmaniasis susceptibility [54]. This deficient production of MIF may reflect the difficulty of these individuals to activate an inflammatory response, with increased release of TNF and IFN-γ, macrophage activation, nitric oxide production and elimination of pathogens.

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