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Study of association of the rs2275913 *IL-17A* single nucleotide polymorphism and susceptibility to cutaneous leishmaniasis caused by *Leishmania braziliensis*

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ABSTRACT

Cutaneous leishmaniasis (CL) caused by *Leishmania braziliensis* is the most spread clinical form of leishmaniasis in Brazil. However, only a few part of the people infected develop clinically perceptive disease, suggesting the influence of human genetic components in the CL pathogeny. The rs2275913 SNP is the nucleotide variant of the *IL17A* gene. The A allele is associated with a vast number of infectious and non-infectious diseases. Here, we investigated the association of the rs2275913 SNP (G/A) from *IL-17A* and two forms of susceptibility to CL in Brazil by case-control study. Furthermore, we evaluated the functional relevance of this SNP during the immune response of the host and analyzed its impact in the parasite elimination. Weak associations of A allele with susceptibility to *L. braziliensis* infection or to symptomatic CL were observed, and a tendency of A allele carriers to be more susceptible to infection and cutaneous disease. Functional analysis of the Th17 cell phenotypes revealed lower frequencies of CD4+ IL-17+ cells in samples of infected people with AA/AG genotypes. Furthermore, people carrying the A allele maintain higher parasite loads, reinforcing the genetic susceptibility findings. This study adds knowledge about the influence of a significant genetic variation on IL-17 promoter on CL pathogenesis, and may contribute to enhance the knowledge about the role of IL-17 in the *L. braziliensis* infections.

1. Background

Cutaneous leishmaniasis (CL) is worldwide the most prevalent clinical form of leishmaniasis which can be caused by many species of *Leishmania*, distributed differently by geographical areas. Around 90% of all CL cases occur in sub-developed or developing countries like Brazil, where *Leishmania braziliensis* is the most spread specie, causing mainly the localized form of CL [1,2]. In Brazil 26,008 cases/year were reported in the last decade [1]. However, seroprevalence studies indicate that only a very few part of the individuals infected with parasites of the *Viannia* subgenus (*L. braziliensis*, *L. guyanensis*, *L. panamensis*, and *L. peruviana*) develop skin lesions that characterize the cutaneous disease [3].

The T CD4+ lymphocytes, also called T-helper (Th) cells, have a central role in the immune responses against *Leishmania* sp. The differential development of Th1 or Th2-type responses translates directly to the spectrum of clinical presentations of CL in patients infected, which can become asymptomatic or develop disease with different severity grades [4]. A successful immune response, which allows parasite elimination without disease development, requires a balance between the activation of IFNγ/TNF-producing Th1 cells, and the secretion of the anti-inflammatory cytokine IL-10 by Th2 cells [5]. It is well established that clinical outcomes of CL caused by *L. braziliensis* are due to exacerbated Th1 activation, leading to decreased parasite load, but causing severe tissue damage [6].

Studies of Th17 cells participation on CL have shown that these cells perform a crucial role in the establishment of infection and in the outcome of infection to disease. Th17 cells act through granulocyte recruitment [7] and unbalance the Th1/Th2 equilibrium [8]. The T CD4+ IL17+ lymphocites are characterized by the production of the

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interleukins (IL)-17A and F, which the master is IL-17A, commonly referred simply as IL-17 [9]. IL-17 cooperates with other cytokines secreted by Th17 (such as IL-21 and IL-22) to induce inflammation, by the recruitment of neutrophils [10]. The presence of CD4 + IL-17 + cells has been found in association with severe CL pathology [8,11] or with self-healing CL in mice [12] and human subclinical *L. braziliensis* infections [13]. Furthermore, although IL-17 has been proven to be important to enhance inflammation, its effectiveness in parasite killing is not a consensus [8,12]. Thus, the participation of IL-17 in the development of symptomatic CL and its effectiveness in parasite control are still not well described.

The host's genetic background influences the TCD4 + cells activity, since mutations can alter the cytokines expression [14]. The 197A allele is a result of a single nucleotide polymorphism (SNP) rs2275913 (-197G/A) in the promoter region of the *IL-17A* gene. The rs2275913 SNP is located within a binding motif for the nuclear factor of activated T cells (NFAT), which is a critical regulator of the *IL-17A* transcription [15]. Thus, this important SNP has been associated with autoimmune [16–19] and infectious diseases as tuberculosis [20,21], hepatitis [22] and Chagas' disease [23]. Although the role of IL-17 in visceral leishmaniasis (VL) and CL has been explored in many studies (reviewed in Gonçalves-de-Albuquerque et al. [24]), the association of the rs2275913 A allele with CL was still not reported.

The aim of this study was to evaluate the potential association between the *IL-17A* rs2275913 (-197G/A) polymorphism and susceptibility to CL in Brazil. Due to the complex immunopathogeny of CL which depends on innate and adaptive immune components to express clinically the disease, different definitions of host susceptibility were assumed: (1) from being infected by *L. braziliensis* to develop clinically relevant disease, and (2) from being exposed to infected sandflies in endemic areas to acquire infection. Moreover, we also evaluated the functional relevance of the rs2275913 IL-17A SNP in the Th17 differentiation and IL-17 secretion, and its impact on parasite loads of the human hosts infected by *L. braziliensis*.

2. Materials and methods

2.1. Subjects and case-control distributions

Individuals coming from infectology and dermatology ambulatories of Oswaldo Cruz Hospital and people living in nine endemic areas of the Pernambuco state, Brazil, were recruited in this study. To study the susceptibility from the infection to the development of disease clinically perceptive, individuals with active disease (i.e. ulcerated localized skin lesions) and/or CL characteristic scars (history of disease development) were assumed as cases (symptomatic/susceptible group - S) and those infected (positive molecular test) but without active or former CL lesions were assumed as controls (asymptomatic/resistant group - A). In a second context of susceptibility, from exposure to *L. braziliensis* (individuals in contact with infected vectors in endemic areas) to infection establishment, healthy non-infected (negative molecular test) people living in endemic areas were set up as the uninfected control group (UC) and were compared to those infected (INF) (all symptomatics and asymptomatics individuals). For the functional study of immune response, a new sample collection of these people that participated in the association study was taken. For this stage, healthy individuals and *L. braziliensis*-infected patients with or without CL lesions, of both sexes and age > 15 years were included. Transplanted, immunosupresed, pregnant and people with chronic disease such as cancer and diabetes were not included. All peolple in this study signed a free informed consent term. This research was approved by the National Commission for Research Ethics (CONEP- n° 1090864).

2.2. Molecular diagnosis of *L. braziliensis* infection and parasite load quantification

The quantitative real-time polymerase chain reaction (qPCR) was used as laboratory diagnostic criteria indicative of infection and as method of quantification of parasite load. In the absence of Montenegro Skin Test (MST) in Brazil, (which had its production stopped since 2013), and due to the low sensitivity of other immunological diagnostic methods to CL [25], the qPCR was used to diagnose previous or actual infection by *L. braziliensis*. For this purpose and for genotyping assays, 4 mL of whole blood were collected and conditioned in tube with EDTA anticoagulant (Vacutainer). The extraction of DNA from blood was realized with QIAamp DNA Blood mini kit (Qiagen) following manufacturer's instructions. The extracted DNA had its quality tested by G3PD amplification [26], which assures the non-degradation of the sample before the diagnosis. For the detection and quantification of parasite loads of *L. braziliensis* 5 μM of primers *kDNA1f/r* were used according to the protocol described by Paiva-Cavalcanti et al. [27], using SYBR Green (Applied Biosystems) PCR master mix. For calculation of the parasite loads, a 1:10 dilution curve of *L. braziliensis* genomic DNA (strain MHOM/BR/1975/M2903) was used as standard. The reactions of qPCR were performed on the ABI PRISM 7500 (Applied Biosystems) equipment. Parasite loads calculation and analysis of the results were done using the software ABI PRISM 7500 version 2.0.6.

2.3. IL-17 rs2275913 SNP genotyping

The *IL-17A*-197G/A genotyping was performed by PCR-restriction fragment length polymorphism (PCR-RFLP) following the protocol of Wu et al. [28]. Primer sequences for *IL-17A*-197G/A are *IL-17f* 5′-AAC AAG TAA GAA TGA AAA GAG GAC ATG GT-3′ and *IL-17r* 5′-CCC CCA ATG AGG TCA TAG AAG AAT C-3′. 100 ng of genomic DNA extracted from patients blood were added in a total volume of 25 μL PCR master mix containing 1 μM of each primer, 200 μM of each dNTP, 2 mM of $MgCl_2$ and 1UTaq DNA polymerase and 10x Taq buffer (Invitrogen) using the Eppendorf Mastercycler gradient (Eppendorf). PCR products were digested 1 h at 37 °C with EcoNI (*XagI*) (Fermentas) and then separated by 3%agarose gel electrophoresis. Digested PCR products generated 102 pb, 68 pb and 34 pb fragments depending on sample's genotype. To confirm the genotyping results, all PCR products were examined by sequencing using the same *IL-17* primer set. The sequences were analysed using the software BioEdit (mbio.ncsu.edu/bioedit/bioedit.html), and compared to the rs2275913 sequence available on dbSNP (ncbi.nlm.nih.gov/SNP).

2.4. Leishmania soluble antigen preparation

Leishmania soluble antigen (LSA) extracts were prepared using a protocol adapted from Chamakh-Ayari et al. [29] and used in cell culture. Promastigote parasite cultures of *L. braziliensis* (strain MHOM/BR/75/M2903) were washed in 1 × PBS, centrifuged at 800*g*/15 min at 4 °C and supernatants were removed. The pellets were resuspended in lysis buffer (50 mM Tris, 5 mM EDTA) 1×10^9 parasites/mL. The parasite solution was subjected to 3 rapid freeze-thaw cycles followed by 6 pulses of 20 s/40 W with sonicator. Samples were centrifuged at 10,000*g*/20 min at 4 °C, and supernatants were collected. Protein quantification was performed using Bradford method, described elsewhere [30].

2.5. Cell cultures

For cell cultures, peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood layered over a Ficoll-Hypaque gradient (Amersham Biosciences). The PBMC concentrate was washed in phosphate buffered saline (PBS) pH 7.2 and ressuspended in Roswell Park Memorial Institute 1640 complete medium with 10% bovine

serum albumin (BSA) (Sigma) and 1% penicilin 100 UI/ml and streptomicin 100 mg/mL at a concentration of 2×10^6 cells/mL. These cells were kept with pure culture medium (unstimulated) or stimulated with 10 μg/mL of phytohemagglutinin (PHA) (Cultilab) as a positive control for 72 h at 37 °C in a 5% CO_2 incubator. To allow later flow cytometry experiments, Brefeldin A (10 μg/mL) (Sigma-Aldrich) was added 4 h prior to the end of culture time. After the incubation, the culture supernatants were collected and stored at −20 °C until analysis.

2.6. Th17 cells and IL-17 flow cytometry detection

Cultured cells were incubated at room temperature with EDTA (20 mmol/L) (Sigma Aldrich) and then washed in filtered PBS added with 0.5% BSA and 0.1% sodium azide (PBS-wash solution). For intracellular cytokine staining, cells were permeabilized with BD Perm/Wash™ buffer (BD Biosciences). Samples were then incubated at room temperature for 30 min with fluorophore-marked antibodies specific against CD4 (PerCP, Life Technologies). Intracellular staining was performed to determine IL-17A (PE, BD Pharmingen) and IFNγ (APC, BD Pharmingen) presence. Cell staining was finished by fixation with BD CytofixTM solution (BD Biosciences). Samples were analysed on a FACS Calibur flow cytometer (Becton Dickson) with a minimum of 20,000 gated events. Lymphocytes were selected in a gate using the SSC × FSC dot plot. A second gate selected CD4/IL-17 double-stained cells (Th17) to generate representative 2-dimensional graphics of IFNγ + (alternative) and IFNγ- (classic) Th17 cells. The measurement of IL-17 secretion in supernatants was performed using the BD™ CBA Human IL-17A Flex Set (Becton Dickinson), acquired on FACS Calibur flow cytometer (Becton Dickinson) equipment according to the manufacturer's instructions. Results were analysed using the FCAP Array 1.0 software (Becton Dickinson).

2.7. Statistics

Sample size calculation was performed by EpiInfo 6.0 software [31] to get a test power of 2.5 in the case-control study. A sample of at least 89 individuals in each group was estimated. The genotype and allele frequencies were obtained by direct counting. Hardy-Weinberg (HW) equilibrium was tested between groups separately, comparing expected and observed frequencies (Chi-Square (X^2) test). Comparisons of the distributions of the allele and genotype frequencies between cases and controls were performed using the Chi-Square test for homogeneity. The level of association between the rs2275913 genotypes with cases and controls were estimated as Odds Ratios (OR) with 95% confidence intervals (CI) [32]. The quantitative data were tested for normality with D'Agostino test. Results were expressed as medians for non-parametric data, and as means ± standard deviation (SD) for parametric data. Mann-Whitney or Kruskal-Wallis (followed by Dunn's multiple comparison post-test) tests were used to analyze differences between non-parametric, and T test/ANOVA for parametric samples groups. All statistical analysis were performed using GraphPad Prism 5.0 (GraphPad Software). P values < 0.05 were considered statistically significant.

3. Results

3.1. Subjects and case-control distributions

To evaluate the association between the SNP *IL-17A* rs2275913 and susceptibility to CL in a Brazillian population, 365 samples of individuals living in endemic areas of Pernambuco, Brazil, were collected between the years 2014 and 2017. 101 (27.6%) of the subjects presented from 1 to 6 active ulcerated lesions and/or scars, that had been previously diagnosed as CL. From the entirety of the samples, 3 were excluded from analysis due to low DNA quality (G3PD) assessed by the molecular test. 160 (44.1%) of the qualified samples were positive in the kDNA1 qPCR. According to the combination of clinical and molecular diagnostics 88 samples were grouped in S, 91 in A and 186 in the uninfected control group (UC). Later re-distribution of qPCR-positive samples resulted in 179 compounding the "infected" (INF) group. Demographic characteristics of groups are disposed in Table 1. The number of recruited individuals of each sex was homogeneous in the S and A groups ($p > 0.05$) but the proportion of male sex was higher among the INF comparing to the UC ($p < 0.05$). Age varies significantly comparing between S and A ($p < 0.05$) but not between INF and UC ($p > 0.05$).

Table 1
Demographic characteristics of symptomatic and asymptomatic *L. braziliensis*-infected patients and uninfected people living in endemic areas recruited for the association study of the SNP rs2275913 with cutaneous leishmaniasis.

		Case	Control	*p* value
		Symptomatic (S)	Asymptomatic (A)	
Age (years)[a]		35.75 ± 16.10	29.70 ± 18.73	*0.030*
Sex[b]	Male	49 (27.37%)	47 (26.25%)	0.588
	Female	39 (21.78%)	44 (24.58%)	
		Infected (INF)	Uninfected (UC)	
Age (years)[a]		32.69 ± 17.69	33.22 ± 17.45	0.776
Sex[b]	Male	96 (26.30%)	69 (18.90%)	*0.001*
	Female	83 (22.73%)	117 (32.05%)	

[a] P values were calculated by the T test for unpaired samples.
[b] P values were calculated by numbers of individuals in Chi-Square test for categorical variables.

3.2. Associations of IL-17A rs2275913 SNP and susceptibility to CL

Samples of 345 subjects were genotyped by the PCR-RFLP and sequencing as described before. The Fig. 1 shows the distribution of genotypic and allelic frequencies observed in study groups (S, A, INF and UC). Chi-Square tests for homogeneity showed that neither S *versus* A nor INF *versus* UC populations were different regarding the proportion of genotypic and allelic frequencies ($p > 0.05$). The A allele and the AA genotype were found in an expected frequency in all case-control groups. Genotypes in all populations were in Hardy-Weinberg (HW) equilibrium (S, $p = 0.114$; A, $p = 0.705$; INF, $p = 0.137$; UC, $p = 0.337$).

Odds ratios were calculated to estimate the association between the *IL-17A*-197 G/A SNP genotypes and the susceptibility to CL in the two case-control settings: at first, susceptibility from being infected by *L. braziliensis* to present clinically perceptive CL lesions (S *versus* A), and later from being exposed to *L. braziliensis*-infected vectors (endemic areas) to acquire infection (INF *versus* UC) (Table 2). No significant association was observed between the rs2275913 with CL in both case-control settings (all ORs were $p > 0.05$).

Although no significant associations were found in the distribution of genotypes between S and A, the analysis of OR in heterozygous A carriers (OR = 1.35) suggests higher risk of developing clinically relevant disease through *L. braziliensis*. Interestingly, AA subjects present lower risk when compared to AG (OR = 1.02). Analyzing all the carriers of the A allele (AG/AA set) and the allelic model of association, lower susceptibilities to disease development are also presented (ORs = 1.27 and 1.12, respectively), compared to the heterozygotes alone (AG). Regarding the infection acquisition (INF *versus* UC), AA individuals presented the high γ susceptibility (OR = 1.47), when compared to AG and whole A carriers (ORs = 1.27 and 1.30, respectively). These data suggest that homozygote A allele owners should be at higher risk of *L. braziliensis* infection acquisition and maintenance, but not in clinically relevant CL development.

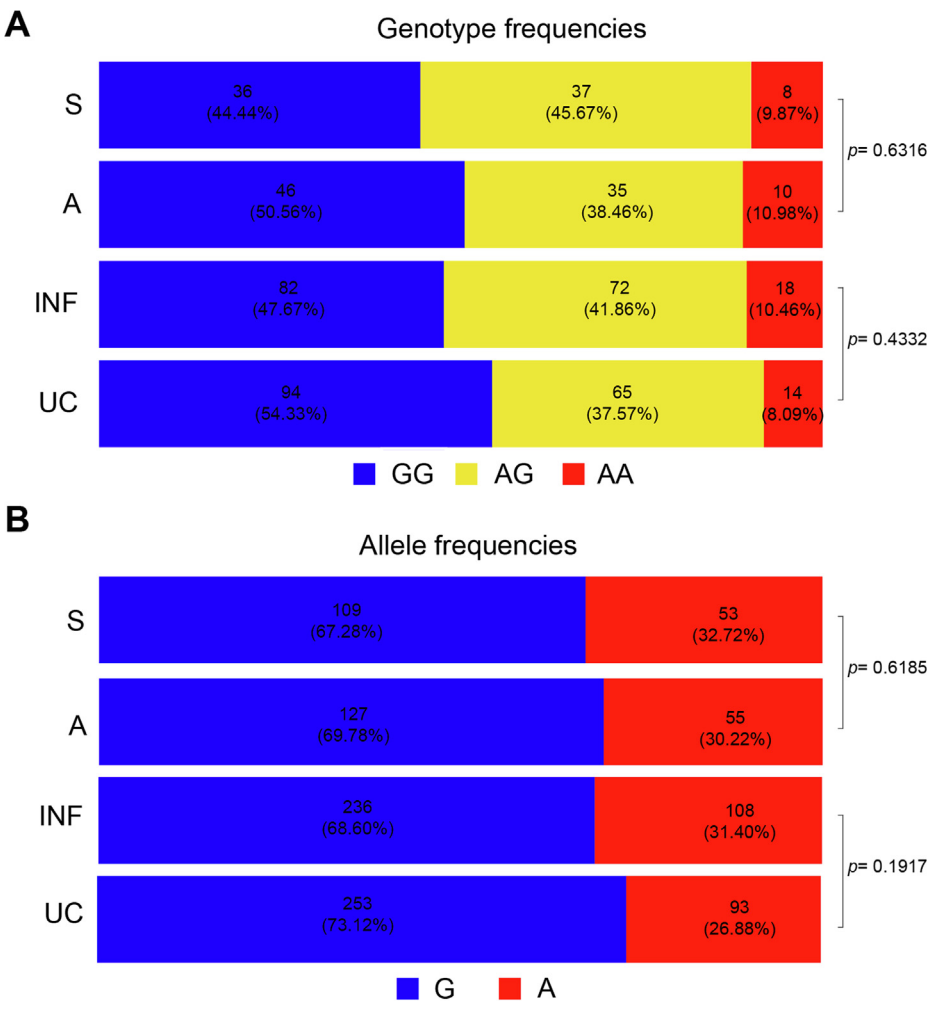

Fig. 1. Genotypic and allelic frequencies of the *IL-17A* rs2275913 SNP in symptomatic and asymptomatic *L. braziliensis*-infected patients and uninfected people in endemic areas of Brazil. Graphic schemes representing the proportions of genotypic (A) and allelic (B) distribution of the rs2275913 SNP in the case-control groups. Each whole bar corresponds to 100% of S (symptomatic), A (asymptomatic), INF (all *L. braziliensis*-infected symptomatic and asymptomatic people) and the UC (uninfected controls) group. The number of individuals and the relative frequencies (in parentheses) are detailed. Chi-Square test was applied to compare the proportions of genotypes and alleles between case-control sets. All groups are in Hardy-Weinberg (HW) equilibrium.

Table 2
Association of the rs2275913 SNP with susceptibility to cutaneous leishmaniasis and infection by *L. braziliensis* in Brazilian population, measured by Odds Ratios.

Allele/Genotype	OR (IC 95%)	*p* value
CL symptomatic/asymptomatic		
A allele(*x* G)	1.12 (0.71–1.77)	0.618
AG/AA	1.27 (0.70–2.33)	0.424
AG	1.35 (0.71–2.55)	0.354
AA	1.02 (0.36–2.85)	0.967
GG	1	
L. braziliensis-infected/uninfected		
A allele(*x* G)	1.24 (0.89–1.73)	0.917
AG/AA	1.30 (0.85–1.99)	0.236
AG	1.27 (0.81–1.98)	0.307
AA	1.47 (0.69–3.14)	0.341
GG	1	

P values calculated comparing the proportion of A allele and genotype A carriers and the GG CI: 95% confidence intervals.

3.3. Th17 phenotypes and IL-17 in individuals carrying the rs2275913 SNP

A subpopulation of 32 individuals from those who participated in the association study was recruited for the analysis of functional alterations caused by the rs2275913 SNP. The description of this new sample of individuals is presented in the Table 3. The proportions of IL-17-producing lymphocytes were compared between A carriers and non-carriers (GG) to evaluate the influence of the rs2275913 SNP in the proportion of Th17 subsets and in the IL-17 secretion by PBMCs of INF and UC subjects. Fig. 2 shows comparisons of classic and alternative Th17 cells percentiles between A allele carriers and non-carriers.

The analysis of CD4+ IL-17+ subtypes by genotypes in UC group revealed significantly higher proportions of Th17 cells in A allele carriers than in GG (Fig. 2D and E). However, in *L. braziliensis*-infected individuals, the presence of the A allele was not sufficient to significantly alter the amount of CD4+ IL-17+ alternative (IFNγ+) and classical (IFNγ-) cells (Fig. 2A and B, respectively). In spite of these differences, the secretion of IL-17 in our PBMC supernatants of AA/AG was not significantly higher both INF and UC groups (Fig. 2C and F, respectively). All *p* values are disposed in the Fig. 2.

To show differential influence of the rs2275913 SNP in the lymphocyte population of INF and UC, the proportions of alternative and classic Th17 cells in A allele carriers with and without *L. braziliensis* infection were compared. Fig. 3 shows comparisons of the whole CD4+ IL-17+ cell population between infected and uninfected individuals carrying the A allele. The Fig. 3A shows that both CD4+ IL-17+ IFNγ+ and CD4+ IL-17+ IFNγ- are less present in the AA/AG individuals of INF group, suggesting that the presence of the rs2275913 SNP downregulates the expression of IL-17A in samples of *L. braziliensis*-infected, but not in samples of UC. Furthermore, this difference was not observed when Th17 cells were compared between INF and UC wild genotypic individuals (GG) (alternative Th17, $p = 0.171$; classic Th17, $p = 0.352$), suggesting that in individuals infected the rs2275913 *IL-17* SNP alters the dynamics of Th17 cells. This alteration appeared to change when the secretion of IL-17 was compared between INF and UC A carriers (Fig. 3B), in spite of the no significance of the statistical

Table 3
Characteristics and genotype frequencies of the subjects recruited for the immunological analyses of *IL-17A* rs2275913 SNP and susceptibility to cutaneous leishmaniasis in endemic areas of Brazil's Northeast.

	S	A	INF	UC	Total
Number of samples	2	10	12**	20	32
Age (years)	47.00 ± 9.89	49.00 ± 19.01	48.67 ± 17.47	29.85 ± 15.46	36.90 ± 18.45
Male/Female	1/1	3/7	4/8**	4/16	8/24
IL-17A rs2275913 SNP					
AA	02	4	6**	5	11
AG	0	1	1**	9	10
GG	0	5	5**	6	11

S: symptomatics - individuals infected with *L. braziliensis* that present clinically the cutaneous leishmaniasis. A: asymptomatics - individuals infected with *L. braziliensis* that do not present clinically the cutaneous leishmaniasis. INF: allindividuals infected with *L. braziliensis* (the sum between symptomatics and asymptomatics). UC: healthy uninfected controls.

** Numbers not counted in the totalsumbecause they correspond to the number of S plus A individuals.

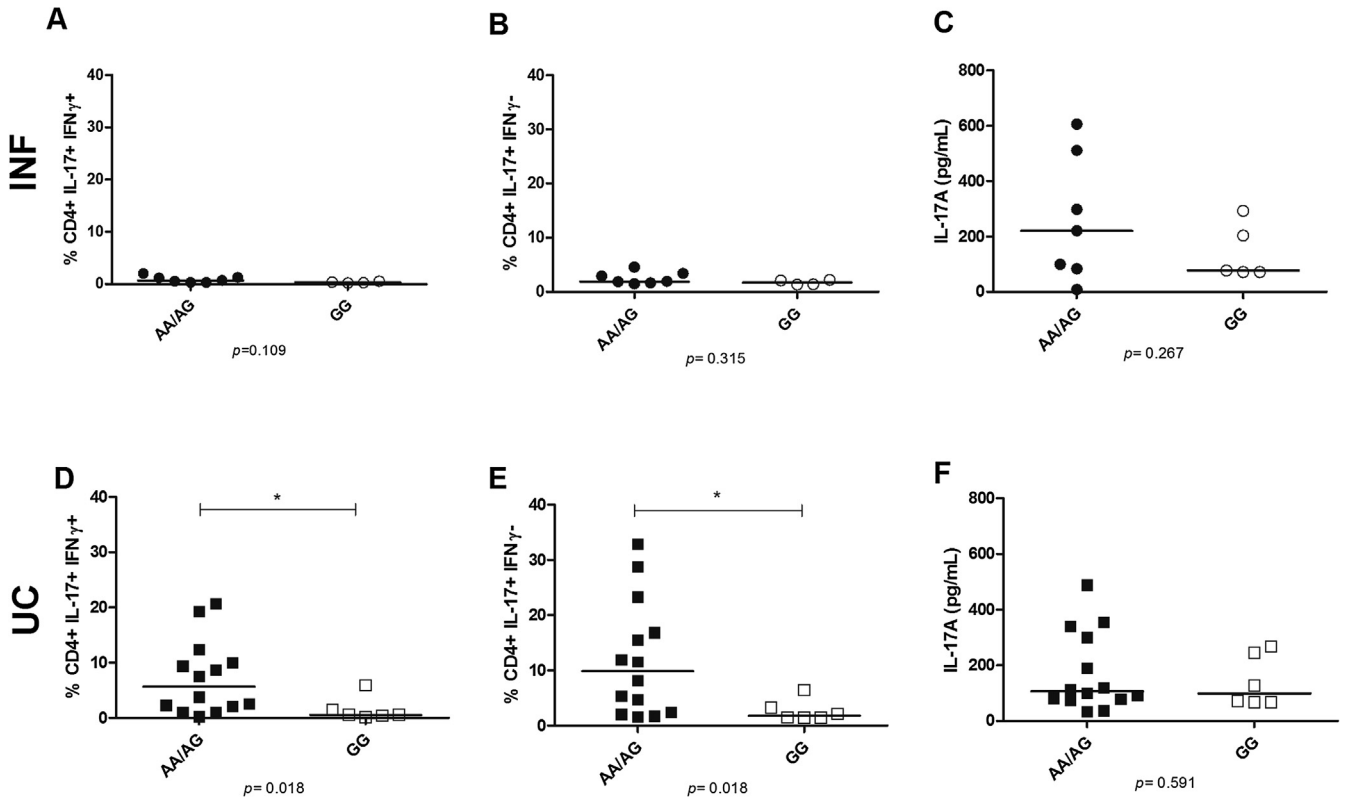

Fig. 2. Th17 phenotypes in PBMC stimulated *in vitro* culture supernatants from A allele carriers (SNP rs2275913) in a population living in cutaneous leishmaniasis endemic areas of Brazil's Northeast. Alternative Th17: CD4+ IL-17+ IFNγ+ (A and D). Classic Th17: CD4+ IL-17+ IFNγ- (B and E). The concentration of IL-17 (pg/mL) secreted in cultured PBMC supernatants (C and F). All Th17 phenotypes were detected in individuals infected with *L. braziliensis* (In, represented by circles) (A, B, C) and in healthy uninfected individuals (UC, represented in squares) (D, E, F). Each circle/square in a graph represents a single sample. Percentiles of alternative or classics CD4+ IL-17+ cells were calculated from the total number of CD4+ lymphocytes. The bars indicate the medians. Medians were compared between A carriers and GG by the Mann-Whitney test. CD: Cluster of Differentiation.

analysis. The secretion of IL-17 in UC and INF GG individuals showed no difference ($p = 0.645$).

3.4. Correlation of rs2275913 SNP and parasite elimination

The parasite loads in all infected individuals were analyzed to evaluate possible effects of different genotypes in parasite elimination. Subjects owning the A allele (AA/AG) were detected with higher parasite loads than those with the wild genotype, although this difference was not statistically relevant in the parameters applied ($p = 0.397$) (Fig. 4A). The influence of the A allele in allowing the maintenance of higher parasite loads was also observed when the individuals of the group S ($p = 0.506$) and the group A ($p = 0.293$) were compared separately (Fig. 4B). This data reinforces the observation that in INF people the expression of IL-17 on Th17 cells should be suppressed, leading to parasite persistence and some higher susceptibility to infection.

4. Discussion

The rs2275913 SNP is the substitution of guanine for adenine in the 197th nucleotide position upstream of the starting codon of the *IL17A* human gene. Since IL-17 has a crucial role in allergic, autoimmune and infectious diseases this mutation has been associated with a vast number of pathologies [16,21,23]. Although the protective role of IL-17 against visceral leishmaniasis is currently accepted [33,34] and its

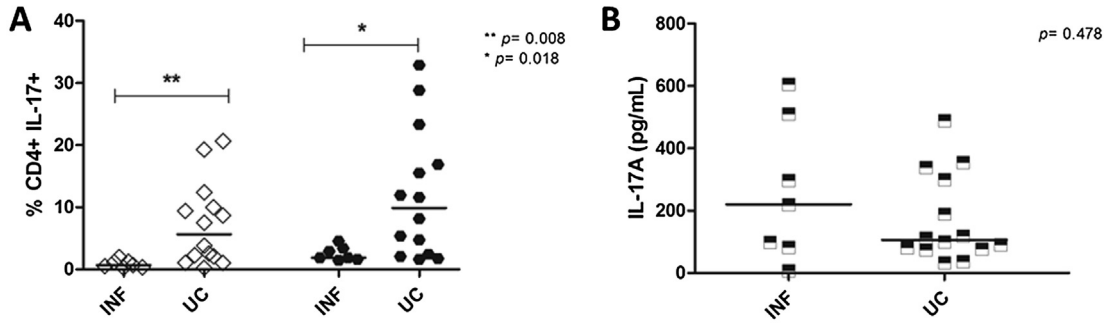

Fig. 3. Th17 cell in PBMC stimulated *in vitro* and IL-17 secreted from A allele carriers (SNP rs2275913) infected by *L. braziliensis* and in healthy uninfected controls from cutaneous leishmaniasis endemic areas of Brazil's Northeast. (A) Alternative (IFNγ+) Th17 in white diamonds; classic (IFNγ-) Th17 in black hexagons. Each diamond/hexagon in the graph represents a single sample. The bars indicate the medians. (B) IL-17 secreted in culture supernatants of INF and UC. Percentiles of alternative or classics CD4+ IL-17+ cells were calculated from the total number of CD4+ lymphocytes. Medians were compared between infected and uninfected people carrying A allele (AA/AG) by the Mann-Whitney test. CD: Cluster of Differentiation.

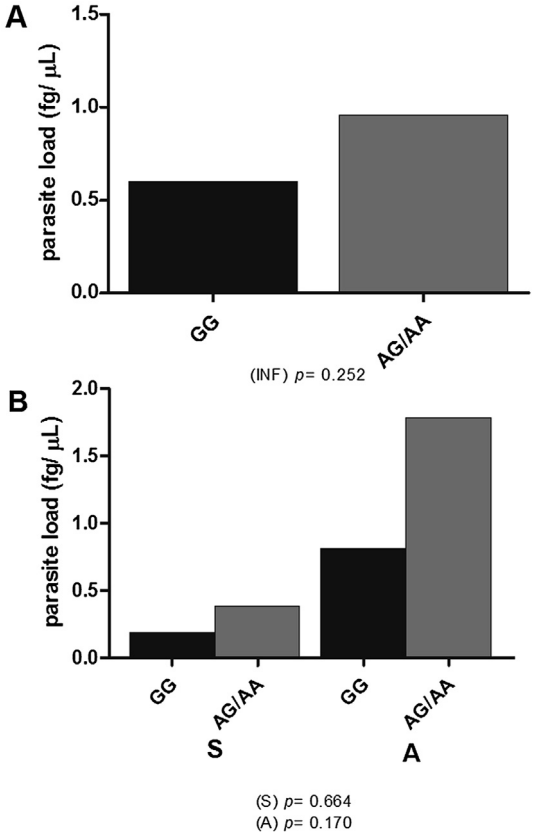

Fig. 4. *IL-17A* rs2275913 SNP and parasite loads of *L. braziliensis* in a population living in endemic areas for cutaneous leishmaniasis of Brazil's Northeast. Medians of parasite loads in subjects with different genotypes in the whole infected group (graph A) and then divided into symptomatics (group S)/ asymptomatics (group A) (graph B) were compared through Kruskall-Walis test and Dunn's post-test, IC = 95%.

involvement in the aggravation of CL is becoming evident [8,35], this study investigated for the first time the association of a genetic variant in *IL-17* with the clinical outcome of the cutaneous localized disease. We evaluated also the functional outcomes of this SNP in T-lymphocytes dynamics in *L. braziliensis*-infected people and healthy uninfected controls.

To assess the *L. braziliensis* infection in individuals living in endemic areas a high sensitivity (91.4%) molecular test was applied [26]. The high sensitivity of qPCR to detect low amounts of parasite DNA in blood samples with high specificity contributes to assure that infection has been established, even when signs and symptoms were not observed. In contrast, previous approaches as Castelluci et al. [36,37] have been using Montenegro skin test as single diagnosis method for the association of IL-6, CXCR1 and SLC11A1 SNPs with cutaneous and mucocutaneous leishmaniasis. Although both methods have high sensitivity, molecular tests present lower chance of cross reactions [24].

Analysis of the gender distribution between cases and controls (symptomatics and asymptomatics) indicated that gender is not influencing the development of clinical disease in the infected people studied. Despite this, it is known that a greater proportion of patients with CL are male, due to their higher occupational exposure (in agriculture, for example) to the vector, with 2.5–3.5 times greater incidence of the disease [38]. This was supported by the observation of data in our study: male sex individuals are more infected than female ($p < 0.001$, Table 1), although they do not necessarily develop CL. The comparison of ages between symptomatics and asymptomatics reveals that younger individuals are less susceptible to the development of clinical disease caused by *L. braziliensis*. A study by Shaw et al. [39], evaluating the susceptibility to infection by *L. peruvian* has estimated that individuals infected at a younger age are more susceptible to CL. The same appears to occur in *L. infantum/donovani* infections, which cause the visceral form of leishmaniasis [40]. A study by Adisson et al. [41] showed that individuals with more advanced ages develop stronger pro-inflammatory responses, and they have less ability to control inflammation. Due to the particularity of *L. braziliensis,* which in strongly stimulates the production of pro-inflammatory cytokines as IFN-γ, TNF and IL-6 than the other *Leishmania* species [42], younger individuals should be favored by their better ability to control inflammation, helping to maintain subclinical infection. Although the exposure to infection is associated to occupational activities, we did not observe differences by age in INF *versus* UC comparisons.

The risk analysis of the A allele calculated by ORs showed that this variant is not strongly associated with the susceptibility to disease development in infected people nor with susceptibility to infection. In leishmaniasis, vector salivary components (vector specie), parasitic virulence (which may vary intraspecies) and nutritional status are other determinants of susceptibility (not controlled in our sampling) [2] that may overlap the genetic background of the host. However, the strict observation of ORs suggests that in both contexts of susceptibility (infection/disease) the A allele could lead to higher risks of infection/clinical disease. The subsequent observation of Th17 depletion in INF group (Fig. 3) suggests that lower ability to control *L. braziliensis* multiplication may be responsible for this tendency in the association study, since the presence of IL-17 is linked to lower parasite burdens in previous studies [12]. Interestingly, the role of IL-17 is crucial for protection against *Trypanosoma cruzi*, which belongs to Kinetoplastids as *Leishmania* sp. In Chagas' disease, that is also a vector-borne

multifactorial disease, the IL-17 has been attributed to parasite control [43,44] or with more severe forms of cardiomyopathy [45]. The analysis of association between the SNP rs2275913 and the development of the cardiac form of Chagas disease showed no significant relationship of the A allele with the cardiomyopathy in the Colombian population studied [23] similarly to our results. Nevertheless, other studies have found strong associations of the same SNP with protection in bacterial diseases (which depends directly to neutrophils activity) in south America, due to higher IL-17 expression in the presence of the allele A [20,21].

The functional analysis of the genotypes showed different immunological behavior in A allele carriers. Higher percent of classic (IFN-γ-) and alternative (IFN-γ+) CD4+ IL-17+ cells were found in A carriers from UC group, compared to healthy controls from GG genotype. A previous well-designed study by Espinoza et al. [19] showed that the -197A allele provides increased NFAT affinity for the promoter site, resulting in longer permanence of the of the transcription factor in the promoter site and leading to more exuberant secretion of IL-17 [19]. The rs2275913 SNP is located in the promoter adjacent to the NFAT binding region. This location suggest that the rs2275913 SNP plays a functional role in the promoter activity of the IL-17 gene through influencing the transcriptional activity of NFAT, affecting specifically the production of IL-17 from T cells. These findings justify that in functional studies of the rs2275913 SNP the IL-22 analysis is not required. Although IL-22 may have synergistic or additive effects to IL-17, there are key differences regarding signaling pathways that determine the release of these two cytokines by immune cells. While NFAT is a critical regulator of the IL-17A transcription [15], it exerts no significant influence on signaling cascade required for IL-22 release [46]. Our results also corroborated the findings of Chen et al. [47] in which lymphocytes from healthy AA/AG patients stimulated *in vitro* by PHA secreted higher IL-17 levels than GG individuals. On the other hand, in INF group the presence of the A allele did not alter significantly the amount of cytokine produced compared to individuals with the wild genotype. Notably, post-translational or secretory regulation mechanisms may influence IL-17 detection on supernatants. Our protocol was not wide enough to determine which of these factors prevented the IL-17 secretion exacerbation on the A allele patients.

The analysis between INF and UC A carriers showed that classic and alternative Th17 cells are less present in the AA/AG individuals of INF group. This data suggests that in infected individuals the presence of A allele contributes to down regulate the Th17 differentiation. Nevertheless, higher IL-17 concentrations were found in PBMC supernatants of INF group. Previous studies have demonstrated that *Leishmania* sp. infections strongly stimulate IL-17 production [35]. Most of the studies measured the secreted IL-17, finding higher levels of the cytokine in supernatants of infected patients [11,48], corroborating our result. In addition, many T cell types are present in the PBMC resulting in diverse immunological interactions which may contribute to enhance or to block Th17 differentiation in *L. braziliensis* infection [49]. However, the mechanism by which the infection could alter the SNP outcome is not clear. Furthermore, other important polymorphisms which were not investigated in this study (i. e. IL-17F 7488T/C rs763780) [50] may be playing a role in cytokine secretion or function.

The functional role of IL-17 was evaluated by the parasite burdens in different genotypes. Higher concentrations of parasite DNA were observed in blood samples of individuals of AG and AA genotypes compared to GG. Aside of the previous analysis, that infected individuals produce less IL-17, this result helps to explain the tendency of association of the rs2275913 with susceptibility. IL-17A plays an important role in potentiating the pro-inflammatory cellular immunity [9,10]. Its participation has been attributed to spontaneous cure in humans and in C5BL/6 mice [12,13] and also with induced cure of *Viannia* subgenus infection [51]. Indeed, other studies related IL-17 only to increased inflammation, without interference on parasites killing, which would depend mainly on the macrophages activated by IFNγ [8,52]. However, positive correlation between IL-17 mRNA expression and IFNγ was reported in human samples [47], indicating that IL-17 may be required to lead to enough macrophage activation that allows the maintenance of subclinical infections.

5. Conclusions

This study evaluated for the first time the association between a genetic variant in IL-17 and the susceptibility to localized CL. In two different assumptions of susceptibility, the case-control association studies demonstrate a tendency of people who carry the A allele of the IL-17 rs2275913 SNP to develop clinically perceptive disease and to acquire infection. The functional evaluation of the SNP through Th17 cell types and IL-17 production in UC reinforced findings from previous studies which observed that the A alelle increases the production of IL-17 in healthy individuals. However it was observed that in individuals infected by *L. braziliensis* carrying the mutant allele the secretion of IL-17 is low, and these patients keep higher parasite loads, corroborating the results of the genetic analyses.

Here we show that the allele A is not strongly associated to susceptibility to CL, but should be linked to higher susceptibility to infection acquisition and to the outcome of infection to cutaneous disease. The results also suggest that in *L. braziliensis*-infected people the A allele suppresses the Th17 cell differentiation, while the same mutation enhances the presence of CD4+ IL-17+ cells in uninfected people. This study may contribute to better understanding of the immunopathogenesis of CL in humans and the role of IL-17 as an important modulator of the cellular response against *L. braziliensis*. The association of genetic characteristics with the development of clinical symptoms may provide subsidies for the rationalization and direction of therapeutic and prophylatic research.

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Declaration of Competing Interest

Authors declare no conflicts of interest.

Authors contributions

SCG-d-A, VMBL, and MP-C designed experiments. SCG-d-A, CNCO and VVAS performed experiments. SG-d-A wrote the paper. VMBL and MP-C revised critically the paper for scientific content. MP-C is the coordinator of the study. All the authors approved the final version to be published. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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