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Human leukocyte antigen-G 3′ untranslated region polymorphism +3142G/C (rs1063320) and haplotypes are associated with manifestations of the American Tegumentary Leishmaniasis in a Northeastern Brazilian population

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ABSTRACT

While the role of cytokine genes has been well documented in the context of *Leishmania (Viannia) braziliensis* infection, no studies have addressed the influence of human leukocyte antigen-G (HLA-G) in susceptibility/resistance to American Tegumentary Leishmaniasis (ATL). Here, we evaluated the influences of *HLA-G*, *IL-10*, *TNF-A* and *IFN-G* in the susceptibility and clinical manifestations of ATL.

DNA of 114 ATL patients and 346 healthy individuals were sequenced for well-documented polymorphisms in *HLA-G* 3′ untranslated region (UTR), in *IL-10* and *TNF-A* promoters and in *IFN-G* intron 1. Soluble HLA-G (sHLA-G) and cytokine levels were evaluated by ELISA and flow cytometry, respectively. Analyses were performed using GraphPad and R-package software.

Individuals bearing *HLA-G* +3142G/G showed an association with increased risk for ATL, whereas those carrying the *HLA-G* +3142C/G and one copy of UTR6 haplotype, showed an association with decreased risk for ATL. sHLA-G was overexpressed in *"susceptible"* patients compared to the *"resistant"* one, and also in patients bearing +3142G/G genotype.

From these results, *HLA-G* +3142G/G may be considered as genotype of susceptibility and UTR6 as marker of protection to ATL. Our findings showed a participation of HLA-G in the pathogenesis of the ATL.

Abbreviations: sHLA-G, soluble human leukocyte antigen-G; HLA, human leukocyte antigen; ATL, american tegumentary leishmaniasis; IFNG, interferon gamma gene; IFN-γ, interferon gamma protein; IL, interleukin; TNFA, tumor necrosis factor gene; TNF-α, tumor necrosis factor protein; DNA, deoxyribonucleic acid; UTR, untranslated region; ELISA, enzyme-linked immunosorbent assay; ILT, immunoglobulin-like transcript; VL, visceral leishmaniasis; CL, cutaneous leishmaniasis; SNPs, single nucleotide polymorphisms; TGF-β, tumor-growth factor-β; PCR, polymerase chain reaction; EM, expectation of maximization algorithm; LD, linkage disequilibrium; MEM-G/9, HLA-G monoclonal antibody, PBS, phosphate buffered saline; HRP, horseradish peroxidase; 1N H_2SO_4, Sulfuric acid one normal; Th, T helper; OR, Odds Ratio; MAF, minor allele frequency; RNA, ribonucleic acid; miRNA, micro-RNA; mRNA, messenger RNA

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1. Introduction

American Tegumentary Leishmaniasis (ATL) is an infectious, non-contagious disease caused by different species of protozoa of the genus *Leishmania* spp., transmitted by a sand fly of the genus *Lutzomyia* spp., that affect skin and mucous membranes of humans and other animals [1]. ATL is considered to be a public health problem; with approximately 700,000 to 1 million people worldwide are infected every year. In Brazil, at least 35,000 new cases are notified per year [1], specifically in the Brazilian Northeastern State of Pernambuco [2], where the *Leishmania (Viannia) braziliensis* transmitted by the *Lu. Wellcomei*, is reported to be the major persistent causative agent [2].

ATL protective immunity in individuals exhibiting spontaneous cure has been associated with a Th1-type immune response with the primary participation of IFN-γ and TNF-α cytokines, which permits macrophage activation and parasitic destruction [3]. IL-17 is also overexpressed in patients with mucosal and cutaneous leishmaniasis [4], and is associated with pathogenesis of the disease in humans and mice [5]. Notwithstanding, the expression of Th1, Th2 and Th17 cytokines may vary considerably depending on the *Leishmania* species (*L. guyanensis, L. naiffi and L. amazonensis*) in ATL patients [6]. Patients who are resistant to the infection also have monocytes that resist the *L. braziliensis* infection, preventing the emergence of skin lesions, despite of the positivity of serological diagnostic tests [7].

The human leukocyte antigen (HLA)-G has well-recognized immunomodulatory properties, since the molecule inhibits the function of cells that participate in the innate and adaptive immune system, through the interaction of the immunoglobulin-like transcript (ILT)-2 and ILT-4 receptors that transduce inhibitory intracellular signals [8]. Several lines of evidence indicate that HLA-G may contribute to leishmaniasis pathogenesis, including: i) high expression of soluble HLA-G (sHLA-G) is reported in visceral leishmaniasis (VL), an observation that may be implicated on the parasite escape from host immunity [9], and ii) HLA-G has been implicated on the shift to a Th2 polarization cytokine profile [10]. Anti-inflammatory cytokines such as IL-10 and tumor-growth factor (TGF)-β have been associated with a poor prognosis for *L. tropica* infection development [11], and TGF-β and IL-17 are synergistically involved on tissue damage in patients infected with human mucosal leishmaniasis [12].

At least three functionally studied variable sites have been described at the 3′ untranslated region (3′UTR) of the *HLA-G* gene that contribute to the posttranscriptional regulation of the gene, including the 14 base pairs insertion/deletion (14-bp INS/DEL), +3142C/G and +3187A/G variation sites [13–15]. In addition, the *HLA-G* polymorphisms and/or haplotypes at 3′UTR were reported to be associated with various parasitic diseases, including *Plasmodium falciparum* malaria and human African and American *trypanosomiasis* [8].

Cytokine gene polymorphisms have also been associated with leishmaniasis, including: i) promoter region *IL-10* –819C/C genotype has been associated with high levels of IL-10 and the presence of severe cutaneous lesions (CL) [16]; ii) a weak association between *TNF-α/β* genes with visceral leishmaniasis (VL) has been reported in a North Brazilian population [17], and the *TNF-A* -308G/A genotype has been associated to *L. braziliensis* infection in Venezuelans [18], iii) the *IFNG* +874A/T (protection) and *IL-4* −590C/T (susceptibility) variable sites have been associated with CL in patients infected by *L. major* [19]; however, there are no studies regarding the *IFNG* +874A/T in ATL caused by *Leishmania (Viannia) braziliensis*. Although the intronic *IFNG* CA microsatellite repeat and *TNF-A* -238A/G [20] have never been evaluated in ATL infection, the CA_{13} microsatellite has been reported as a high risk marker for rheumatoid arthritis [21] and *TNF-A* −238A allele as protection marker for chronic graft-versus-host disease [22]. Since the differential cytokine expression may contribute to susceptibility/resistance against protozoan infections, and considering that several variable sites observed at coding and non-coding regions of cytokine genes have also been associated with the differential expression of cytokines [23], genetic host variability is a relevant issue on disease susceptibility.

Considering that cytokines, particularly IL-10, TNF-α and IFNs, may modulate HLA-G expression [23], we evaluated all variation sites at the *HLA-G* 3′UTR, and selected the most relevant and studied cytokine single nucleotide polymorphisms (SNPs) for the immune response [24] at the promoter region of *IL-10*, *TNF-A* and intron 1 region of *IFN-G* genes in ATL patients from the Brazilian Pernambuco State. To further understand the relationship between genotypes with protein levels, we evaluated sHLA-G and cytokine levels (IL-10, TNF-α and others) in ATL patients classified according to the major clinical outcome.

Table 1
Characteristics of leishmaniasis cases and healthy blood donors from Brazilian Northeastern (NE) populations from Recife, State of Pernambuco (PE).

Study population features	[a]Cases		[a]Control		Patient status
	n	(%)	n	(%)	
Genetic association study	**114**	**100**	**346**	**100**	
Sex					
Female	32	28.07	152	43.90	
Male	77	67.54	187	54.00	
Not available (NA)	5	4.39	7	2.00	
Age, years					
Minimum	12		18		
Median	29		35		
Maximum	80		63		
Skin color					
White	21	22.11	91	26.30	
Mulatto	59	62.11	210	60.70	
Black	15	15.79	37	10.70	
Amerindian ancestry	0	0.00	1	0.30	
Not available (NA)	19	20.00	7	2.00	
Immunological analysis	**83**	**100**			
Sex					
Female	22	26.51			
Male	61	73.49			
Age, years					
Minimum	13				
Median	29				
Maximum	76				
Clinical and laboratory features					
Ulcerated	62	74.70			Sensible[b]
Cutaneous or mucocutaneous lesion	10	12.05			Spontaneous healed
Without lesion	11	13.25			Resistant

[a] Adjusted by sex, skin color and age.
[b] The survey was performed before the patients beginning a specific treatment.

2. Patients and methods

2.1. Study population

We studied 114 ATL patients (Table 1) diagnosed at the Leishmaniasis Reference Service of the Oswaldo Cruz Foundation of Pernambuco (FIOCRUZ-PE), using clinical, epidemiological, immunological and molecular criteria. The intra-dermoreaction of Montenegro (IDRM) (rapid diagnostic test) was used to confirm ATL diagnosis [25]. ATL patients were classified into: i) susceptible when exhibiting typical active lesions; ii) spontaneous healed, when presented a previous history of disease and characteristic lesion scaring without receiving specific chemotherapy, and iii) resistant, patients who were exposed to the infection, exhibited no skin lesion for more than five years of follow-up. In parallel, we studied 346 age and gender matched healthy blood donors (residing in a non-endemic area of the State of Pernambuco) (Table 1).

Among the 114 patients, plasma cytokines and sHLA-G levels were available for 83 untreated patients, of whom 62 patients were classified

as susceptible, 10 exhibited spontaneous healing and 11 were resistant (Table 1).

All individuals were informed about the details of the study and signed consent forms. The study protocol was approved by the Ethics Committee of the Aggeu Magalhães Institute (protocol # CAAE 11083812.7.0000.5190). In cases where the potential participant was < 18 years of age, their parents or guardians were also informed and signed the consent form allowing their participation.

2.2. HLA-G, IL-10, TNF-A and IFN-G variation sites

Genomic DNA was extracted from peripheral blood mononuclear cells using the DNAzol reagent (Invitrogen, Carlsbad, CA). DNA was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), and adjusted to a concentration of 200 ng/μL.

The 3′UTR of the *HLA-G* gene was PCR-amplified using the HLAG8F: 5′-TGTGAAACAGCTGCCCTGTGT-3′ and HLAG8R: 5′-GTCTTCCATTTA TTTTGTCTCT-3′R) primers, as previously described [26].

The typing of the *IL-10* promoter sequence (−819C/T:rs1800871 and −657A/G:rs non-available) followed the protocol reported by Perrey et al [27].

The *TNFA* promoter region (−308A/G:rs1800629 and −238A/G:rs361525) was amplified using primers (TNF promo F: 5′ CTCAGA GAGCTTCAGGGATAT 3′ and TNF promo R: 5′ TCTGTCTCGGTTTCTT CTCCA 3′) to generate a PCR-fragment between positions −1117 and −138. Sample reactions were prepared with 200 mM Tris-HCl (pH 8.4), 500 mM KCl, 1.5 mM $MgCl_2$, 0.3 mM dNTP, 0.5 μM each primer and 1 U *Taq* DNA Polymerase (Invitrogen). The reaction conditions were 30 cycles of 94° C for 45 s, 60° C for 30 s and 72° C for 45 s, with initial denaturation of three minutes at 94° C and final extension of seven minutes at 72° C.

The intronic region (position +601 to +1114) of *INF-G* gene was amplified using a generic primer, as previously reported [28], and an additional primer (intron 1: 5′ TTCTGCTTCTCTATCTATATTA 3′), designed to amplify a fragment that encompasses the two most studied polymorphisms [+874A/T (rs2430561) and a CA dinucleotide repeat (CA_{12}, CA_{13}, CA_{14} or CA_{15})] [19,21,28]. PCR samples were prepared with 200 mM Tris-HCl (pH 8.4), 500 mM KCl, 1.85 mM $MgCl_2$, 0.3 mM dNTP, 0.5 μM each primer and 1 U Taq DNA Polymerase (Invitrogen); and the reaction conditions included one cycle at 94 °C for 3 min, followed by 30 cycles of 94 °C for 45 s, 55 °C for 30 s and 72 °C for 45 s, and a final extension of 7 min at 72 °C.

All PCR-products were sequenced using both forward and reverse primers using the BigDye protocol in a 96-capillary automatic sequencer (Applied Biosystem, Foster City, CA). The genetic polymorphisms were identified using the Seqman software (Roche 454, Life ScienceTM, Basel, Switzerland).

2.3. Plasma levels of sHLA-G and cytokines

The soluble HLA-G1 and HLA-G5 isoforms were detected by enzyme-linked immunosorbent assay (ELISA), as reported elsewhere [29]. sHLA-G isoforms were captured by incubating patient's plasma onto a monolayer of immobilized-monoclonal antibody MEM-G/9 (Exbio, Prague, Czech Republic) for 18 h at 4 °C. Non-specific binding was blocked using 2% albumin in phosphate-buffered saline (PBS) for 30 min. After washing the plates 3 times with PBS containing 0.05% tween 20, the plates were incubated for 1 h with HRP-anti-b2-microglobulin peroxidase-conjugated detection antibody and with the substrate (ortho-phenyl-n-diaminodihydroxychloride) (DAKO, Santa Clara, CA). The reaction was terminated by the addition of 1 N H_2SO_4 and immediately read at 490 nm. The sHLA-G levels were estimated by a five-point standard curve (12.5–200 ng/mL) performed using hybridoma M8-HLA-G5-expressing cell line culture supernatant. All plasma samples were tested in duplicate.

Cytokine levels were determined using the Th1/Th2/Th17 cytometric bead array (CBA) kit (Becton Dickinson, San Jose, CA) as recommended by the manufacturer, considering the following detection limits: 2.4 pg/mL (IL-6), 2.6 pg/mL (IL-2), 3.7 pg/mL (IFN-γ), 3.8 pg/mL (TNF-α), 4.5 pg/mL (IL-10), 4.9 pg/mL (IL-4), and 18.9 pg/mL (IL-17A).

2.4. Statistical analysis

Allelic and genotypic frequencies were determined by direct counting, and the comparisons of frequencies between patients and controls were calculated using 2 × 2 contingency tables. The strength of association was evaluated by the odds ratios (OR – with 95% confidence interval-CI), and p-values were obtained using the two-tailed Fisher's exact test. Haplotype inferences were performed by the EM algorithm using Arlequin v3.5 software [30,31], and by the Bayesian method using the Phase 2.1 software [32,33]. To identify ATL associations with a specific haplotype in zygotic model, we created and tested different models of specific-haplotype gametes (in trend/additive, recessive and dominant models). In the additive model, haplotype-specific tests were performed by fixing each specific haplotype and comparing it against others, and in the dominant and recessive models, specific-haplotype frequencies were estimated using 2 × 2 contingency tables. For multiple comparisons encompassing the variation sites along the studied genes, we adopted the p-value < 0.005 [34].

Adherence to Hardy-Weinberg equilibrium was tested separately for each population (case and control) by the exact test of Guo and Thompson [35], using the ARLEQUIN v3.5.2 software. The Hardy-Weinberg equilibrium was reached if p-value > 0.05. The linkage disequilibrium (LD) pattern was evaluated by calculating r^2, and LD plots were obtained using the Haploview 4.2 software [36].

The continuous variables were analyzed for homoscedasticity and normality, using the Bartlett and Shapiro-Wilk tests, respectively. When the assumption of homogeneity and normality was not violated, the ANOVA followed by the *post hoc* Tukey tests were used (multiple comparisons). Otherwise, the Kruskal-Wallis followed by the *post hoc* Dunn tests (multiple comparisons) and the Mann-Whitney tests were used. The Spearman correlation test was used to evaluate the relationship between variables. Analyses were performed using Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, www.graphpad.com) and the R-package software [37].

3. Results

3.1. Hardy-Weinberg equilibrium and linkage disequilibrium (LD)

Variable sites at the *HLA-G, IL-10 and TNF-A* genes adhered to the Hardy-Weinberg equilibrium, except the *HLA-G* +3035C/T and *HLA-G* +3142C/G variation sites that exhibited deficit of heterozygosity in patients (Table 2).

The *IFN-G* CA microsatellite exhibited a deficit of heterozygosity in controls, and the *IFN-G* +874A/T exhibited a deficit of heterozygosity in patients and controls (Supplementary Table 1).

Eight variation sites were observed at the *HLA-G* 3′UTR: 14-bp INS/DEL (rs371194629), +3003C/T (rs1707), +3010C/G (rs1710), +3027A/C (rs17179101), +3035C/T (rs17179108), +3142C/G (rs1063320), +3187A/G (rs9380142) and +3196C/G (rs1610696). The LD profile evaluated only variable sites exhibiting minor allele frequency (MAF) ≥ 1%, as follows: patients (r^2 = 64), controls (r^2 = 98) and in the pooled group (r^2 = 91), in whom a strong LD (r^2 > 50) was observed between the +3010C/G and +3142C/G variation sites (Fig. 1A, B and C). In contrast, in the control group, a strong LD was observed between INS/DEL and +3010C/G (r^2 = 53), and with +3142C/G (r^2 = 52). In the same control group, the +3142C/G showed moderate LD with +3187A/G (r^2 = 50) (Fig. 1B).

Table 2
Comparisons of the 3′UTR *HLA-G* allele and genotype frequencies between patients with leishmaniasis and healthy blood donors from Brazilian Northeastern (NE) populations from Recife, State of Pernambuco (PE).

Variables	Study population					p-HW[b]	
	Case (%)	Cont. (%)	OR	95% CI	[a]p-value	Case	Cont.
14-bp INDEL						0.444	0.145
DEL	58.33	57.36	1.04	0.77–1.41			
INS	41.67	42.64	0.96	0.71–1.30	0.816		
Genotypes							
DEL/DEL	35.96	34.83	1.05	0.67–1.64	0.821		
DEL/INS	44.74	45.05	0.99	064–1.52	1.000		
INS/INS	19.30	20.12	0.95	0.56–1.62	0.893		
+3003C/T						1.000	0.705
C	8.77	7.51	1.19	0.69–2.04			
T	91.23	92.49	0.84	0.49–1.45	0.568		
Genotypes							
C/C	00.00	0.60	0.58	0.03–12.16	1.000		
C/T	17.54	13.81	1.33	0.75–2.36	0.359		
T/T	82.46	85.59	0.79	0.45–1.40	0.450		
+3010C/G						0.564	0.259
C	58.77	58.26	1.02	0.75–1.39			
G	41.23	41.74	0.98	0.72–1.33	0.938		
Genotypes							
C/C	35.96	35.44	1.02	0.66–1.60	0.910		
C/G	45.61	45.65	1.00	0.65–1.53	1.000		
G/G	18.42	18.92	0.97	0.56–1.67	1.000		
+3027A/C						1.000	0.168
A	3.98	4.80	0.82	0.39–1.75			
C	96.02	95.20	1.22	0.57–2.60	0.715		
Genotypes							
A/A	00.00	0.60	0.58	0.03–12.27	1.000		
A/C	7.96	8.41	0.94	0.43–2.06	1.000		
C/C	92.04	90.99	1.14	0.53–2.49	0.848		
+3035C/T						**0.036**	0.409
C	83.93	84.38	0.97	0.64–1.46			
T	16.07	15.62	1.04	0.68–1.57	0.916		
Genotypes							
C/C	73.21	71.77	1.08	0.66–1.74	0.809		
C/T	21.43	25.23	0.81	0.48–1.35	0.447		
T/T	5.36	3.00	1.83	0.65–5.15	0.249		
+3142C/G						**0.000**	0.178
C	29.22	41.29	**0.59**	**0.40–0.86**			
G	70.78	58.71	**1.70**	**1.17–2.49**	**0.006**		
Genotypes							
C/C	20.78	18.92	1.12	0.61–2.08	0.749		
C/G	16.88	44.74	**0.25**	**0.13–0.47**	**< 0.0001**		
G/G	62.34	36.34	**2.90**	**1.74–4.84**	**< 0.0001**		
+3187A/G						0.227	0.103
A	68.18	72.07	0.83	0.48–1.43			
G	31.82	27.93	1.20	0.70–2.08	0.567		
Genotypes							
A/A	51.52	53.75	0.91	0.45–1.87	0.856		
A/G	33.33	36.64	0.86	0.41–1.84	0.850		
G/G	15.15	9.61	1.68	0.61–4.65	0.358		
+3196C/G						1.000	0.542
C	93.75	71.62	5.94	1.41–25.13			
G	6.25	28.38	0.17	0.04–0.71	0.004		
Genotypes							
C/C	87.50	51.95	6.47	1.45–28.94	0.008		
C/G	12.50	39.34	0.22	0.05–0.98	0.035		
G/G	00.00	8.71	0.31	0.02–5.35	0.380		

[a] Fisher's exact test; INS/DEL represent Insertion/Deletion of 14 base pare respectively.
[b] Hardy-Weinberg p-value.

3.2. HLA-G +3142G allele and G/G genotype were over-represented in ATL patients exhibiting high sHLA-G levels

Compared to controls, the +3142G allele (p = 0.006, OR = 1.70, 95%CI 1.17–2.49) and the +3142G/G genotype (p < 0.0001; OR = 2.90, 95% CI 1.74–4.84) were over-represented in patients (Table 2). The comparisons of the other *HLA-G* 3′UTR polymorphic sites between patients and controls did not reach significance, except +3196C/G but with large 95% CI, at allelic level (Table 2). The further evaluation of the relationship between *HLA-G* polymorphic sites and sHLA-G permitted the observation that ALT patients carrying the +3142G/G genotype exhibited increased sHLA-G levels when compared to other genotypes (p = 0.050, Fig. 2).

The most frequent *HLA-G* 3′UTR haplotypes detected in the studied population were: UTR-1 (26.50%), UTR-2 (25.40%), UTR-3 (15.30%), UTR-4 (7.00%), UTR-5 (9.60%), UTR-6 (5.40%) and UTR-7 (4.50%) and others (UTR pool) (6.30%), and the comparisons between patients and controls were performed only for haplotypes with minimum

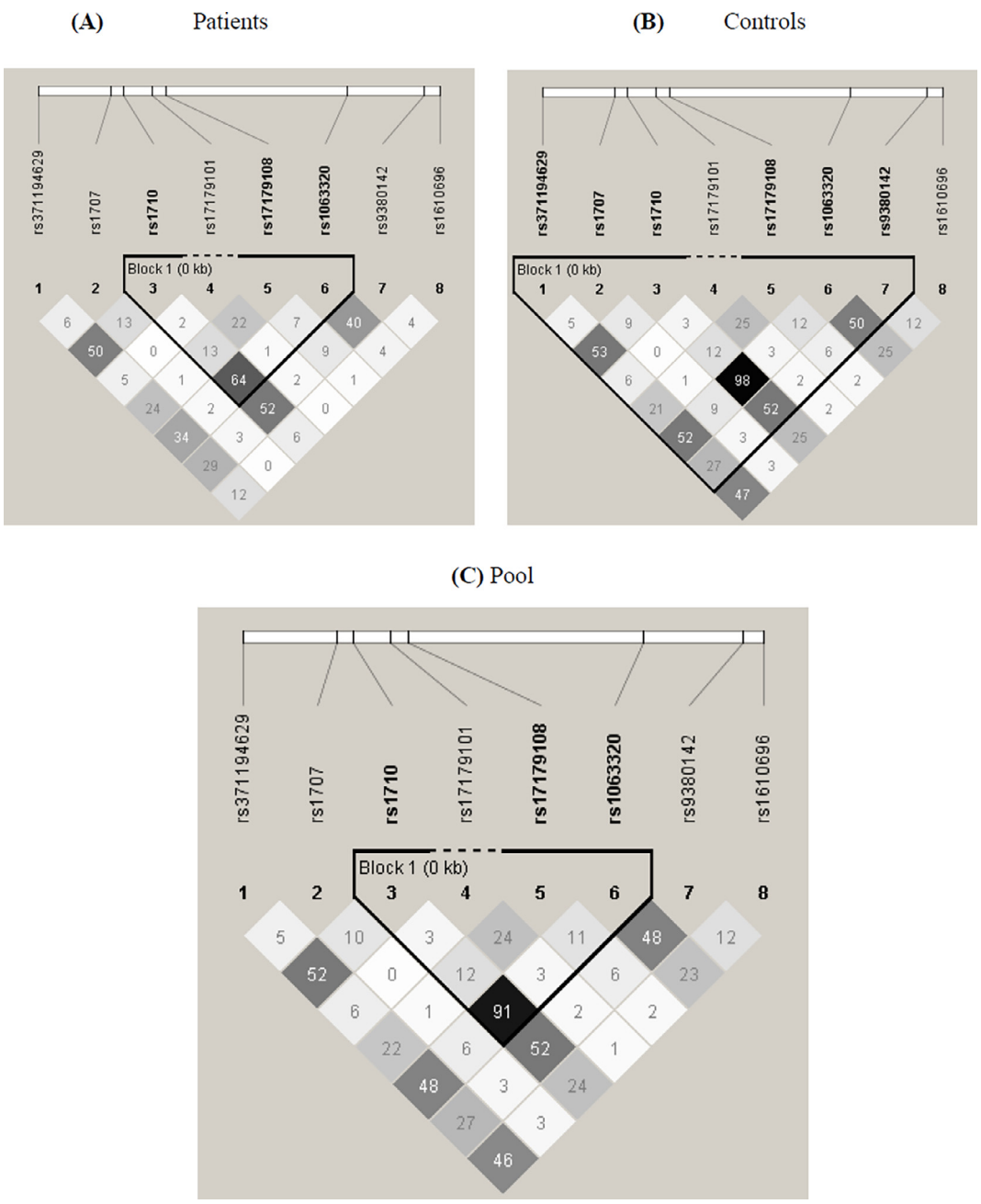

Fig. 1. The Linkage Disequilibrium (LD) patterns for the 3′UTR *HLA-G* region encompassing positions +2960 to +3196 in (**A**) patient with leishmaniasis, (**B**) healthy blood donors from Brazilian Northeastern (NE) populations from Recife, State of Pernambuco (PE) and (**C**) pooled group. From left to right, the positions of each ''rs'' is shown on the figures as follows: 14-bp INS/DEL (rs371194629), +3003C/T (rs1707), +3010C/G (rs1710), +3027A/C (rs17179101), +3035C/T (rs17179108), +3142C/G (rs1063320), +3187A/G (rs9380142) and +3196C/G (rs1610696). The LD pattern was evaluated by calculating r^2. **LD r^2** measures correlation between a pair of loci. Its value variates from zero to 1; zero (no LD) and 1 (complete LD). LD plot was generated and visualized by Haploview v4.2. The r^2 values (×100) for the marker pairs are listed in the corresponding boxes. The LD plot was generated using SNPs with a minimum allele frequency (MAF) of 1%.

frequency of 5% (Fig. 3). Contrary to the +3142G allele, the +3142C allele was associated with protection against ATL (p = 0.006, OR = 0.59, 95% CI 0.40–0.86, Table 2), and this association was stronger for patients exhibiting the +3142GC genotype (Table 2) or those carrying the UTR6 haplotype under dominant model (*14-bpDEL/+3003T/+3010G/+3027C/+3035C/**+3142C**/+3187A/+3196C*) (Fig. 3 and Table 3).

The stratification of patients according to clinical outcome (susceptible, spontaneous healed and resistant) revealed that susceptible patients exhibited increased sHLA-G levels when compared to spontaneous healed (p = 0.071) and to resistant patients (p = 0.030, Fig. 4). Noteworthy, patients exhibiting spontaneous healing and those classified as resistant exhibited closely similar sHLA-G levels (Fig. 4); however, susceptible patients exhibited significant increased sHLA-G levels, when compared to the pooled group (spontaneous healed plus resistant) (p = 0.027, Fig. 5), indicating that spontaneous healed and resistant presented a similar behavior in terms of sHLA-G production. In terms of sHLA-G expression, no significant difference was observed between patient UTRs carrying the +3142C with those carrying the +3142G allele (Supplementary Fig. 1).

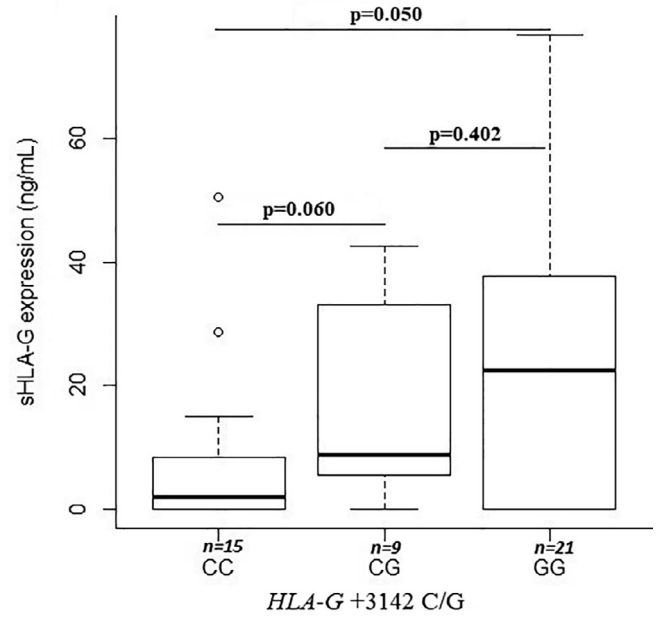

Fig. 2. Soluble HLA-G (sHLA-G) level and +3142C/G polymorphism {CC genotype **(n = 15)**, CG genotype **(n = 9)** and GG genotype **(n = 21)**} in whole patient. All associations between different groups were performed on R software v3.4.2. Kruskal-Wallis rank sum test (Dunn.test) was performed to compare the median expression of sHLA-G in different groups. p-value of Bartlett test of homogeneity of variances was 0.04911.

3.3. Negative correlation between IL17-A and sHLA-G in the susceptible ATL group

Considering ATL patients as a whole group, the comparisons of the *IL-10* and *TNF-A* promoter polymorphisms between patients and controls showed no significant differences (Supplementary Tables 2 and 3). No significant differences were observed regarding the +874A/T *IFN-G* polymorphism; however, we observed that the CA_{12} and CA_{15} microsatellite alleles were underrepresented in patients. Notwithstanding, the microsatellite alleles did not fit the Hardy-Weinberg equilibrium (Supplementary Table 1).

The comparisons of the plasma levels of TNF-α, IL-10 and others (IFN-γ, IL-6, IL-4 and IL-2) among the different clinical ATL phenotypes showed no significant differences (data not shown).

In addition, when we correlated IL-17A levels in patients stratified according to ATL outcome, no significant differences were obtained (Supplementary Fig. 2); however, the IL-17A plasma levels were weakly and negatively correlated to sHLA-G in the susceptible group (Supplementary Fig. 3).

4. Discussion

A previous study evaluated classical HLA class I and class II alleles in a series of Southern Brazilian patients, reporting that the *HLA-B*27, HLA-B*35, HLA-B*44* and *HLA-DRB1*13* allele groups were associated with cutaneous or mucocutaneous lesions, whereas the *HLA-B*45* group protected against the clinical manifestations of leishmaniasis [38,39]. Notwithstanding, little attention has been devoted to the role of non-classical HLA class I genes in ATL. Considering that individuals infected with leishmania may differentially respond to the parasite exhibiting or not clinical manifestations, and considering that the non-classical immune checkpoint HLA-G molecule/gene modulates the innate/adaptive immune response rather than antigen presentation, the study of HLA-G and of some factors associated with the modulation of HLA-G expression [23] is of relevance. Although 61 coding alleles have been described (IPD-IMGT/HLA v.3.34.0, 2018-10-18), only 6 to 7 distinct proteins have been frequently observed in worldwide populations [40], reflecting the evolutionary maintenance of the coding region structure, since one of the major functions of the molecule is its expression in the placenta to help on the prevention of the mother immune attack against the semi-allogenic fetus [41]. On the other hand, the *HLA-G* gene control regions exhibit a great diversity of variation sites that can be combined into several haplotypes. In this context, in the present study we combined some aspects associated with the control of the HLA-G in Brazilian ATL patients, followed up in a tertiary reference center. For instance, we studied the levels and the gene polymorphisms associated with the induction of HLA-G expression [23] as well as the *HLA-G* variability at the 3′UTR.

4.1. LD pattern between 3′UTR HLA-G variable sites

In terms of the healthy population, the variation sites observed at the *HLA-G* 3′UTR are in LD in several worldwide populations so far studied [42], emphasizing the LD observed for the +3142C/G and +3010C/G variation sites, observed in this study, which has been previously reported in healthy Brazilian samples from State of São Paulo [42], and from the State of Pernambuco (Northeastern of Brazil) [43]. In addition, the *HLA-G* 3′UTR haplotype frequencies observed in healthy individuals from Southern and Northeastern Brazilian samples were also closely similar [42,43].

4.2. HLA-G +3142G allele and G/G genotype were over-represented in ATL patients exhibiting high sHLA-G levels

Considering ATL patients, individuals carrying the *HLA-G* +3142G allele at single or double doses +3142G/G genotype exhibited an increased risk to ATL development (Table 2). Although no study has evaluated the functional role of the +3010C/G variation site, the +3142C/G variation site presents a posttranscriptional regulation due to the action of the miR-148a, miR-148b, and miR-152 microRNAs [13]. Besides the unequivocal association of the +3142G/G genotype, we also observed elevated level of sHLA-G in patients carrying the

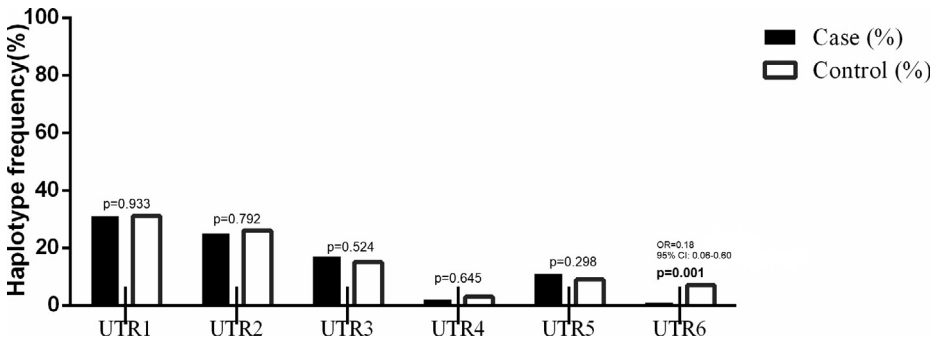

Fig. 3. Comparisons of the *HLA-G* 3′UTR haplotype frequencies between patients with leishmaniasis and healthy blood donors from the Brazilian Northeastern (NE) populations from Recife, State of Pernambuco (PE), Brazil. Only haplotypes exhibiting minimum frequency of 5% are shown. The sequences of the variation sites follow the pattern: 14-bp DEL/INS/+3003C/T/+3010C/G/+3027A/C/+3035C/T/+3142C/G/+3187A/G/+3196C/G; highlighting in bold the changes among haplotypes: UTR1 (**DEL**T**G**CC**CG**C), UTR2 (**INS**TCCCGA-**G**), UTR3 (DELTCCCGAC), UTR4 (DEL**CG**CC**C**AC), UTR5 (**INS**TCC**T**GAC), UTR6 (DELT**G**CC**C**AC). INS/DEL Insertion/Deletion of 14 base pairs.

Table 3
Comparisons of the 3′UTR *HLA-G* specific-haplotype (gametes) frequencies between patients with leishmaniasis and healthy blood donors from Brazilian Northeastern (NE) populations from Recife, State of Pernambuco (PE).

[a]Models	Study population				
	Case (%)	Cont. (%)	OR	95% CI	[b]p-value
Trend or additive					
UTR1 (DELTGCCCGC)					
Others	54.39	51.05	1.14		
UTR1 + / −	28.95	36.64	0.71	0.44–1.12	0.140
UTR1 + /UTR1 +	16.67	12.31	1.42	0.79–2.57	0.265
UTR2 (INSTCCCGA**G)**					
Others	57.89	56.76	1.05		
UTR2 + / −	35.09	35.14	1.00	0.64–1.56	1.000
UTR2 + /UTR2 +	7.02	8.11	0.86	0.38–1.94	0.840
UTR3 (DELTCCCGAC)					
Others	68.42	72.97	0.80		
UTR3 + / −	29.82	24.32	1.32	0.82–2.12	0.264
UTR3 + /UTR3 +	1.75	2.70	0.44	0.09–2.09	0.514
UTR4 (DEL**CG**CCCAC)					
Others	95.61	93.99	1.39		
UTR4 + / −	4.39	6.01	0.72	0.26–1.96	0.640
UTR4 + /UTR4 +	0.00	0.00	2.91	0.06–147.8	1.000
UTR5 (INSTCCTGAC)					
Others	78.95	82.58	0.79		
UTR5 + / −	19.30	16.82	1.18	0.68–2.04	0.568
UTR5 + /UTR5 +	1.75	0.60	2.96	0.41–21.24	0.269
UTR6 (DELTGCC**C**AC)					
Others	98.25	86.49	8.75		
UTR6 + / −	0.88	13.51	**0.06**	**0.01–0.42**	**< 0.0001**
UTR6 + /UTR6 +	0.88	0.00	8.82	0.36–218.1	0.255
Dominant					
UTR1 (DELTGCCCGC)					
Others	54.39	51.05	1.14		
UTR1 + / − or UTR1 + /UTR1 +	45.61	48.95	0.87	0.57–1.34	0.587
UTR2 (INSTCCCGA**G)**					
Others	57.89	56.76	1.05		
UTR2 + / − or UTR2 + /UTR2 +	42.11	43.24	0.95	0.62–1.47	0.912
UTR3 (DELTCCCGAC)					
Others	68.42	72.97	0.80		
UTR3 + / − or UTR3 + /UTR3 +	31.58	27.03	1.25	0.78–1.98	0.398
UTR4 (DEL**CG**CCCAC)					
Others	95.61	93.99	1.39		
UTR4 + / − or UTR4 + /UTR4 +	4.39	6.01	0.72	0.26–1.96	0.640
UTR5 (INSTCCTGAC)					
Others	78.95	82.58	0.79		
UTR5 + / − or UTR5 + /UTR5 +	21.05	17.42	1.26	0.74–2.15	0.401
UTR6 (DELTGCC**C**AC)					
Others	98.25	86.49	8.75		
UTR6 + / − or UTR6 + /UTR6 +	1.75	13.51	**0.11**	**0.03–0.48**	**0.0001**

[a] UTR: (14-bp DEL/INS; +3003C/T; +3010C/G; +3027A/C; +3035C/T; +3142C/G; +3187A/G; +3196C/G); INS/DEL represent Insertion/Deletion of 14 base pare respectively; **UTR + / − or UTR + /UTR +** represent single heterozygous or homozygous carriers respectively.
[b] Fisher's exact test.

+3142G/G genotype (Fig. 2). In addition, after stratification in ATL patients, susceptible patients did exhibit increased sHLA-G levels when compared to resistant patients (Fig. 4 and Fig. 5). Once susceptible patients present a poor prognosis of the disease, exhibiting active cutaneous lesions, the increased sHLA-G observed in genetically predisposed patients may down-regulate the host innate/adaptive immune responses against the parasite and may permit parasite escape mechanisms, as have been reported for VL [9]. The increased sHLA-G level observed in our ATL patients carrying the +3142G/G genotype, contrasts with the functional study which demonstrated that the presence

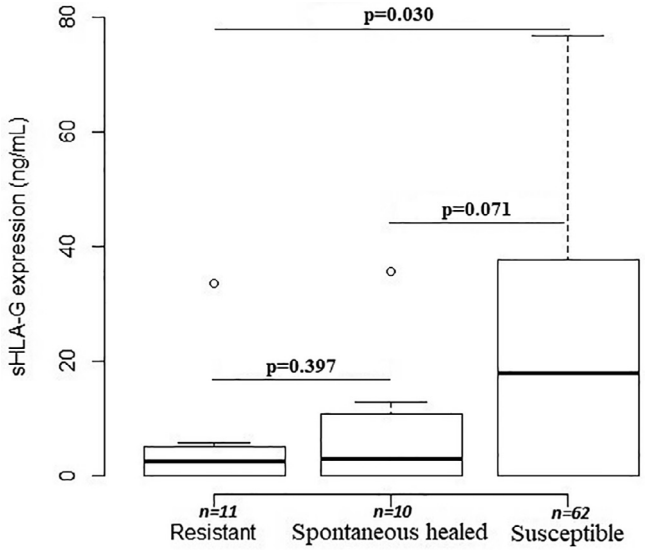

Fig. 4. Soluble HLA-G (sHLA-G) level in whole patient group {**"Susceptible (n = 62), Spontaneous healed (n = 10) and Resistant (n = 11)"**}. **"Susceptible"** and **"Spontaneous healed"** were patients detected positives for *Leishmania*, had ulcerated wound, but the patients belong to **"Spontaneous healed"** group only were spontaneous cured without any treatment, the patients belong to **"Susceptible"** group were not. The **"Resistant"** were asymptomatic patients. All associations between different groups were performed on R software v3.4.2. The Kruskal-Wallis rank sum test (Dunn.test) was performed to compare the median expression of sHLA-G in different groups. p-value of Bartlett test of homogeneity of variances was 0.001901.

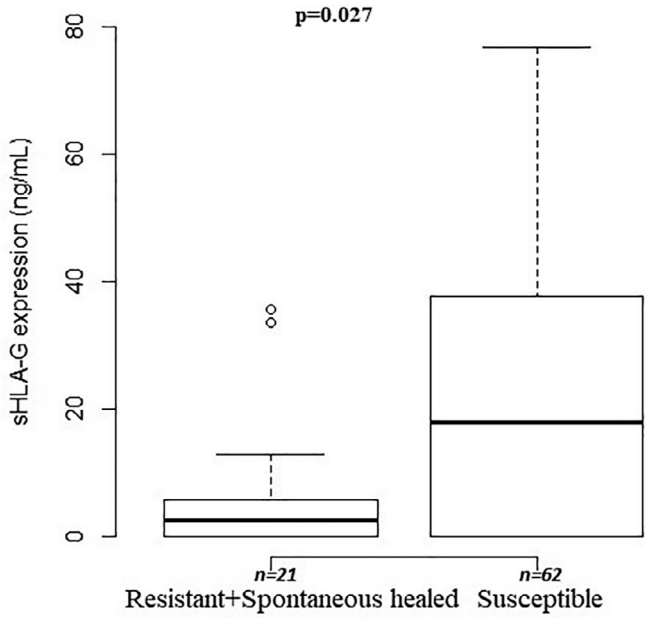

Fig. 5. Soluble HLA-G (sHLA-G) level in whole patient group {**"Resistant" (n = 21) and ''Susceptible" (n = 62)**}. As **''Resistant'' and "Spontaneous healed"** patients showed the same profile in Fig. 4, we pooled them with **"Resistant"** group and compared to susceptible patient called here **''Susceptible"** (Fig. 5). All associations between different groups were performed on R software v3.4.2. The Wilcoxon rank sum test (Mann-Whitney Test) was used to compare the median expression of sHLA-G in different groups. p-value for Shapiro-Wilk normality test was < 0.05.

of G instead of C at position +3142 leaded to high miRNA affinity and consequently to low sHLA-G levels [13]; however, the microRNAs present at the ATL milieu or at systemic level might not be the same used to evaluate the functional effect of the variation site. Besides

miRNAs, other transcriptional and posttranscriptional microenvironment factors may influence the sHLA-G expression [44].

4.3. Negative correlation between IL17-A and sHLA-G in the susceptible ATL group

Considering the factors that may induce HLA-G expression, we did not observe cytokine variation sites or cytokine plasma levels associated with sHLA-G levels; however, we observed a trend negative correlation between IL-17A and sHLA-G in the susceptible patient group, suggesting a possible downregulating effect of sHLA-G on effector Th17 and other Th1 cells that are important for parasite control [45]. Noteworthy, the small number of individuals in each ATL group and the restricted number of variation sites in cytokine genes may not be sufficient to reach significance, and larger groups encompassing the whole gene variation sites may further clarify this issue.

4.4. Conclusions

We showed for the first time the influence of the *HLA-G* in the susceptibility/resistance to ATL. The *HLA-G* +3142G allele in double doses was associated with: i) ATL susceptibility and ii) increased levels of sHLA-G. Increased sHLA-G levels may be detrimental to the host during ATL infection, possibly by inhibiting the immune response against the parasite and permitting parasite escape from host immune response.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2019.08.001.

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