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IL2RA Genetic Variants Reduce IL-2–Dependent Responses and Aggravate Human Cutaneous Leishmaniasis

Pablo R. S. Oliveira,*†‡ Hélia Dessein,*† Audrey Romano,*† Sandrine Cabantous,*† Maria E. F. de Brito,§ Ferrucio Santoro,*† Maira G. R. Plta,*† Valéria Pereira,§† Lain C. Pontes-de-Carvalho,‡ Virmondes Rodrigues, Jr.,§ Sima Rafati,† Laurent Argiro,*† and Alain J. Dessein*†

The outcome of Leishmania infections varies substantially, depending on the host and the parasite strain; infection may be asymptomatic or cause mild or severe skin ulcers (cutaneous leishmaniasis [CL]), limited or disseminated lesions, or lethal visceral disease. We previously reported an association between IL-2R mutations and susceptibility to visceral leishmaniasis in children infected with Leishmania donovani. In the present study, we evaluated the possible role of IL-2 signaling in human CL. We first showed that the transcripts of several genes of the IL-2 pathway were abundant in skin lesions caused by Leishmania braziliensis. We then carried out a genetic analysis, focusing on major genes of the IL-2 pathway. We used a family-based approach and found that polymorphisms of several genes appeared to be associated with CL in a Brazilian population. Moreover, two polymorphisms of the IL2RA gene were significantly and independently associated with CL. We confirmed this result in a second Brazilian sample (also exposed to L. braziliensis) and in Iranians infected with Leishmania tropica: IL2RA rs10905660 T (Pcombined = 6 x 10⁻⁷) and IL2RA rs706778 T (Pcombined = 2 x 10⁻⁷) were associated with greater susceptibility to lesion development. These alleles were also correlated with a poor IFN-γ response and poor FOXP3⁺ regulatory T cell activation. Thus, IL-2 plays a crucial role in protection against the cutaneous ulcers caused by Leishmania, and the IL-2 pathway is a potential target for strategies aiming to control Leishmania-related diseases. The Journal of Immunology, 2015, 194: 000–000.

Leishmaniasis is a group of diseases caused by intracellular protozoan parasites of the genus Leishmania, which includes several species that are widespread in the tropics, subtropics, and the Mediterranean basin (1). Leishmaniasis is a major public health problem in the regions in which it is endemic, with 1.5–2 million new cases and 70,000 deaths each year, and 350 million people at risk for developing the disease (2). Leishmaniasis is transmitted to humans by phlebotomine sandflies and causes a wide spectrum of clinical manifestations, from severe visceral disease to asymptomatic skin lesions. The most serious forms of the disease are visceral leishmaniasis (VL), also called kala-azar (KA), and cutaneous leishmaniasis (CL), which may heal within a few days or last for months (3, 4). Dermotropic Leishmania species (such as Leishmania braziliensis and Leishmania tropica) cause severe cutaneous lesions, but asymptomatic infections also occur and animal studies have shown that both the parasite and the immunologic/genetic background of the host determine the outcome of infection (4, 5).

Experimental studies have indicated that several components of innate and adaptive immunity, including phagocyte cells, NK cells, effector CD4⁺ and CD8⁺ T cells, and regulatory T (Treg) cells, are involved in the control of leishmaniasis (5–7). These studies have also indicated that a fine balance between effector and regulatory immune responses may be required for the efficient control of Leishmania without extensive collateral tissue damage (8). In this respect, Treg cells may play a crucial role in controlling Th1 and Th2 responses in infected animals (9, 10). The extent to which these findings can be extended to human infections remains unclear, because most of these animal studies were carried out with a small number of Leishmania strains that are not among the most pathogenic. Furthermore, infection conditions in the laboratory are different from those for infection by sandflies in the field.

Genetics is a powerful tool for exploring immunological pathways involved in human susceptibility to infectious diseases. Certain polymorphisms in genes of the immune system have been shown to alter the risk of leishmaniasis, but only a few of the associations identified have been validated in genetically different populations and replicated in individuals infected with different Leishmania strains (11, 12). We have shown that susceptibility to KA caused by Leishmania donovani in Sudan is linked to Chr22q12 (13) and that a mutation of the IL2RB gene, which encodes the β-chain of the IL-2R, may partly account for this linkage (14). The IL-2R is composed of three subunits: IL-2Rα (CD25, encoded by IL2RA), which is specific for IL-2 and confers high affinity to the receptor; IL-2Rβ
IL-2 PATHWAY AND SUSCEPTIBILITY TO CUTANEOUS LEISHMANIASIS

Leishmania was activated in recently activated by Ag (16). IL-2R is abundant only on Treg cells and on T lymphocytes formed this analysis on skin biopsies from L. braziliensis found that the same with susceptibility to CL caused by L. braziliensis and L. tropica in populations from Brazil and Iran, respectively. Finally, we found that the same IL2RA variant increasing disease risk was associated with a downregulation of IL-2–dependent responses.

Materials and Methods

Ethics statement

All individuals agreeing to participate in this research were informed about the nature of the study and signed an informed consent form. All procedures were approved by Local Ethics Committees. For children under the age of 18 y, informed written consent for participation was obtained from the parents. The study protocol was approved by Local Ethics Committees at the Aggeu Magalhães (Recife, Pernambuco) and Gonçalo Moniz (Salvador, Bahia) Research Centers (Oswaldo Cruz Foundation). Approval was also obtained from the Brazilian National Committee for Ethics in Research. The collection and use of the Iranian samples were approved by the Ethics Committee of the Pasteur Institute of Tehran.

Gene expression analysis

Eight lesion biopsies from patients infected with L. braziliensis (from the border of the ulcers, before treatment) and eight normal skin samples from uninfected donors were collected and immediately stored in RNA later solution (Life Technologies). Tissue (20 mg) was placed in microtubes containing 1.4-mm-diameter ceramic beads (CK14; Bertin Technologies) and 350 μl RTL lysis buffer (Qiagen) supplemented with 3.5 μl 2-ME (Sigma-Aldrich). Complete disruption of the tissue was achieved with a Precellys 24 homogenizer (Bertin Technologies). We added 400 μl TRIZol reagent (Life Technologies) and 150 μl chloroform (Sigma-Aldrich) and the tubes were then vigorously vortexed and incubated for 5 min at room temperature. The aqueous phase was recovered, mixed with 500 μl 70% ethanol, and the RNA was purified using the RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. The recovered RNA was quantified by spectrophotometric analysis (NanoVue Plus spectrophotometer; GE Life Sciences) and its integrity was assessed with a 2100 Bioanalyzer (Agilent Technologies). All 16 samples included in the gene expression profiling experiment were suitable for microarray analysis in terms of RNA quality (RNA integrity number ≥ 8.0).

Some samples underwent labeling, and hybridization were performed according to the Agilent one-color microarray-based gene expression analysis protocol (Agilent Technologies). The microarray used was the SurePrint G3 human gene expression v2 array (G4851B; Agilent Technologies). Data were quantile-normalized with GeneSpring GX software (Agilent Technologies). Microarray data were deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/; accession no. GSE69391). A two-class unsupervised significance analysis of microarray was carried out to identify genes differentially expressed in cutaneous lesions and normal skin. A false discovery rate based on expected versus observed values (false discovery rate 90th percentile < 0.001%) was used to limit false-positive results. Only genes displaying a ≥2-fold increase or decrease in expression were considered to be differentially regulated. The Pearson correlation coefficient was used to define the hierarchical clustering of samples.

Global canonical pathway analysis was performed with the Ingenuity database, version 8.7 (Ingenuity pathway analysis; http://www.ingenuity.com). The entire dataset was used to calculate relative p value (right-tailed Fisher exact test) for the IL-2 pathway.

Population samples (genetic association studies)

The general characteristics of the study populations are given in Table I. In the Brazilian sample (family-based design), the study was carried out on subjects living in an area in which CL (caused by L. braziliensis) is endemic, in the northeast of Brazil. We recruited a total of 1079 individuals (453 trios from 289 nuclear families) from rural zones located near the Atlantic Forest in the state of Bahia (Brazilian discovery sample) and from sugar cane plantations (city of Cortés) in the state of Pernambuco (Brazilian replication sample). CL was diagnosed by local clinicians and was treated with meglumine antimoniate (Glucantime) at local health centers. The selected cases had been living in an area of endemic CL for at least 5 y, had one or more characteristic lesions or scars, and had received at least 10 meglumine antimoniate injections on 10 different days. Subjects who had taken traditional medicine or were cured without treatment were excluded. Trios consisted of an affected child and both parents. In the Iranian sample (population-based design), the cases (n = 118) and controls (n = 126) were from the city of Mashhad (Razavi Khorasan Province) in northeastern Iran. CL, mostly caused by L. tropica, is endemic in this region (17). Cases were subjects living in the endemic region, with positive skin tests for L. tropica Ags and characteristic skin ulcers. The controls had never had skin lesions that could have been caused by Leishmania, were living in a region in which leishmaniasis was highly endemic, and reacted positively in a skin test with L. tropica Ags.

DNA extraction

Genomic DNA was extracted from 2 ml whole blood by the standard salting-out method (18) or with the QIAamp DNA Blood Midi Kit (Qiagen), according to the manufacturer’s instructions, and was stored at −20°C until use. DNA concentration and purity were determined by UV spectrophotometry.

Tag–single nucleotide polymorphism selection

Given the genetic heterogeneity of the Brazilian population, we carried out linkage disequilibrium calculations and tag–single nucleotide polymorphism (SNP) selection separately for American, African, and European populations from the 1000 Genomes Project (19). Only SNPs with a minor-allele frequency ≥5% in at least one of the reference panels were included in the analysis. An optimal set of markers covering all genes (including an extra 5 kb at each end) was selected on the basis of r2 ≥ 0.8 between SNPs using PLINK software (20).

Polymorphism genotyping and quality control

In the discovery study (SNP array), we genotyped 754 individuals from the Brazilian discovery sample for 133 tag-SNPs covering the IL2, IL2RA, IL2RB, JAK3, STAT5A, and STAT5B genes using an Infinium iSelect BeadChip assay (Illumina). After genotyping, quality control was carried out before testing for association. All procedures were conducted automatically with PLINK software. In addition to two SNPs displaying significant deviation from Hardy–Weinberg equilibrium (p < 0.0001; based on founders only), another three SNPs and 16 individuals with high rates of missing genotype data (>10%) were excluded from the analysis. Mendelian inconsistencies were also eliminated.

In the other studies (TaqMan genotyping), SNPs were genotyped with validated TaqMan probe assays (Applied Biosystems). In brief, each reaction contained 12.5 ng genomic DNA, 900 nM each primer, 200 nM each fluorescently labeled probe, and TaqMan Universal PCR Master Mix (Applied Biosystems) in a total volume of 5 μl. PCR was conducted under the following conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of amplification (95°C for 15 s and 60°C for 1 min). Allelic discrimination was achieved with the 7900HT Fast Real-Time PCR System (Applied Biosystems). Quality control criteria similar to those applied in the discovery study were used following TaqMan genotyping. In the Brazilian replication sample, only six individuals were excluded, on the basis of high missing genotype rates (>10%). In the Iranian cohort, all samples and SNPs satisfied the quality control criteria.

Cell culture

PBMCs from individuals living in a region in which CL (due to L. braziliensis) is endemic (southern Bahia, Brazil) were purified, cultured, and stimulated with parasite Ags (5 μg/ml) or with PHA (5 μg/ml; Sigma-Aldrich), as previously described (21). IFN-γ levels were determined by ELISA on culture supernatants, using the Ready-SET-Go! ELISA kit (BD Biosciences) according to the manufacturer’s protocol. To evaluate Treg cell activation, freshly isolated PBMCs from healthy donors were activated with bead-bound anti-CD3/CD28 Abs (1:20 or 1:200 bead/cell ratio; Miltenyi Biotec) and cultured for 72 h in the presence of TGF-β1 alone (2 ng/ml human TGF-β1; PeproTech) or in combination with IL-2 (10 U/ml human IL-2; BD Biosciences).

Flow cytometry analysis

Cultured cells were labeled as previously described (22) with PE-Cy7–anti-CD4 (SK3), allophycocyanin–anti-CD25 (M-A251), and PE–anti-FOXP3 (259D/C7) or with appropriate isotype-matched control Abs (all from BD Pharmingen).
between Treg (cTreg) and resting Treg (rTreg) cells. Surface staining was performed for 20 min with the corresponding mixture of fluorescently labeled Abs. Cells were fixed and permeabilized for the intracellular staining of FOXP3, according to the manufacturer’s recommendations (BD Cytofix/Cytoperm kit; BD Biosciences). Data were acquired with a FACSCalibur (BD Biosciences) and analyzed with FACSdiva software (BD Biosciences).

**Sequence annotations**

Comparative genomic data and regulatory features in the IL2RA region (10:6052652–6119288; GRCh37/hg19 reference sequence) were obtained from both the Ensembl (http://www.ensembl.org) and University of California Santa Cruz (http://genome.ucsc.edu) genome browsers. SNP positions were cross-referenced with sequence annotations, including genomic evolutionary rate profiling–constrained elements for 36 eutherian mammals (EPO low coverage) (23), chromatin segmentation state, and enrichment for marks of open chromatin (DNase I hypersensitive sites). These last two types of information were obtained from the ENCODE project (24).

**Statistical analysis**

For the family-based studies (both Brazilian samples), associations between SNPs and disease were evaluated with the transmission disequilibrium test, using FBAT software (25), under three different genetic models (additive, dominant, or recessive). Ten thousand permutations were carried out in the Brazilian discovery study to obtain empirical p values. A simple multiple test correction was applied to the empirical p values to control the probability of observing false-positive results. Corrected empirical p values < 0.05 were taken as significant. Genetic associations in the Iranian cohort (population-based design) were assessed by carrying out χ² tests (also under additive, dominant, and recessive genetic models). The significance threshold applied in our replication/validation studies was p = 0.05.

The conditional extended transmission disequilibrium method (26), implemented in the UNPHASED software package (27), was used to assess the independence of SNP effects on disease susceptibility. Fixed-effect meta-analysis on all population samples and SNPs from further analysis (as detailed in Materials and Methods), a set of 128 polymorphisms remained and was analyzed in 738 individuals (317 affected trios from 205 nuclear families).

We performed a family-based transmission disequilibrium test and the strongest association signals (p ≤ 4 × 10⁻⁷) were observed for two SNPs in intron 1 of the IL2RA gene [rs10905669 (p = 3 × 10⁻⁷) and rs706778 (p = 3 × 10⁻⁵)] (Table II). This initial screening stage also revealed other SNPs in the IL2, IL2RA, IL2RB, and JAK3 genes that were suggestively associated with CL (p < 0.05). Phenotype permutations on these markers, followed by multiple test correction, confirmed significant associations only for IL2RA rs10905669 and rs706778 (both SNPs showed corrected empirical p values of <0.05).

We then attempted to replicate the strongest association signals found in the discovery study in a second Brazilian sample, composed of 325 subjects (130 affected trios from 80 families). We used validated TaqMan assays to genotype rs10905669 and rs706778. Postgenotyping quality control criteria similar to those applied in the initial screening phase were used in this second-stage analysis (see details in Materials and Methods). The association of rs706778 with CL was replicated in this second Brazilian sample (p = 0.04), with the T allele increasing disease risk (Table III). The rs10905669 polymorphism tended to be associated with CL in this second population sample (p = 0.08).

**IL2RA variants are also associated with CL in Iranians infected with L. tropica**

We investigated whether the IL2RA polymorphisms were also associated with CL in a genetically distinct population infected with other Leishmania species. L. tropica also causes severe CL and can lead to skin ulcers that can last for several months. We recruited a population-based cohort, composed of 236 Iranians (116 cases and 120 controls) living in a zone in which CL caused by L. tropica was highly endemic (Table I). We found that both rs10905669 (p = 0.03) and rs706778 (p = 0.04) were also associated with CL in this population (Table III). Importantly, the IL2RA alleles associated with a high disease risk were the same in all studied datasets from Brazil and Iran.

We then carried out a fixed-effect meta-analysis on all population samples. This analysis confirmed strong associations with disease for both rs10905669 (p = 6 × 10⁻⁷) and rs706778 (p = 2 × 10⁻⁵) (Table IV). These results provide strong support for the notion that genetic variants of the IL2RA gene or its surrounding sequences are risk factors for human CL.

**IL2RA variants rs10905669 and rs706778 are independently associated with CL**

We then investigated whether rs10905669 and rs706778 captured a single signal or were independently associated with CL. The rs10905669 and rs706778 variants were poorly correlated (r² between SNPs < 0.2) in our Brazilian discovery dataset. Nevertheless, these SNPs may be in linkage disequilibrium with the same causal variant. We tested this hypothesis by carrying out conditional tests on these two polymorphisms, and we found that rs10905669 and rs706778 were independently associated with the cutaneous disease (p < 0.05 for both SNPs after conditional tests).

**Only polymorphisms in the IL2RA region account for the genetic association**

The tag-SNP may not necessarily be the causal mutation. It may instead be in linkage disequilibrium with a functional variant. We therefore analyzed all SNPs moderately or strongly correlated with a combination of several populations from the American, African, and European continents, available from the 1000 Genomes Project. After applying stringent quality control to remove low-quality samples and SNPs from further analysis (as detailed in Materials and Methods), a set of 128 polymorphisms remained and was analyzed in 738 individuals (317 affected trios from 205 nuclear families).
rs10905669 (Fig. 2A) and rs706778 (Fig. 2B) to exclude the possibility of polymorphisms of other genes close to IL2RA being responsible for the genetic associations. Pairwise correlation values were obtained from the 1000 Genomes Project ($r^2$ threshold = 0.6 in a 1 Mb window). Closer examination of this region indicated that all polymorphisms captured by either rs10905669 or rs706778 were located in a region extending from 4 kb directly 5' to IL2RA to 18 kb into intron 1 of the gene.

![FIGURE 1. Whole-genome expression profiling of cutaneous lesions from patients infected with L. braziliensis revealing activation of the IL-2 pathway.](image)

Significance analysis of microarray was carried out to evaluate whether gene expression differed significantly between cutaneous lesions (CL) and normal skin samples from uninfected donors (NS). (A) Heat map showing the expression profiles of 56 genes related to the IL-2 pathway in eight CL samples and eight NS samples. Global canonical pathway (GCP) analysis based on the Ingenuity database was used: the entire dataset was used to calculate relative $p$ value (right-tailed Fisher exact test) for the IL-2 pathway. The Pearson correlation coefficient was used to define hierarchical clustering of the samples. Asterisks indicate significant differences in gene expression between groups. (B) Plot showing the mean fold change in expression for 14 genes differentially expressed in CL and NS.
The IL2RA allele conferring predisposition to CL is correlated with a poor IFN-γ response and poor FOXP3\(^+\) Treg cell activation

Despite the pleiotropic role of the IL-2 pathway in the immune system, we focused our functional analysis on IFN-γ and the Treg cell responses because: 1) the role of IFN-γ in sterile immunity is well established in leishmaniasis; 2) IL-2 signaling plays a non-redundant role in the development/function of Treg cells; and 3) human CL (principally that due to \(L.\) \textit{braziliensis}) is frequently associated with an intense inflammatory response (immunopathology), which could be regulated by Treg cells.

We first evaluated the IFN-γ production of PBMCs from Brazilians exposed to \(L.\) \textit{braziliensis} and carrying different IL2RA genotypes. Patients with active lesions were not included in the analysis because treatment could interfere with the results. IFN-γ levels were lower in cultures stimulated with \(L.\) \textit{braziliensis} extract (\(p = 0.04\)) or with PHA (\(p = 0.07\), trend toward significance) from subjects carrying the rs706778 TT genotype than in cultures from individuals carrying other genotypes (CT and CC) (Fig. 3A).

Table II. Markers showing suggestive or significant associations with CL (due to \(L.\) \textit{braziliensis}) in the Brazilian discovery study

<table>
<thead>
<tr>
<th>Tag-SNP</th>
<th>Gene</th>
<th>Allele</th>
<th>Freq</th>
<th>Fam</th>
<th>(O_t)</th>
<th>(E_t)</th>
<th>(p)</th>
<th>(P_p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2069762</td>
<td>IL2RA (^{intergenic})</td>
<td>A</td>
<td>0.79</td>
<td>76</td>
<td>183</td>
<td>167</td>
<td>0.009(^a)</td>
<td>NS</td>
</tr>
<tr>
<td>rs4833248</td>
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<td>G</td>
<td>0.79</td>
<td>76</td>
<td>183</td>
<td>167</td>
<td>0.009(^a)</td>
<td>NS</td>
</tr>
<tr>
<td>rs7609976</td>
<td>IL2RA (^{intronic})</td>
<td>A</td>
<td>0.97</td>
<td>19</td>
<td>58</td>
<td>51</td>
<td>0.04(^a)</td>
<td>NS</td>
</tr>
<tr>
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<td>IL2RA (^{intronic})</td>
<td>C</td>
<td>0.89</td>
<td>9</td>
<td>16</td>
<td>12</td>
<td>0.01(^d)</td>
<td>NS</td>
</tr>
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<td>0.86</td>
<td>11</td>
<td>18</td>
<td>14</td>
<td>0.02(^d)</td>
<td>NS</td>
</tr>
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<td>114</td>
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<td>241</td>
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<tr>
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<td>0.88</td>
<td>9</td>
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<td>NS</td>
</tr>
<tr>
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<td>IL2RA (^{intronic})</td>
<td>T</td>
<td>0.18</td>
<td>80</td>
<td>84</td>
<td>62</td>
<td>0.0003(^e)</td>
<td>0.03</td>
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<tr>
<td>rs706778</td>
<td>IL2RA (^{intronic})</td>
<td>T</td>
<td>0.41</td>
<td>75</td>
<td>76</td>
<td>53</td>
<td>0.0003(^f)</td>
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<td>0.70</td>
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<td>180</td>
<td>0.04(^a)</td>
<td>NS</td>
</tr>
<tr>
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<td>T</td>
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<td>G</td>
<td>0.68</td>
<td>97</td>
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<td>207</td>
<td>0.004(^e)</td>
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<tr>
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<td>0.82</td>
<td>64</td>
<td>151</td>
<td>138</td>
<td>0.01(^a)</td>
<td>NS</td>
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<td>0.65</td>
<td>43</td>
<td>51</td>
<td>42</td>
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<td>NS</td>
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<tr>
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<td>0.18</td>
<td>11</td>
<td>13</td>
<td>7</td>
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<tr>
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<td>JAK3 (^{intronic})</td>
<td>C</td>
<td>0.78</td>
<td>76</td>
<td>178</td>
<td>166</td>
<td>0.04(^a)</td>
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<td>0.66</td>
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<td>11</td>
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<td>10</td>
<td>0.03(^d)</td>
<td>NS</td>
</tr>
</tbody>
</table>

For asymptotic \(p\) values (\(P\)), superscript letters indicate the following: a, additive genetic model; d, dominant genetic model; r, recessive genetic model. Allele, reference allele; Freq, frequency of the reference allele; Fam, number of informative families; \(O_t\), observed transmissions; \(E_t\), expected transmissions; \(P\), asymptotic \(p\) value; \(P_p\), empirical \(p\) value (10,000 permutations), corrected for all tests.
because the frequency of its TT genotype was too low in our study samples.

Discussion
We first showed that several genes of the IL-2 pathway (especially those of the JAK3/STAT5 axis) were activated in skin ulcers caused by *L. braziliensis*, indicating that this pathway may play an important role in sterile immunity, infection-induced immunopathology, or both. We then found that at least two tag-SNPs in the *IL2RA* gene were independently associated with CL in Brazilian families exposed to *L. braziliensis*. These associations were extended to an Iranian population affected by *L. tropica*, which also causes severe cutaneous lesions. Thus, these SNPs were found to be associated with CL caused by two different *Leishmania* species. Between-study heterogeneity (as assessed with Cochran’s Q test) was negligible for both SNPs: rs10905669 (p = 0.50) and rs706778 (p = 0.77) (Table IV). The consistent effects of these two polymorphisms in populations from South America and the Middle East, which may have undergone different regional adaption and selection processes, suggest functional relevance or strong linkage to a causal variant yet to be identified. The data presented in Fig. 2 ruled out the possibility of these associations with *IL2RA* actually being due to SNPs in another gene, including genes not encoding IL-2/IL-2R components.

To support our results, we carried out an in silico analysis of the SNPs correlated (r² ≥ 0.6) with either rs10905669 or rs706778 (in American, African, and European reference panels, 1000 Genomes Project) to identify the most likely regulatory variants in each region. We evaluated various functional annotations (see *Materials and Methods*) in our sets of polymorphisms, including predicted chromatin state segmentation, predicted DNAse hypersensitivity, and sequence conservation across mammals (Supplemental Fig. 1). In the rs10905669 linkage disequilibrium block, we identified rs942201, rs1107345, rs10905668, and rs10905669 as the best candidate regulatory SNPs on the basis of their location within an accessible (open chromatin) regulatory element. This analysis also revealed that rs3134883 was the best candidate for a causal variant in the rs706778 linkage disequilibrium block. This SNP is located within an evolutionarily conserved open chromatin region (DNase I hypersensitivity site), which also has histone marks for promoter elements. We confirmed that all the cited SNPs were indeed associated with CL in Brazil and Iran (data not shown). We are currently investigating the molecular mechanisms and the effects of these mutations in the *IL2RA* gene.

IL-2/IL-2R signaling promotes T and B cell growth and survival and is involved in primary and memory immune responses in vivo (29, 30). It also controls growth and the cytolytic activity of NK cells (31). IL-2 also regulates the fate of T effector cells, as it induces Th2 differentiation, promotes optimal IFN-γ production by Th1 cells, and limits Th17 differentiation (32). Conversely, IL-2 signaling plays a nonredundant role in immune homeostasis by promoting the development and suppressive function of Treg cells (15). *IL2RA* polymorphisms may therefore affect leishmaniasis in several ways. We have shown in the present study that the rs706778 T allele is associated with a poor IFN-γ response in PBMCs from individuals living in a region of endemic CL. We have also shown that the rs706778 TT genotype is associated with a low proportion of CD4+ CD45RA− FOXP3 hi effector Treg cells in cultures of stimulated PBMCs in both the presence and absence of exogenous IL-2.

There is strong evidence to suggest that IFN-γ enhances anti-*Leishmania* immunity (6), and the impairment of the IFN-γ response due to mutations of the *IL2RA* gene may hinder the control of parasite replication in infected individuals. Our data also indicate that the increased risk of CL may be associated with impaired Treg cell activation. These results indicate that the allele increasing disease risk decreases the signaling of IL-2 through its receptor. However, they do not conclusively demonstrate that Treg cells are protective in CL. Indeed in vitro–induced Treg cells have been shown to be different from in vivo–induced Treg cells. In particular, their phenotype is not stabilized by site-specific demethylation (22). Additionally, it could be argued that the effects of the *IL2RA* variants on CL susceptibility are unrelated to their effects on Treg cells. Previous studies (33, 34) have suggested that Treg cells may aggravate *L. braziliensis* infections.

### Table III. Polymorphisms in intron 1 of the *IL2RA* gene are associated with CL in other independent cohorts

<table>
<thead>
<tr>
<th>Marker</th>
<th>Risk Allele</th>
<th>Freq</th>
<th>Fam</th>
<th>Oₙ</th>
<th>Eₙ</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs10905669</td>
<td>T</td>
<td>0.16</td>
<td>20</td>
<td>18</td>
<td>13</td>
<td>0.08 a</td>
</tr>
<tr>
<td>rs706778</td>
<td>T</td>
<td>0.42</td>
<td>43</td>
<td>58</td>
<td>51</td>
<td>0.04 a</td>
</tr>
</tbody>
</table>

**Table IV. Meta-analysis of the studied population samples from Brazil and Iran**

<table>
<thead>
<tr>
<th>SNP</th>
<th>Risk Allele</th>
<th>Brazilian Discovery Study</th>
<th>SE</th>
<th>OR</th>
<th>SE</th>
<th>OR</th>
<th>Iranian Extension Study</th>
<th>OR</th>
<th>Q</th>
<th>OR</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs10905669</td>
<td>T</td>
<td>0.15</td>
<td>2.1</td>
<td></td>
<td></td>
<td></td>
<td>0.27</td>
<td>1.5</td>
<td>2</td>
<td>0.50</td>
<td>2.0</td>
</tr>
<tr>
<td>rs706778</td>
<td>T</td>
<td>0.13</td>
<td>1.8</td>
<td>0.18</td>
<td>2.0</td>
<td>0.24</td>
<td>1.5</td>
<td>3</td>
<td>0.77</td>
<td>1.8</td>
<td>2.10^-9</td>
</tr>
</tbody>
</table>

In the Brazilian replication study, rs10905669 did not reach the significance threshold and was not included in the analysis. SE, SE of odds ratio (OR); N, number of valid studies; Q, p value for the Cochran’s heterogeneity statistic; P, p value for fixed effect meta-analysis.
Our data are consistent with those of recent studies showing that IFN-\(\gamma\) mediates parasite killing (sterile immunity) rather than tissue injury (35), whereas immunopathology results mostly from the cytolytic activity of CD8\(^+\) T cells (35, 36), which can be restrained by Treg cells. Overall, our observations suggest that IL2RA variants may alter the risk of leishmaniasis through their pleiotropic effects on various IL-2–dependent responses.

Our search for variants of the IL2RB gene conferring predisposition to cutaneous lesions identified several SNPs that appeared to be associated with CL in Brazilians. However, the SNP associated with KA in Sudan (14) was not associated with CL in Brazilians. Further studies are therefore required to determine whether disease dissimilarities and/or different linkage disequilibrium structures between these two populations could account for such results. Nevertheless, this work on L. braziliensis and L. tropica and our previous study in a L. donovani–infected population (14) show that polymorphisms of genes of the IL-2R alter human susceptibility to the leishmaniasis caused by various Leishmania species.

Other studies have reported associations between common non-coding polymorphisms at the IL2RA locus and susceptibility to several autoimmune diseases (37–39), probably through an impairment of Treg cell responses (40). Genetic variants in the IL2 and IL2RB regions have also been associated with immune dysregulation (41–43). To our knowledge, this study provides the first demonstration
that mutations of the $IL2RA$ gene (encoding the only receptor subunit specific for IL-2) influence susceptibility to an infectious disease. Given that $IL2RA$ variants could potentially impair IL-2-dependent responses in general, but with a more restrictive effect in some pathologic conditions, this work should prompt studies assessing these mutations in many other infectious diseases, particularly those caused by intracellular pathogens.

In conclusion, we present strong evidence for a link between mutations of $IL2RA$ gene and susceptibility to CL through our demonstration that certain allelic variants of $IL2RA$ is more frequent in Brazilian subjects with cutaneous ulcers caused by $L. braziliensis$. These associations were confirmed in a second Brazilian cohort and extended to a cohort from Iran infected with $L. tropica$ (providing evidence that our results are robust and ruling out the possibility of spurious associations due to statistical/methodological artifacts). We also demonstrated that the causal polymorphisms concerned $IL2RA$ (rather than genes in the surrounding regions) by ruling out the possibility of polymorphisms outside of $IL2RA$ but in linkage disequilibrium with the associated polymorphisms being responsible for the observed effects. Furthermore, we demonstrated that the alleles increasing disease risk were those correlated with decreased IL-2–dependent responses, thereby impairing IFN-γ production and T cell activation/induction in vitro. We conclude that IL-2 modulates disease susceptibility by increasing protection against cutaneous lesions. This identifies the IL-2 pathway as a suitable target for strategies aiming to control Leishmania-related diseases.

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Disclosures
The authors have no financial conflicts of interest.

References


