FUNDAÇÃO OSWALDO CRUZ
CENTRO DE PESQUISAS GONÇALO MONIZ

Curso de Pós-Graduação em Biotecnologia em Saúde e Medicina Investigativa

TESE DE DOUTORADO

GENÉTICA DA LEISHMANIOSE CUTÂNEA EM HUMANOS: IDENTIFICAÇÃO DE VIAS IMUNOLÓGICAS QUE CONTRIBUEM PARA A SUSCETIBILIDADE À DOENÇA

PABLO RAFAEL SILVEIRA OLIVEIRA

Salvador-Bahia
2015

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PABLO RAFAEL SILVEIRA OLIVEIRA

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Tese apresentada ao Curso de Pós-Graduação em Biotecnologia em Saúde e Medicina Investigativa, como parte dos requisitos necessários para a obtenção do grau de Doutor.

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2015

DEDICATÓRIA

Para o meu grande amor, Natália... por ter sido a minha paz durante esses anos tão difíceis.

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OLIVEIRA, Pablo Rafael Silveira. Genética da leishmaniose cutânea em humanos: identificação de vias imunológicas que contribuem para a suscetibilidade à doença. 211 f. il. Tese (Doutorado) – Fundação Oswaldo Cruz, Centro de Pesquisas Gonçalo Moniz, Salvador, 2015.

RESUMO

A leishmaniose cutânea (LC) é a forma clínica mais comum do complexo de doenças causadas por protozoários do gênero *Leishmania*. Interessantemente, alguns indivíduos infectados com espécies dermotrópicas do parasito não desenvolvem a LC, enquanto outros desenvolvem lesões crônicas. Os mecanismos envolvidos nesta variação permanecem amplamente desconhecidos, embora fatores genéticos do hospedeiro podem influenciar o risco de desenvolver a doença. No primeiro estudo apresentado nesta tese, foi mostrado que a sinalização IL-2/IL-2R desempenha um papel crucial na resposta imune contra espécies dermotrópicas de *Leishmania*. Os transcritos de vários genes da via de sinalização IL-2 são mais abundantes em úlceras cutâneas causadas por *Leishmania braziliensis* do que em amostras de pele normal de dadores não infectados. Um estudo de associação em famílias brasileiras (209 famílias nucleares) identificou dois polimorfismos no gene *IL2RA* associados à LC causada por *L. braziliensis* [rs10905669 (p = $3x10^{-4}$) e rs706778 (p = $3x10^{-4}$)]. Estes resultados foram replicados em uma segunda amostra brasileira (80 famílias nucleares) [rs10905669 (p = 0.08) e rs706778 (p = 0.04)] e em iranianos infectados com *Leishmania tropica* (coorte do tipo caso-controle composta por 236 indivíduos) [rs10905669 (p = 0.03) e rs706778 (p = 0.04)]. Uma metanálise dos três estudos confirmou que os alelos rs10905669 T ($p_{combinado}$ = $6x10^{-7}$) e rs706778 T ($p_{combinado}$ = $2x10^{-9}$) são fortemente associados a uma maior predisposição à LC. O alelo T do SNP rs706778 também foi associado a uma menor produção de IFN-γ por células mononucleares após a estimulação com extrato de *Leishmania* e com uma redução na ativação de células T regulatórias (T_{reg}) $FoxP3^+$ *in vitro*. Em conjunto, os dados apresentados no estudo 1 suportam a hipótese de que a via de sinalização IL-2 é implicada no desenvolvimento da LC, com possíveis consequências no controle da replicação do parasito e na imunopatologia associada à infecção. No segundo estudo desta tese, foi realizada uma análise de ligação genômica em famílias brasilieras expostas à *L. braziliensis*. Esta análise revelou um novo *locus* de suscetibilidade para a LC na região 10q21-q23 (LOD sugestivo = 2.39). Em toda esta região, os genes mais fortemente induzidos em lesões cutâneas (em relação aos controles) foram *PRF1* (*fold-change* = 49.3) e *SRGN* (*fold-change* = 21.8). Interessantemente, ambos os genes codificam moléculas envolvidas nos mecanismos de citotoxicidade de células T $CD8^+$ e de células *natural killer*. Por fim, dois polimorfismos na região do gene *SRGN* foram associados ao risco de LC em famílias brasileiras expostas à *L. braziliensis* [estudo primário (209 famílias): rs10998538 (p = 0.001) e rs12437 (p = 0.003); estudo de replicação (80 famílias): rs10998538 (p = 0.01) e rs12437 (p = 0.007)]. Por fim, os dados apresentados nesta tese apontam a serglicina (codificada pelo gene *SRGN*) e a via de sinalização IL-2 como alvos potenciais de novas estratégias de tratamento ou prevenção contra a LC em humanos.

Palavras-chave: Leishmaniose cutânea; Genética; Polimorfismo*;* Vias de sinalização.

OLIVEIRA, Pablo Rafael Silveira. Genetics of cutaneous leishmaniasis in humans: Identification of pathways that play crucial roles in disease susceptibility. 211 f. il. Tese (Doutorado) – Fundação Oswaldo Cruz, Centro de Pesquisas Gonçalo Moniz, Salvador, 2015.

ABSTRACT

Cutaneous leishmaniasis (CL) is the most common clinical form of leishmaniasis and can be caused by several dermotropic *Leishmania* species. Interestingly, some infected individuals do not develop cutaneous lesions, while others are severely affected. The basis of this variation remains largely unknown, although host genetic factors seem to influence disease risk. In the first study presented in this thesis, it was shown that IL-2 plays a crucial role in human immunity against dermotropic *Leishmania* species. It was observed that the transcripts of several genes of the IL-2 pathway were more abundant in skin ulcers caused by *Leishmania braziliensis* than in normal skin samples. A primary association study on Brazilians (754 individuals from 209 families) identified two polymorphisms in the *IL2RA* gene associated with CL caused by *L. braziliensis* [rs10905669 (p = 3x10^{-4}) and rs706778 (p = 3x10^{-4})]. This result was confirmed in a second Brazilian sample (325 subjects from 80 nuclear families) [rs10905669 (p = 0.08) and rs706778 (p = 0.04)] and in Iranians infected with *Leishmania tropica* (236 individuals) [rs10905669 (p = 0.03) and rs706778 (p = 0.04)]. A meta-analysis confirmed that rs10905669 T allele ($p_{combined}$ = 6x10^{-7}) and rs706778 T allele ($p_{combined}$ = 2x10^{-9}) were strongly associated with increased susceptibility to CL. The T allele of rs706778 was also associated with lower IFN-γ production by peripheral blood mononuclear cells after *Leishmania* antigen stimulation and with reduced FoxP3$^+$ T_{reg} activation *in vitro*. Altogether, these data support the notion that the IL-2 signaling pathway is implicated in the development of cutaneous leishmaniasis and could be involved in the control of both parasite replication and infection-induced immunopathology. In the second study, a genome wide linkage (GWL) scan conducted in Brazilian multiplex families revealed a new susceptibility *locus* for CL on chromosome 10q21-q23 (suggestive LOD = 2.39). Interestingly, in this entire region, the most strongly induced transcripts were from *PRF1* (fold change = 49.3) and *SRGN* (fold change = 21.8) genes, both encoding molecules involved in the cytotoxic mechanisms by a variety of cell types, including CD8$^+$ T cells and natural killer cells. Finally, two polymorphisms in the *SRGN* region were associated with susceptibility to CL in Brazilian families exposed to *L. braziliensis* [Discovery study (209 families): rs10998538 (p = 0.001) and rs12437 (p = 0.003); Replication study (80 families): rs10998538 (p = 0.01) and rs12437 (p = 0.007)]. These data highlight the serglycin (encoded by the *SRGN* gene) and the IL-2 pathway as suitable targets for new strategies aimed to treat or prevent cutaneous leishmaniasis.

Key-words: Cutaneous leishmaniasis; Genetics; Polymorphism; Signaling pathway.

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LISTA DE ABREVIATURAS

1KG	Projeto 1000 genomas
ACP	Análise de componentes principais
AFR	Populações africanas
AGVC	Análise global de vias canônicas
AKT	Proteína quinase B alfa
AMR	Populações americanas
APC	Aloficocianina
APCs	Células apresentadoras de antígeno
CCL	Ligante de quimiocina (motivo C-C)
CD	Agrupamento de diferenciação
cM	Centimorgan
CNV	Variante de número de cópias
CP1	Componente principal 1
CP2	Componente principal 2
cRNA	RNA complementar
CXCR	Receptor de quimiocina (motivo C-X-C)
DL	Desequilíbrio de ligação
DNA	Ácido desoxirribonucleico
dNTPs	Desoxinucleotídeo trifosfato
ELISA	Ensaio imunoenzimático
eT_{reg}	Célula T regulatória efetora
EUR	Populações européias
FC	*Fold change* (diferença de expressão)
FDR	Taxa global de resultados falso-positivos
FITC	Isotiocianato de fluoresceína
FoxP3	*Forkhead box P3*
γc	Receptor comum γc
GWA	Análise de associação genômica
GWL	Análise de ligação genômica
GZMB	Granzima B
H3K27Ac	Acetilação da lisina 27 da histona H3
H3K4Me1	Metilação da lisina 4 da histona H3
H3K4Me3	Trimetilação da lisina 4 da histona H3
HLA	Antígeno leucocitário humano
IFN-γ	Interferon gama
IL-	Interleucina
IL-2R	Receptor de alta afinidade para a interleucina 2
IL-2Rα	Subunidade alfa do receptor para a interleucina 2
IL-2Rβ	Subunidade beta do receptor para a interleucina 2

Indel	Inserção e deleção
JAK	Janus quinase
LC	Leishmaniose cutânea
LOD	Logaritmo de *odds*
LV	Leishmaniose visceral
MAPK	Proteína quinase ativada por mitógeno
MLB	Método da probabilidade binomial mista
NK	Célula *Natural killer*
NKT	Célula *Natural killer* T
PBMC	Célula mononuclear do sangue periférico
PCR	Reação em cadeia da polimerase
PE	Ficoeritrina
PE-Cy7	Ficoeritrina acoplada à cianina 7
PHA	Fitoemaglutinina
PI3K	Fosfatidilinositol 3-quinase
PRF1	Perforina 1
PSAP	Prosaposina
RIN	Valor de integridade de RNA
RNA	Ácido ribonucleico
rT_{reg}	Célula T regulatória não ativada (*resting*)
SAM	Análise de significância para microarranjos
SAR1A	*Secretion associated, Ras related GTPase 1A*
SLC29A3	*Solute carrier family 29, member 3*
SNP	Polimorfismo de base única
SRGN	Serglicina
STAT	Transmissor de sinal e ativador de transcrição
SUPV3L1	*Suppressor of var1 3-like protein 1, ATP-dependent RNA helicase*
TCR	Receptor de célula T
TDT	Teste de desequilíbrio de transmissão de alelos
TGF-β1	Fator de crescimento transformador beta 1
T_H	Célula T auxiliar
TNF	Fator de necrose tumoral
T_{reg}	Célula T regulatória
TTC18	*Tetratricopeptide repeat domain 18*
USP54	*Ubiquitin specific peptidase 54*
UTR	Região não codificante
UV	Luz ultravioleta
VPS26A	*Vacuolar protein sorting 26 homolog A*

LISTA DE URLs

ENCODE	http://genome.ucsc.edu/ENCODE/
ENSEMBL	http://ensembl.org/
GEO	http://ncbi.nlm.nih.gov/geo/
NCBI	http://ncbi.nlm.nih.gov/
PLINK	http://pngu.mgh.harvard.edu/~purcell/plink/
Projeto 1000 genomas	http://1000genomes.org/
Sistema Ingenuity	http://ingenuity.com
String	http://string-db.org/
Target Explorer – Sistema Ingenuity	http://targetexplorer.ingenuity.com/
UCSC	http://genome.ucsc.edu

SUMÁRIO

1 REVISÃO BIBLIOGRÁFICA

1.1 ASPECTOS GERAIS DAS LEISHMANIOSES (ÊNFASE EM LEISHMANIOSE CUTÂNEA)

Protozoários do gênero *Leishmania* foram descritos de forma independente por Willian Leishman e Charles Donovan em 1903, apesar de terem sido observados anteriormente por David Cuningham em 1885 e Peter Borovsky em 1889 (VANNIER-SANTOS *et al*., 2002). Do início do século 20 até os dias atuais foram identificadas cerca de 20 espécies de *Leishmania* que infectam humanos e causam um complexo de doenças conhecidas como leishmanioses (HERWALDT, 1999; DESJEUX, 2001; DESJEUX, 2004).

A transmissão do parasito ocorre através de vetores hematófagos (Diptera: Psychodidae) do gênero *Phlebotomus* no Velho Mundo e *Lutzomyia* no Novo Mundo (SACKS E KAMHAWI, 2001). Os parasitos se multiplicam no trato digestivo do vetor na forma de promastigotas. Quando transmitidas para o hospedeiro vertebrado, as promastigotas são fagocitadas principalmente por macrófagos, células dendríticas ou neutrófilos (PRINA *et al*., 2003; JOHN e HUNTER, 2008; LASKAY *et al*., 2008; PETERS *et al*., 2008). No modelo de interação direta *Leishmania*-macrófago, o parasito é fagocitado diretamente pelos macrófagos e se multiplica dentro do vacúolo parasitóforo na forma de amastigotas, disseminando a infecção (MURRAY *et al*., 2005). De acordo com o modelo de interação *Leishmania*-neutrófilo-macrófago, os neutrófilos são as primeiras células que capturam os parasitos no sítio de infecção e atuam como pontes entre a *Leishmania* e a sua célula hospedeira final, o macrófago (PETERS e SACKS, 2009). De modo geral, após o estabelecimento da infecção nos macrófagos, a progressão da doença dependerá: (i) do perfil imunológico/genético do hospedeiro, associado ao tipo de resposta de células T (LARA *et al*., 1991; PETZL-ERLER *et al*., 1991; BLACKWELL, 1999; CASTELLUCCI *et al*., 2006) e (ii) da espécie de *Leishmania*, associada à sua capacidade de interagir com as células apresentadoras de antígeno (APCs) (SILVEIRA *et al*., 2008; BREWIG *et al*., 2009), produzindo manifestações clínicas variadas.

As leishmanioses têm ampla distribução e podem ser encontradas em zonas intertropicais dos continentes americano e africano, e estender-se para regiões temperadas da América do Sul, sul da Europa e Ásia (LAINSON e SHAW, 1977; GRIMALDI JR. *et al*., 1989). A distribuição geográfica da doença depende dos aspectos ecológicos da espécie de flebotomíneo que atua como vetor do parasito (DESJEUX, 2001). A leishmaniose cutânea (LC) é a forma mais comum da doença. Estima-se a ocorrência de 1.5 milhões de novos casos de LC anualmente, com cerca de 12 milhões de pessoas vivendo sob o risco de desenvolver a doença em todo o mundo (DESJEUX, 2004; ALVAR *et al*., 2012). Mais de 90% dos casos de LC ocorrem na América do Sul e no Oriente Médio. A *Leishmania braziliensis* apresenta a maior distribuição geográfica entre as espécies de *Leishmania* que ocorrem na América do Sul. Doenças causadas por *L. braziliensis* já foram descritas em 14 países (SHAW, 2000). No Brasil, a *L. braziliensis* é responsável pela maioria dos casos de leishmaniose cutânea e é uma das espécies mais patogênicas (SILVEIRA *et al*., 2009). A infecção por *L. braziliensis* pode causar úlceras localizadas na pele, lesões cutâneas disseminadas, além da leishmaniose mucocutânea, que pode desfigurar a face dos pacientes afetados (BOAVENTURA *et al*., 2006). No Oriente Médio, a LC é causada principalmente por *Leishmania major* e *Leishmania tropica* (KLAUS *et al*., 1999). A *L. tropica* pode causar lesões cutâneas graves e a sua transmissão é comum em regiões periféricas de grandes cidades e em aldeias nas zonas rurais (MOHAGHEGH *et al*., 2013). A leishmaniose lupóide, que é uma manifestação tardia associada à infecção com *L. tropica*, ocorre em cerca de 6% dos pacientes iranianos (KLAUS e FRANKENBURG, 1999). Os pacientes com a forma lupóide apresentam nódulos no entorno das lesões ou das cicatrizes, muitas vezes anos após a cicatrização da lesão inicial.

O primeiro sinal de infecção com uma espécie dermotrópica de *Leishmania* é a formação de um pequeno eritema no sítio primário da infecção, que se desenvolve em uma pápula, em seguida em um nódulo que ulcera progressivamente até se tornar uma lesão característica da LC (indolor, com bordo elevado e fundo necrótico). As lesões cutâneas variam em gravidade, aparência clínica e tempo de cura (BARRAL *et al*., 1995; TURETZ *et al*., 2002). Primariamente, a LC compromete a pele, mas a

linfadenopatia regional é comum, podendo preceder o aparecimento das lesões cutâneas (BARRAL *et al*., 1995).

1.2 IMUNOLOGIA DA LEISHMANIOSE CUTÂNEA

1.2.1 Estudos iniciais e complexidade na relação parasito-hospedeiro

Estudos iniciais em modelos murinos de leishmaniose mostraram que as células T $CD4^+$ estão envolvidas no controle da carga parasitária e na progressão da doença e que as células T $CD8^+$ contribuem para o desenvolvimento da memória imunológica contra a *Leishmania* (MITCHELL, 1983; TYTUS *et al*., 1987). Posteriormente, outros estudos mostraram que a resistência/suscetibilidade de camundongos à *L. major* é determinada pela atividade de diferentes subclasses de células T auxiliares (SACKS e NOBEN-TRAUTH, 2002). A progressão da doença é associada ao predomínio de linfócitos T_H2, que produzem IL-4, enquanto a resistência à infecção é associada à ativação de células T_H1, que produzem IFN-γ, estimulando a atividade leishmanicida de macrófagos. No entanto, foi observado que outros componentes do sistema imunológico também desempenham papéis essenciais durante as leishmanioses (SACKS e ANDERSON, 2004; ALEXANDER e BROMBACHER, 2012). A IL-12, produzida por APCs, é um fator central na estimulação da resposta T_H1 anti-*Leishmania* (SYPEK *et al*., 1993). Células dendríticas (MOLL *et al*., 1993) e neutrófilos (TACCHINI-COTTIER *et al*., 2000) regulam a imunidade adaptativa durante as leishmanioses. Além disso, as células T regulatórias (T_{regs}) controlam as respostas de células T_H1 e T_H2 em camundongos infectados com *Leishmania* (BELKAID *et al*., 2002; SUFFIA *et al*., 2005). Recentemente, as células T_H17 (e a citocina IL-17) (KATARA *et al*., 2013; GONZALEZ-LOMBANA *et al*., 2013), além da IL-1β, produzida após a ativação do inflamassomo (LIMA-JUNIOR *et al*., 2013), foram implicadas na imunopatogênese da leishmaniose cutânea. Estes e outros estudos (discutidos a seguir) revelaram a complexidade da resposta imune ativada durante as leishmanioses.

1.2.2 A via de sinalização IL-2 e as leishmanioses

A IL-2 é produzida principalmente por células T $CD4^+$ e, em menor magnitude, por células T $CD8^+$ e células *natural killer* (NK) (SETOGUCHI *et al*., 2005; MALEK, 2008). O receptor de alta afinidade para a IL-2 (IL-2R) é composto pelas moléculas IL-2Rα (CD25), IL-2Rβ (CD122) e γc (CD132) (Figura 1). A subunidade IL-2Rα não participa da sinalização intracelular, mas aumenta a afinidade da IL-2 ao seu receptor em até 100 vezes (TANIGUCHI e MINAMI, 1993). O receptor trimérico IL-2R é expresso de forma transitória na superfície de células T $CD4^+$ e $CD8^+$, pouco tempo após a ativação pelo TCR (receptor de células T) (BOYMAN e SPRENT, 2012). Ao contrário de outras subclasses de células T, as T_{regs} expressam IL-2Rα constitutivamente (MALEK, 2008). IL-2Rβ e γc são os componentes funcionais do IL-2R, e ambos apresentam motivos de sinalização nas suas caudas citoplasmáticas. A transmissão do sinal ocorre através de várias vias intracelulares, incluindo as vias reguladas por JAK-STAT, PI3K-AKT e MAPK (WALDMANN, 2006). A subnidade γc confere resposta não só à IL-2, mas também a outras citocinas, como: IL-4, IL-7, IL-9, IL-15 e IL-21 (ROCHMAN *et al*., 2009).

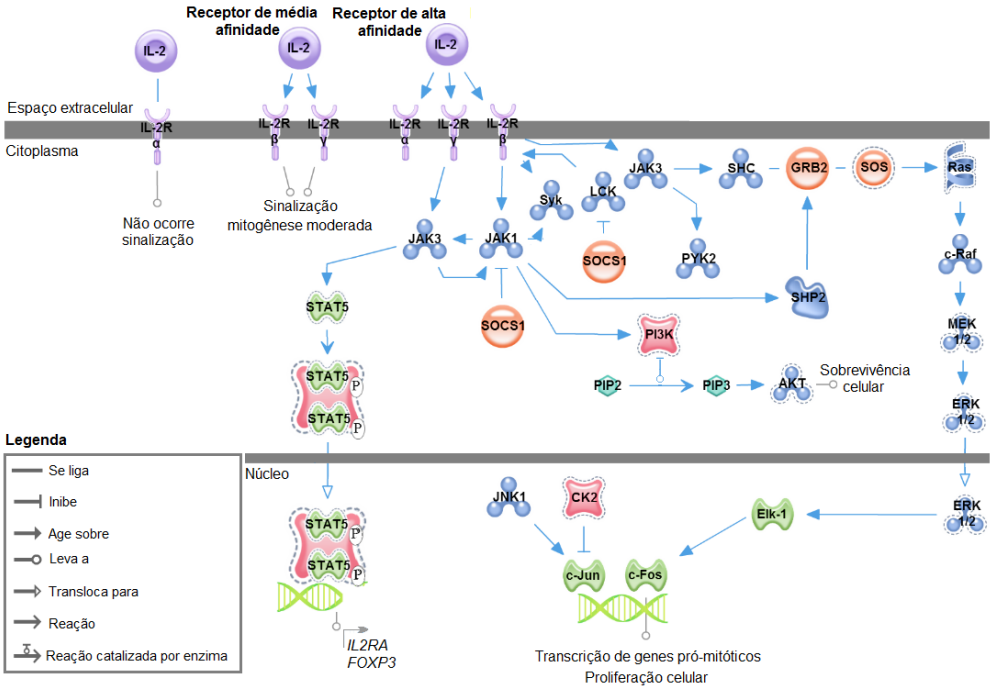


Figura 1. Via de sinalização IL-2 (adaptado de Target Explorer – Ingenuity Systems; http://targetexplorer.ingenuity.com/).

A sinalização IL-2/IL-2R promove a proliferação e a sobrevivência de células T e B, além de participar do desenvolvimento das respostas imunológicas primária e de memória *in vivo* (SAPAROV *et al*., 1999; WILLIAMS *et al*., 2006). A sinalização IL-2 também controla a proliferação e a atividade citolítica de células NK (FEHNIGER *et al*., 2003). A IL-2 regula o desenvolvimento e a função de células T efetoras, induzindo a diferenciação de células T_H2, aumentando a produção de IFN-γ por células T_H1 e inibindo a diferenciação de células T_H17 (LIAO *et al*., 2011). Por outro lado, muitas evidências indicam que a sinalização IL-2/IL-2R desempenha um papel não-redundante no desenvolvimento e função de T_{regs} $FoxP3^+$ (BAYER *et al*., 2005; SETOGUCHI *et al*., 2005; FONTENOT *et al*., 2005; BURCHILL *et al*., 2007). Estudos *in vitro* mostraram que a ativação de células $CD4^+CD25^+FoxP3^+$ requer IL-2 (THORNTON *et al*., 2004) e que esta citocina é necessária para a sobrevivência e para a expansão da população de T_{regs} em tecidos periféricos (SAKAGUCHI *et al*., 2006). Além disso, a sinalização através do IL-2R é necessária para a indução e manutenção da expressão de FoxP3, que é essencial para a função supressora de T_{regs} (FONTENOT *et al*., 2005; PASSERINI *et al*., 2008).

Interessantemente, nosso grupo identificou variantes do gene *IL2RB* (IL-2Rβ) que predispõem indivíduos infectados com *Leishmania donovani* para a leishmaniose visceral (LV) (BUCHETON *et al*., 2007), indicando que a via de sinalização IL-2 participa do desenvolvimento das leishmanioses. Além disso, a maioria dos fatores regulados pela sinalização IL-2/IL-2R é ativado durante a infecção por *Leishmania*. Por exemplo, o papel do IFN-γ produzido por células T_H1 na resposta protetora contra o parasito é bem estabelecido (MOUGNEAU *et al*., 2011) e as T_{regs} e as suas principais citocinas, IL-10 (SALHI *et al*., 2008) e TGF-β (BARRAL-NETO *et al*., 1992), modulam as respostas de células T durante as leishmanioses (BELKAID, 2007). As T_{regs} encontradas em lesões cutâneas (SUFFIA *et al*., 2005; CAMPANELLI *et al*., 2006) controlam a imunidade e a persistência do parasito em um modelo murino de infecção com *L. major* (BELKAID *et al*., 2002). Além disso, foi mostrado que as T_{regs} são centrais na regulação da inflamação causada pela infecção por *Leishmania amazonensis* (JI *et al*., 2005). Estudos recentes também indicam que as T_{regs} estão envolvidas na imunopatogênese

da leishmaniose cutânea causada por *L. braziliensis* (FALCÃO *et al*., 2012; COSTA *et al*., 2013; RODRIGUES *et al*., 2014).

1.2.3 Os papéis das células T $CD8^+$ e NK nas leishmanioses

Linfócitos citotóxicos (células T $CD8^+$, células NK e células NKT) são componentes cruciais da resposta imune efetora para a eliminação de células tumorais, células autoreativas e células infectadas com patógenos intracelulares (RUSSELL e LEY, 2002). As células citotóxicas induzem a eliminação de células-alvo através de dois mecanismos distintos. A primeira via envolve a sinalização de FASL (na membrana da célula efetora) através de seu receptor cognato FAS (na superfície da célula-alvo), que resulta na destruição do alvo celular por apoptose (NAGATA e GOLSTEIN, 1995). É importante salientar que a via FASL-FAS é envolvida principalmente na eliminação de linfócitos autoreativos (VAN PARIJS e ABBAS, 1996). No segundo mecanismo (ilustrado na Figura 2), grânulos citotóxicos são secredados pela célula efetora e induzem a apoptose da célula-alvo (DE SAINT BASILE *et al*., 2010). Os grânulos citotóxicos contém moléculas efetoras como a granzima B (serina protease) e a perforina 1 (que forma poros na membrana da célula-alvo). Um proteoglicano conhecido como serglicina é responsável pelo armazenamento destes fatores nos grânulos citotóxicos (RAJA *et al*., 2002). O complexo macromolecular formado por granzima B/perforina 1/serglicina é secretado no espaço extracelular pela célula citotóxica e é endocitado pela célula-alvo. Quando liberada no citoplasma, a granzima B induz a apoptose do alvo celular pela ativação da cascata de caspases diretamente (YANG *et al*., 1998) ou indiretamente, através de uma via depedente do citocromo C na membrana interna das mitocôndrias (MACDONALD *et al*., 1999).

Além de sua atividade citotóxica, as células NK também produzem citocinas efetoras, tais como IFN-γ e TNF, que participam do desenvolvimento da resposta T_H1 e da ativação de macrófagos (STETSON *et al*., 2003; LAOUAR *et al*., 2005; BIHL *et al*., 2010; PRAJEETH *et al*., 2011). Muitas evidências indicam que as células NK contribuem para o controle da infecção. Esta conclusão baseia-se nas observações obtidas em camundongos infectados com *L. major* ou *L. amazonensis*, que apresentam

carga parasitária aumentada e agravamento da doença após a depleção de células NK. Também foi observada uma melhora clínica após a transferência de células NK exógenas para estes animais (LASKAY *et al*., 1993; SCHARTON e SCOTT, 1993; LAURENTI *et al*., 1999; SANABRIA *et al*., 2008). Ademais, as células NKT parecem controlar a infecção por *L. major* na pele (MATTNER *et al*., 2006).

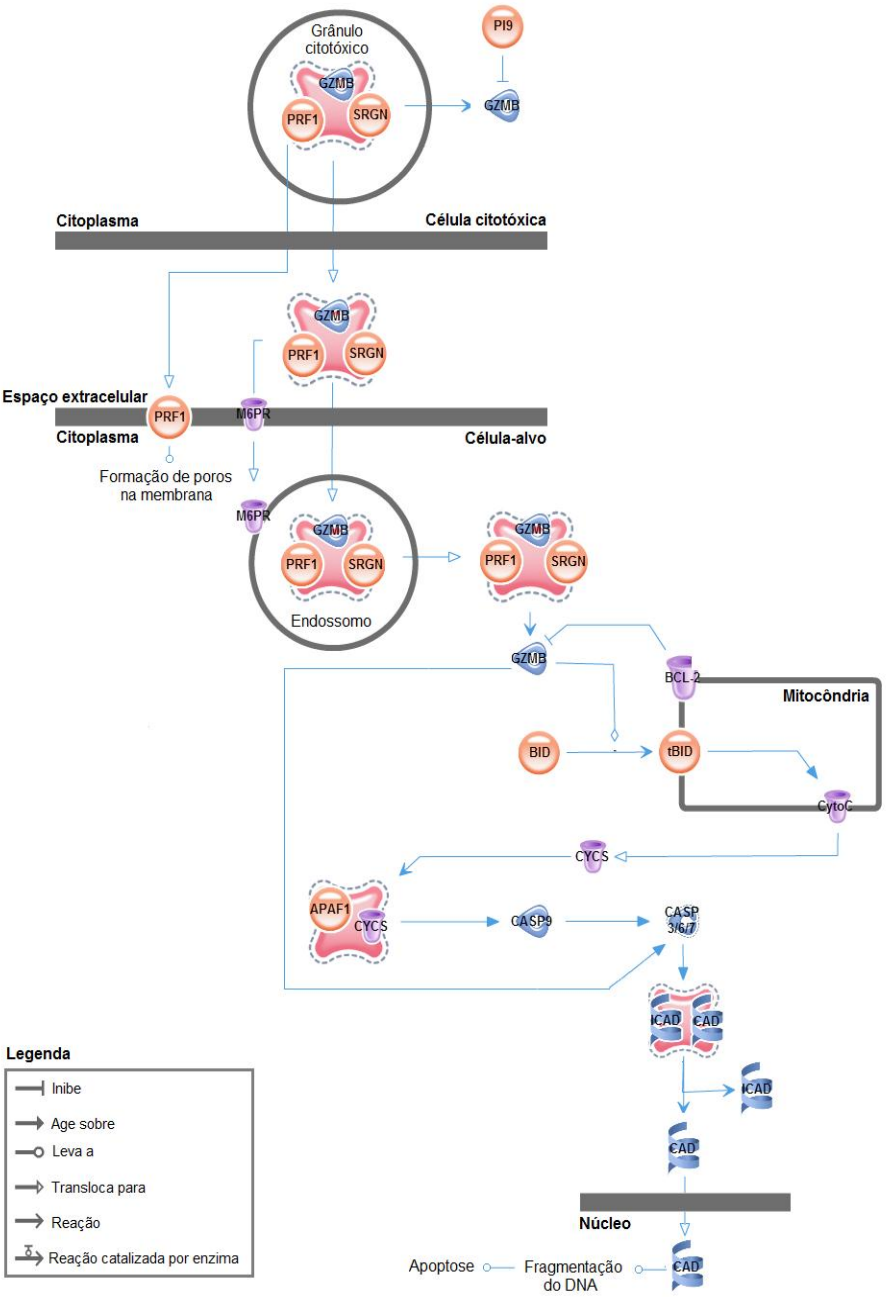


Figura 2. Eliminação de células-alvo mediada por grânulos de células citotóxicas (adaptado de Target Explorer – Ingenuity Systems; http://targetexplorer.ingenuity.com/).

Durante as leishmanioses, as células T $CD8^+$ contribuem para o controle do parasito através da produção de IFN-γ (UZONNA *et al*., 2004). Neste sentido, camundongos deficientes em células T $CD8^+$ são mais suscetíveis à infecção por *L. major* (MULLER *et al*., 1991). Por outro lado, foi mostrado recentemente que a depleção de células T $CD8^+$ em camundongos infectados com *L. braziliensis* tem efeito protetor em relação ao desenvolvimento de lesões cutâneas (NOVAIS *et al*., 2013). Embora os dados apresentados acima pareçam contraditórios, as evidências obtidas por Novais e colaboradores (2013) sugerem que, de modo diferente da infecção por *L. major*, na qual o IFN-γ produzido por células T $CD8^+$ exerce uma função protetora, na infecção por *L. braziliensis* a atividade citolítica de células T $CD8^+$ atua como principal fator envolvido na destruição tecidual nos sítios de infecção. Estes resultados são consistentes com observações que mostram uma correlação positiva entre o número de células T citolíticas e a gravidade da doença cutânea em pacientes infectados com *L. braziliensis* (FARIA *et al*., 2009; SILVA *et al*., 2013).

1.3 FATORES GENÉTICOS DO HOSPEDEIRO E SUSCETIBILIDADE ÀS LEISHMANIOSES

De modo geral, as doenças complexas, como as doenças parasitárias e infecciosas, estão sob a influência de um grande número de variantes genéticas, com efeitos leves ou moderados (CHAPMAN e HILL, 2012). Mutações com efeitos funcionais drásticos e penetrâncias elevadas são responsáveis por algumas formas familiares, além de formas com início precoce. Porém, elas são geralmente responsáveis por uma proporção muito pequena dos casos. As evidências de que fatores genéticos do hospedeiro influenciam a suscetibilidade às leishmanioses são substanciais e incluem agregação familiar de fenótipos clínicos (CASTELLUCCI *et al*., 2005; CABELLO *et al*., 1995), ampla variabilidade clínica entre indivíduos infectados com a mesma espécie/cepa de *Leishmania* (ZIJLSTRA *et al*., 2003) e risco aumentado entre irmãos (PEACOCK *et al*., 2001). Embora as bases genéticas de resistência/suscetibilidade às leishmanioses não sejam completamente compreendidas,

diferentes mutações que contribuem para o risco da doença já foram identificadas (LIPOLDOVÁ e DEMANT, 2006).

A influência de fatores genéticos do hospedeiro sobre as leishmanioses foi estudada principalmente em populações infectadas por espécies de *Leishmania* do Novo Mundo, embora alguns estudos com espécies do Velho Mundo também tenham sido realizados. Muitas análises de ligação ou associação genômica já foram conduzidas para avaliar a arquitetura genética da leishmaniose visceral em humanos. Por outro lado, a identificação de *loci* que influenciam o risco de leishmaniose cutânea em humanos tem sido baseada em análises de genes candidatos.

Como citado acima, nossa equipe mostrou que a suscetibilidade à leishmaniose visceral (causada por *L. donovani*) no Sudão (grupo étnico Aringa) é controlada pelo *locus* 22q12 (BUCHETON *et al*., 2003). Foi mostrado também que mutações no gene *IL2RB*, localizado no *locus* 22q12, poderiam ser responsáveis, pelo menos em parte, por este sinal de ligação genética (BUCHETON *et al*., 2007). Subsequentemente, polimorfismos no gene *NRAMP1* (*natural resistance-associated macrophage protein-1*) foram associados a uma maior predisposição à LV no Sudão (MOHAMED *et al*., 2004). Ainda no Sudão, porém avaliando indivíduos do grupo étnico Masalit, Miller e colaboradores (2007) identificaram um importante *locus* de suscetibilidade (1p22) para a LV. Foi mostrado também que a doença visceral em brasileiros infectados com *Leishmania chagasi* é controlada principalmente por três regiões genômicas (6q27, 7q11.22 e 17q11.2-q21.3). Além disso, polimorfismos nos genes que codificam as quimiocinas CCL1 e CCL16 poderiam ser responsáveis pelo sinal de ligação genética observado na região 17q11.2-q21.3 (JAMIESON *et al*., 2007). Recentemente, um estudo de associação do genoma completo, cuja sigla em inglês é GWA (*genome wide association*), identificou que a região que contém os genes HLA-II *DRB1* e *DQA1* (6p21) é associada com a suscetibilidade à leishmaniose visceral em populações da Índia e do Brasil (LEISHGEN CONSORTIUM *et al*., 2013).

Estudos iniciais em populações humanas infectadas com espécies dermotrópicas de *Leishmania* revelaram que as regiões dos genes que codificam as moléculas HLA I e II controlam o desenvolvimento de lesões cutâneas associadas às infecções por *Leishmania guyanensis* (BARBIER *et al*., 1987) e *L. braziliensis* (LARA *et al*., 1991;

PETZL-ERLER *et al*., 1991). Mutações no gene *TNF* (que codifica o fator de necrose tumoral) foram associadas ao risco de leishmaniose mucocutânea em pacientes venezuelanos infectados com *L. braziliensis* (CABRERA *et al*., 1995). Além disso, polimorfismos que diminuem a expressão da IL-6 foram associados a uma maior predisposição à leishmaniose mucocutânea no Brasil (CASTELLUCCI *et al*., 2006). Nossa equipe também evidenciou uma mutação no gene *IL10* que aumenta o risco de leishmaniose cutânea em famílias brasileiras expostas à *L. braziliensis* (SALHI *et al*., 2008). Além disso, outro estudo identificou mutações nos genes *CXCR1* e *NRAMP1* que aumentam a predisposição à LC no Brasil (CASTELLUCCI *et al*., 2010).

Apesar de todos estes esforços, não se sabe ao certo como estas mutações contribuem para a variação que existe na suscetibilidade humana às leishmanioses e quais os seus efeitos na heterogeneidade dos quadros clínicos observados.

1.3.1 Abordagens para o estudo de fatores genéticos em doenças complexas

Estudos genéticos (de ligação ou de associação) são ferramentas poderosas para explorar o envolvimento de fatores/vias no desenvolvimento de doenças infecciosas em humanos. Além disso, as associações genótipo-fenótipo representam evidências diretas sobre a implicação de genes/moléculas em um determinado traço.

1.3.1.1 Análise de ligação

Em humanos, a frequência de *crossing-overs* (cruzamentos cromossômicos) durante a meiose é relativamente baixa (média de 35 pontos no genoma gamético), fazendo com que a maior parte da informação genética seja herdada em conjunto (SHAM e CHERNY, 2011). Portanto, marcadores genéticos podem ser utilizados como ferramentas para a identificação de padrões genômicos ligados a diferentes traços em populações humanas (DAWN TEARE e BARRETT, 2005). Como a ligação genética pode operar ao longo de grandes distâncias, é possível avaliar todo o genoma humano utilizando um número relativamente pequeno de marcadores altamente polimórficos, como os marcadores microssatélites (STRACHAN, 1999). Os estudos de ligação

genética em famílias são baseados na probabilidade (LOD, ou logaritmo de *odds*) de que uma região genômica e um traço fenotípico sejam herdados em conjunto (cosegregação) (DAWN TEARE and BARRETT, 2005). Esta análise é parametrizada pela frequência do alelo em questão e por sua penetrância (probabilidade de um indivíduo com um determinado genótipo expressar o fenótipo correspondente) (RISCH, 1990). Nas doenças mendelianas (monogênicas), esses parâmetros são facilmente determinados a partir de estudos sobre a prevalência da doença na população investigada e através de análises de segregação alélica simples. Estes estudos de ligação tiveram grande sucesso na identificação de *loci* que controlam diversas doenças monogênicas ou oligogênicas (HALL *et al*., 1990; BELL *et al*., 1991). No entanto, os estudos de ligação clássicos são menos eficientes para a identificação de fatores genéticos que influenciam a maioria das doenças complexas (EGELAND *et al*., 1987; SHERRINGTON *et al*., 1988). Este fenômeno pode ser explicado pelo fato de que a maioria das mutações envolvidas em tais doenças têm efeitos discretos sobre a determinação dos fenótipos clínicos. Visto isso, o método da probabilidade binomial mista, cuja sigla em inglês é MLB (*mixed-likelihood-binomial method*), foi desenvolvido para otimizar a capacidade de detecção de sinais de ligação em doenças poligênicas (KRUGLYAK *et al*., 1996; ABEL e MULLER-MYHSOK, 1998). Este método é o mais apropriado para o estudo de doenças complexas como a esquistossomose e a leishmaniose (MARQUET *et al*., 1996; BUCHETON *et al*., 2003), uma vez que ele não assume a existência de um *locus* principal controlando o desenvolvimento do fenótipo avaliado.

1.3.1.2 Análise de associação

Os estudos de associação têm maior poder estatístico do que os estudos de ligação para detectar variantes genéticas com efeitos moderados sobre um determinado traço, desde de que o marcador genético avaliado apresente um forte desequilíbrio de ligação (DL) com o polimorfismo funcional (RISCH e MERIKANGAS, 1996). Desequilíbrio de ligação é a associação não-aleatória de alelos em dois ou mais *loci* e pode ser influenciado por muitos fatores, tais como taxa de recombinação,

características demográficas (tamanho e taxa de crescimento da população) e história evolutiva (deriva genética, efeito fundador, gargalos populacionais, mutação, seleção e fluxo gênico) (REICH *et al*., 2001). A determinação dos padrões de DL em populações humanas é extremamente útil para os estudos de associação genética, pois com estas informações é possível selecionar um subconjunto de variantes que são marcadores para a maioria das mutações em uma determinada região do genoma. Os estudos de associação podem ser realizados com qualquer tipo de variante genética [polimorfismo de base única (SNP), variantes de número de cópias (CNV) ou inserção e deleção (indel)], sendo que o mais comumente utilizado é o SNP (MCCARTHY *et al*., 2008). Isso ocorre devido a abundância desse tipo de polimorfismo, que representa cerca de 94% de toda a variação encontrada no genoma humano (1000 GENOMES PROJECT CONSORTIUM, 2012). Os estudos de associação podem ser baseados: (i) na genotipagem de um determinado polimorfismo ou de um conjunto de marcadores (como os tag-SNPs) em genes candidatos, geralmente selecionados a partir de evidências biológicas; (ii) na genotipagem de um conjunto de SNPs cobrindo todas as regiões do genoma, como ocorre no GWA (que avalia até 5 milhões de marcadores); ou (iii) no sequenciamento completo do genoma ou de regiões específicas (GOLDSTEIN *et al*., 2013), como éxons (BAMSHAD *et al*., 2011) ou *loci* previamente associados a um determinado fenótipo.

Na epidemiologia genética, o formato mais comumente utilizado nos estudos de associação é o caso-controle (estudo de base populacional). Nesse tipo de estudo, as frequências de determinados alelos são comparadas entre um conjunto de casos e um conjunto de controles adequados, a fim de identificar variantes genéticas associadas ao traço avaliado. Casos e controles são geralmente recrutados de forma independente, a partir de um grupo de indivíduos com a mesma origem étnica. A seleção de controles inadequados e a estratificação populacional são problemas frequentemente enfrentados por estudos genéticos de base populacional (BENYAMIN *et al*., 2009). A estratificação populacional tem origem quando diferentes proporções de casos e controles são recrutados de populações geneticamente diferentes, produzindo associações espúrias devido à heterogeneidade da amostra, e não à doença de interesse. Os testes de associação baseados em agrupamento familiar são alternativas aos estudos de base

populacional. Por exemplo, na presente tese, as amostras brasileiras (com história recente de miscigenação) foram analisadas por método que avalia distorções na transmissão de alelos dos progenitores para os indivíduos afetados pela doença (teste de desequilíbrio de transmissão de alelos, TDT) (LAIRD *et al*., 2000). Este teste previne eventuais conflitos de associação genótipo/fenótipo devido ao uso de controles inapropriados ou de estratificação populacional.

2 PACIENTES, MATERIAL E MÉTODOS

2.1 ASPECTOS ÉTICOS

Todos os indivíduos que participaram destes estudos foram informados sobre os motivos da pesquisa e assinaram um termo de consentimento livre e esclarecido (Apêndice). O protocolo de estudos foi aprovado pelos comitês de ética dos Centros de Pesquisas Aggeu Magalhães (Recife, Pernambuco) e Gonçalo Moniz (Salvador, Bahia) (CEPs CpqAM e CPqGM; número do protocolo: 01/10). O estudo também foi aprovado pela Comissão Nacional de Ética em Pesquisa (CONEP/CAAE: 00082.0.095.000-09; número do protocolo: 15/2011). O recrutamento e a utilização das amostras iranianas foram aprovados pelo comitê de ética do Instituto Pasteur de Teerã, Irã (2005).

2.2 PERFIS GLOBAIS DE EXPRESSÃO GÊNICA

Análises transcriptômicas foram conduzidas com o objetivo de avaliar as alterações que ocorrem na pele após o desenvolvimento da leishmaniose cutânea e de auxiliar na interpretação dos resultados obtidos nos estudos genéticos. Oito biópsias de lesões cutâneas (coletadas da borda das úlceras, antes do início do tratamento) de pacientes infectados com *L. braziliensis* (Cortês, Pernambuco) e 8 amostras de pele normal de doadores não-infectados (Marselha, França) foram coletadas e imediatamente conservadas em solução *RNAlater* (Life Technologies). Fragmentos de tecido (20 mg) foram transferidos para microtubos contendo esferas de cerâmica de 1.4 mm (*CK14*; Bertin Technologies) e 350 µL de tampão de lise *RTL* (Qiagen), suplementado com 3.5 µL de β-mercaptoetanol (Sigma-Aldrich). O tecido foi macerado com o auxílio de um homogeneizador *Precellys 24* (Bertin Technologies). Após a adição de 400 µL de Trizol (Life Technologies) e 150 µL de clorofórmio (Sigma-Aldrich), as soluções foram homogeneizadas (em um vórtex) e incubadas por 5 minutos à temperatura ambiente. Em seguida, a fase aquosa foi coletada, misturada com 500 µL de etanol a 70% (Sigma-Aldrich) e o RNA foi purificado com o auxílio do *RNAeasy Mini*

kit (Qiagen), de acordo com as instruções do fabricante. O RNA purificado foi quantificado por espectrofotometria (UV; espectrofotômetro NanoVue Plus, GE Healthcare) e a sua integridade foi determinada com o auxílio de um bioanalizador (*2100 Bioanalyzer*, Agilent Technologies). Todas as 16 amostras incluídas no estudo apresentaram ótima qualidade [valor de integridade de RNA ≥ 8.0, cuja sigla em inglês é RIN (*RNA Integrity Number*)].

A amplificação, marcação e hibridização das amostras foram realizadas de acordo com protocolo padrão para análise de expressão gênica baseada em microarranjos (uma cor), disponibilizado pela Agilent Technologies. Em resumo, 1 µg de RNA foi convertido em RNA complementar acoplado à cianina-3 (cRNACy3) com o auxílio do *Quick Amp Labeling Kit* (Agilent Technologies). Em seguida, o cRNACy3 foi purificado através de colunas *RNAeasy* (Qiagen). A incorporação da sonda e a quantidade de cRNA recuperado foram conferidos em espectrofotômetro (NanoDrop ND-1000, Thermo Scientific). O cRNACy3 (1 µg) foi fragmentado (60° C por 30 minutos) em uma solução contendo tampão de *fragmentação* e *agente bloqueador* (ambos da Agilent Technologies). Após a fragmentação do material, 250 mL de tampão de *hibridização* (Agilent Technologies) foram adicionados às amostras, que foram então hibridizadas ao microarranjo *SurePrint G3 Human Gene Expression v2* (G4851B – 50599 sequências de RNA; Agilent Technologies). Em seguida, as lâminas foram lavadas com os tampões *GE wash 1* (1 minuto à temperatura ambiente) e *GE wash 2* (1 minuto a 37° C) (ambos da Agilent Technologies). As lâminas foram então escaneadas com o auxílio de um *SureScan Microarray Scanner* (Agilent Technologies) e as imagens foram convertidas em valores de expressão com o programa *Feature Extraction v9.5.3* (Agilent Technologies). Os resultados brutos dos transcriptomas foram depositados no banco de dados GEO (http://ncbi.nlm.nih.gov/geo/; número de acesso: GSE63931).

Os dados foram normalizados pela mediana usando o programa GeneSpring GX (Agilent Technologies). A análise de significância para microarranjos, cuja sigla em inglês é SAM (*significance analysis of microarray*) (TUSHER *et al*., 2001), foi utilizada para a identificação de genes diferencialmente expressos entre lesões cutâneas e amostras de pele normal. Mil permutações de fenótipos foram conduzidas para avaliar se a expressão dos genes foi significativamente associada aos diferentes fenótipos

avaliados. Os resultados foram corrigidos pela taxa global de resultados falso-positivos (FDR, do inglês *false discovery rate*), com o objetivo de limitar a ocorrência de associações significativas atribuídas ao acaso (FDR 90% < 0.001%). Somente genes com expressões aumentadas ou diminuídas em mais de 2 vezes (*fold change* ≥ 2.0) foram considerados diferencialmente regulados. O coeficiente de correlação de Pearson foi utilizado para definir a relação hierárquica entre as amostras. Além disso, a variabilidade dos dados foi determinada através de análise de componentes principais.

Uma lista contendo todos os genes modulados foi utilizada para a identificação de vias canônicas associadas à leishmaniose cutânea, com o auxílio do banco de dados IPA (IPA, versão 8.7; http://ingenuity.com). As redes de interação de genes foram construídas com base nos bancos de dados IPA e String (versão 9.1; http://string-db.org/) (Tabelas A4 e A5, Apêndice). Foram consideradas interações diretas (físicas) ou indiretas (funcionais) entre moléculas. O *p*-valor relativo para uma rede de interação foi calculado pelo teste exato de Fisher (cauda direita), comparando a proporção de genes modulados em uma determinada rede com a proporção de genes modulados nas demais redes avaliadas.

2.3 AMOSTRAS POPULACIONAIS (ESTUDOS DE ASSOCIAÇÃO GENÉTICA)

Amostras brasileiras (*estudos de base familiar - trios*): as análises de associação primária e de replicação, descritas nos Estudos 1 e 2, foram conduzidas em famílias que vivem em zonas endêmicas para a leishmaniose cutânea (causada pela *L. braziliensis*), localizadas na região nordeste do Brasil (Tabela A1, Apêndice). Mil e setenta e nove indivíduos (453 indivíduos afetados de 289 famílias nucleares) foram recrutados entre os anos de 2001 e 2012, nas zonas rurais de Itabuna, São José da Vitória, Buerarema e Una, localizadas na região sul do estado da Bahia (amostra primária Brasil 1), e na cidade de Cortês (próximo a plantações de cana-de-açucar), na região da Mata Meridional do estado de Pernambuco (amostra de replicação Brasil 2). Os indivíduos selecionados para o estudo tinham entre 11 e 73 anos, de ambos os sexos, não importando a origem étnica. Os trios foram constituídos por um indivíduo

afetado e ambos os progenitores. As famílias selecionadas tinham pelo menos um filho afetado pela leishmaniose cutânea (independentemente do fenótipo dos pais).

Os indivíduos afetados viviam por no mínimo cinco anos em uma área na qual a LC é endêmica, apresentavam uma ou mais lesões características (úlceras indolores com bordas elevadas e fundo necrótico) ou cicatrizes compatíveis com a LC e haviam recebido pelo menos 10 injeções de antimoniato de meglumina durante 10 dias. O diagnóstico foi baseado no aspecto da lesão ou da cicatriz e na reação positiva ao teste de Montenegro (teste de hipersensibilidade tardia), 48 horas após a injeção intradérmica do extrato de *L. braziliensis*. Os indivíduos que fizeram uso de tratamentos não-convencionais (por exemplo, extrato de plantas) ou que tiveram cura espontânea (sem nenhum tipo de tratamento) não foram incluídos no estudo.

Amostra iraniana (*estudo de base populacional*): os casos (*n* = 118) e os controles (*n* = 126) utilizados na análise de extensão do Estudo 1 foram recrutados entre os anos de 2005 e 2006, na cidade de Mashhad (província de Razavi Khorasan), no nordeste do Irã. A *L. tropica* é o principal agente etiológico da leishmaniose cutânea nesta região (MOHAGHEGH *et al.*, 2013). Os casos e os controles foram selecionados entre jovens (com 10 a 29 anos, de ambos os sexos), que haviam se deslocado pouco pelo país, para limitar a possiblidade de infecção por *L. major*. Os casos foram selecionados seguindo critérios similares aos utilizados no Brasil, ou seja, foram positivos ao teste intradérmico realizado com o extrato de *L. tropica* e apresentaram úlceras ou cicatrizes características da LC. Pacientes com leishmaniose lupóide não foram incluídos no estudo. Os controles nunca apresentaram lesões na pele causadas por *Leishmania*, viveram por pelo menos cinco anos em uma zona na qual a LC é endêmica e reagiram positivamente ao teste cutâneo com o extrato de *L. tropica*.

2.4 EXTRAÇÃO DE DNA

O DNA genômico dos indivíduos incluídos nos estudos genéticos foi extraído a partir de amostras de sangue total usando o *QIamp DNA Blood Midi Kit* (Qiagen), de acordo com as instruções do fabricante. Resumidamente, 100 µL de proteinase K (600 mAU/mL) e 2.4 mL de *tampão AL* foram adicionados a 2 mL de sangue (temperatura

ambiente). A solução foi homogeneizada (em um vórtex) e incubada a 70° C por 10 minutos. Posteriormente, 2 mL de etanol a 99% (Sigma-Aldrich) foram adicionados à solução, que foi homogeneizada. As amostras foram transferidas para colunas *QIAamp Midi* acopladas a tubos de 15 mL e centrifugadas a 1850 x g durante 3 minutos (temperatura ambiente). As colunas foram lavadas com 2 mL de *tampão AW1* (centrifugadas a 4500 x g por 1 minuto) e em seguida com 2 mL de *tampão AW2* (centrifugadas a 4500 x g por 15 minutos). As colunas foram então transferidas para tubos de 15 mL limpos e 300 µL de água destilada foram aplicados diretamente sobre a membrana. Em seguida, o material foi centrifugado a 4500 x g por 2 minutos para eluição do DNA purificado. A concentração e a pureza das amostras de DNA foram determinadas por espectrofotometria (UV; espectrofotômetro NanoVue Plus, GE Healthcare).

2.5 SELEÇÃO DE TAG-SNPS

Os estudos de associação apresentados nesta tese foram baseados na genotipagem de marcadores genéticos (tag-SNPs). Esta abordagem é fundamentada no desequilíbrio de ligação (DL) entre polimorfismos, possibilitando a seleção de um conjunto reduzido de marcadores genéticos que capturam os sinais da maioria das mutações comuns em uma determinada região genômica. Como citado anteriormente, DL é a associação não-aleatória de alelos em dois ou mais *loci*. O DL pode ser representado por r^2, que é influenciado por fatores como efeitor fundador e recombinação, sendo extremamente útil para detectar correlações entre polimorfismos. O r^2 é definido pela equação abaixo:

$$r^2 = \frac{(f_{AB} - f_A f_B)^2}{f_A f_a f_B f_b}$$

Considerando um par de SNPs, em que o primeiro tem alelos A e a, e o segundo tem alelos B e b, as frequências destes alelos são representadas por f_A e f_a, f_B e f_b,

respectivamente. f_{AB} representa a frequência do haplótipo AB. O valor de r^2 poderá variar entre 0 e 1. Neste sentido, dois polimorfismos com $r^2 = 1$ podem prever um ao outro perfeitamente quando a informação sobre um deles é desconhecida. Desta forma, um número reduzido de SNPs que são marcadores (tag-SNPs) para a maioria dos polimorfismos comuns nas suas proximidades pode ser usado para estudar genes ou sequências de interesse.

A região na qual as famílias brasileiras foram recrutadas (nordeste do Brasil) é habitada por uma população geneticamente heterogênea, resultado de cruzamentos entre populações de três continentes diferentes: nativos americanos (ameríndios), africanos e europeus. Como não existe um painel de referência público que descreve a ocorrência e a frequência de polimorfismos na população brasileira, os cálculos de desequilíbrio de ligação (incluindo os mapas regionais de DL mostrados nas Figuras 9 e 14) e a seleção de tag-SNPs foram conduzidos com o auxílio de painéis de referência de populações relacionadas à população brasileira (senso amplo), obtidos do projeto 1000 genomas – *phase 1* (1000 GENOMES PROJECT CONSORTIUM, 2012; http://1000genomes.org/). Do continente americano foram incluídos mexicanos, porto riquenhos e colombianos; do continente africano foi utilizado o painel de referência para os Iorubás de Ibadan, Nigéria; além de populações da Europa (americanos de origem européia, ibéricos e toscanos). Somente SNPs comuns (com frequência ≥ 5%) em pelo menos um dos painéis de referência foram incluídos no processo de seleção de tag-SNPs. Um conjunto de marcadores abrangendo todos os genes selecionados (incluindo as suas regiões regulatórias) foi determinado com base no coeficiente de correlação quadrada ($r^2 \geq 0.8$) entre SNPs, com o auxílio do programa PLINK (PURCELL *et al.*, 2007; http://pngu.mgh.harvard.edu/~purcell/plink/).

2.6 GENOTIPAGEM DE SNPS E CONTROLE DE QUALIDADE

Os indivíduos da amostra primária Brasil 1 (*n* = 754) foram genotipados para 133 tag-SNPs cobrindo os genes *IL2*, *IL2RA*, *IL2RB*, *JAK3*, *STAT5A* e *STAT5B* (Estudo 1 – Tabela A1, Apêndice), e para 45 tag-SNPs abrangendo as regiões dos genes *PRF1* e *SRGN* (Estudo 2 – Tabela A2, Apêndice), usando o microarranjo de DNA *Infinium*®

iSelect Beadchip (Illumina), de acordo com as instruções do fabricante. Resumidamente, 4 µL de tampão de *reconstituição* e 20 µL de tampão *MA1* (Illumina) foram adicionados a 200 ng de DNA (em 10 µL de água). Em seguida, a solução foi homogeneizada (em um vórtex) e incubada por 30 minutos à temperatura ambiente. Quatro microlitros de NaOH a 0.1 N foram adicionados às amostras, que foram homogeneizadas e incubadas à temperatura ambiente por 10 minutos. Em seguida, foram adicionados 34 µL de tampão *MA2* (Illumina) e 38 µL de tampão *MSM* (Illumina) e o DNA foi desnaturado e amplificado isotermicamente a uma temperatura de 37° C (*overnight*). O DNA foi fragmentado por incubação durante 1 hora a 37° C com 25 µL de tampão *FMS* (Illumina) e então precipitado pela adição de 155 µL de isopropanol a 99% (Sigma-Aldrich) e de 50 µL de tampão *PM1* (Illumina) (a 4° C por 30 minutos). A solução foi homogeneizada e centrifugada por 20 minutos a 3000 x g (4° C). Todo o sobrenadante foi removido e o DNA fragmentado foi reconstituído em 23 µL de tampão *RA1* (Illumina) (37° C por 1 hora). Para a hibridização, as amostras foram aquecidas a 95° C por 20 minutos e mantidas à temperatura ambiente por mais 30 minutos. Em seguida, 400 µL de tampão *PB2* (Illumina) e 15 µL das amostras foram cuidadosamente aplicados nos reservatórios apropriados das lâminas de genotipagem (37° C, *overnight*). Para a marcação e a extensão das sequências de DNA, o material foi incubado a 44° C e diferentes reagentes foram aplicados em sequência através das lâminas: 150 µL de tampão *RA1* (30 segundos, repetido 5 vezes); 450 µL de tampão *XC1* (Illumina) (10 minutos); 450 µL de tampão *XC2* (10 minutos); 200 µL de tampão *TEM* (Illumina) (15 minutos); e 450 µL de formamida/EDTA 95% (1 minuto). As lâminas foram então resfriadas a 37° C e tratadas com 3 ciclos de 450 µL de tampão *XC3* (Illumina) (1 minuto) seguido por 250 µL de tampão *STM* (Illumina) (10 minutos). O material foi então lavado por imersão nos tampões *PB1* e *XC4* (Illumina) (ambos por 5 minutos). Por fim, as câmaras foram vedadas (a vácuo) e escaneadas com o auxílio de um *iScan* (Illumina).

Os SNPs foram genotipados nas coortes de replicação (Brasil 2) e de extensão (Irã 1) com o uso de sondas *TaqMan* (Applied Biosystems). Em resumo, as reações foram conduzidas com 12.5 ng de DNA genômico, 900 nM de cada primer, 200 nM de cada sonda (VIC ou FAM) e o *TaqMan Universal PCR Master Mix* (Applied Biosystems),

em um volume final de 5 µL. A PCR (reação em cadeia da polimerase) foi realizada sob as seguintes condições: 50° C por 2 minutos, 95° C por 10 minutos, seguido por 40 ciclos de amplificação (95° C por 15 segundos e 60° C por 1 minuto). Os genótipos dos indivíduos (discriminação alélica) foram determinados com o auxílio de um *7900HT Fast Real-Time PCR System* (Applied Biosystems).

Um controle de qualidade foi realizado antes da condução dos testes de associação. Todos os procedimentos foram conduzidos automaticamente com o auxílio do programa PLINK. SNPs apresentando desvio significante do equilíbrio de Hardy-Weinberg ($p < 10^{-4}$; estudo de base familiar: baseado nos progenitores/fundadores; estudo de base populacional: baseado nos controles) e marcadores ou amostras com mais de 10% dos genótipos não determinados foram excluídos das análises de associação. Erros mendelianos também foram eliminados dos estudos de base familiar. O número de SNPs e de indivíduos excluídos no controle de qualidade são apresentados na seção “Resultados” e nas Tabelas A1 e A2 (Apêndice).

2.7 CULTURA DE CÉLULAS

Experimentos *in vitro* foram conduzidos no Estudo 1 para avaliar o impacto de mutações no gene *IL2RA* sobre respostas dependentes da sinalização IL-2/IL-2R. Para isto, células mononucleares do sangue periférico (PBMCs) de indivíduos de uma região endêmica (Bahia) para a leishmaniose cutânea (causada por *L. braziliensis*) e de doadores sadios (não endêmicos) foram purificadas através de gradiente de Ficoll (Ficoll-Paque, GE Healthcare). As células (10^6 células/poço) foram cultivadas em meio RPMI-1640 (2 mM L-glutamine) contendo 10% de soro fetal bovino, 25 mM de HEPES, 10 U/mL de estreptomicina e 100 µg/mL de penicilina (todos da Invitrogen), à 37° C e 5% de CO_2. As células foram estimuladas com 5 µg/mL do extrato total de formas promastigotas do parasito (por 96 horas) ou com 5 µg/mL de fitoemaglutinina (PHA; Sigma-Aldrich) (por 72 horas). A concentração de IFN-γ nos sobrenadantes das culturas foi determinado por ELISA (ensaio imunoenzimático), com o uso do *Ready-Set-Go ELISA kit* (BD Biosciences), de acordo com as instruções do fabricante. Para avaliar a capacidade de ativação de células T regulatórias *in vitro*, PBMCs de doadores sadios

foram ativadas com anticorpos anti-CD3/CD28 (1:20 ou 1:200 – razão esfera:célula; Miltenyi Biotec) e cultivadas por 72 horas na presença de TGF-β1 (2 ng/mL hTGF-β1; PeproTech) ou de TGF-β1 combinado à IL-2 (10 U/mL hIL-2; BD Biosciences).

2.8 CITOMETRIA DE FLUXO

Para definir as subpopulações de linfócitos FoxP3$^+$, as células cultivadas foram marcadas segundo protocolo descrito por Miyara e colaboradores (2009), com anticorpos fluorescentes PE-Cy7-anti-CD4 (SK3), APC-anti-CD25 (M-A251) e PE-anti-FoxP3 (259D/C7) ou com controles isotípicos adequados (todos da BD Biosciences). O anticorpo FITC-anti-CD45RA (HI100) foi usado para discriminar as T_{regs} efetoras (eT_{regs}) das T_{regs} não ativadas (rT_{regs}). As células foram incubadas com o coquetel de anticorpos para marcação de superfície (20 minutos à temperatura ambiente, protegidas da luz). Em seguida, as células foram fixadas e permeabilizadas para a marcação intracelular de FoxP3 (*BD Cytofix/Cytoperm kit*; BD Biosciences). Os dados foram adquiridos com o auxílio de um FACSCalibur (BD Biosciences) e analisados através do programa DIVA (BD Biosciences).

2.9 ANÁLISE DE LIGAÇÃO GENÔMICA (GENOTIPAGEM DE MICROSSATÉLITES)

Uma análise de ligação genômica, cuja sigla em inglês é GWL (*genome wide linkage*), foi conduzida para identificar *loci* que influenciam a suscetibilidade à leishmaniose cutânea em famílias brasileiras expostas à *L. braziliensis*. A análise primária (*wide screening*, apresentada no Estudo 2) foi realizada em um conjunto de 32 famílias multicaso, recrutadas na região sul do estado da Bahia. As amostras de DNA foram processadas no Centro Nacional de Genotipagem (CNG, Evry, França) com o conjunto de 400 marcadores microssatélites *PRISM Linkage Mapping Set-MD-10* (Applied Biosystems). Esses microssatélites abrangem todos os cromossomos autossômicos com um espaçamento médio de 10 cM (centimorgan) entre os marcadores e uma taxa média de heterozigosidade de 75%. A localização de cada microssatélite foi obtida no banco de dados NCBI (http://ncbi.nlm.nih.gov/). PCRs

multiplex (panéis de 28 marcadores) foram realizadas com 75 ng de DNA em solução contendo 3 mM de $MgCl_2$, tampão de PCR, 1mM de dNTPs, 1.5 unidades de Taq DNA polimerase *Platinum* (todos da Life Technologies) e a concentração apropriada de cada par de primers em um volume total de 10 µL. As condições de PCR foram as seguintes: 94° C por 2 minutos, seguido por 40 ciclos de amplificação (94° C por 30 segundos, 55° C por 30 segundos e 72° C por outros 30 segundos), com uma extensão final das sequências a 72° C por 10 minutos. Os produtos amplificados foram purificados através do gel *Sephadex G50* (Amersham Pharmacia Biotech). Para a determinação dos genótipos, as amostras foram adicionadas ao tampão *capillary loading* (Applied Biosystems), injetadas nos capilares e submetidas à eletroforese em um sequenciador ABI 3700 (Applied Biosystems).

Outras 21 famílias multicaso, recrutadas na região sul da Bahia, foram incluídas no estudo com o objetivo de realizar uma análise mais detalhada (*fine-mapping*) dos *loci* apresentando sinais de ligação sugestivos com a LC no estudo primário (1q31-q32, 18q22-q23 e 10q21-q23). Os painéis de *fine-mapping* foram compostos por 10 a 12 microssatélites adicionais, regularmente espaçados nas regiões selecionadas (espaçamento médio de 0.92 cM entre os marcadores e taxa média de heterozigosidade de 78%). Os microssatélites utilizados nesta segunda fase foram selecionados a partir dos bancos de dados NCBI e Ensembl (http://ensembl.org/). As condições de PCR foram as mesmas descritas acima.

2.10 ANOTAÇÃO FUNCIONAL DE SEQUÊNCIAS

Os polimorfismos com potencial regulatório foram identificados através de análises *in silico* das regiões dos genes *IL2RA* (10:6052652-6119288) e *SRGN* (10:70800523-70971325) (sequência de referência GRCh37/hg19). Informações sobre genômica comparativa e epigenômica foram obtidas dos bancos de dados públicos Ensembl e UCSC (http://genome.ucsc.edu). A posição dos polimorfismos foi cruzada com anotações de sequência de DNA, incluindo elementos conservados em 36 mamíferos placentários (GERP; COOPER *et al.*, 2005), evidências de regiões promotoras/*enhancers* (estado de metilação/acetilação de histonas) e

hipersensibilidade à DNAse I (indicativo de acessibilidade da cromatina). Os dois últimos parâmetros foram obtidos do projeto ENCODE-MultiCell (ENCODE PROJECT CONSORTIUM, 2012; http://genome.ucsc.edu/ENCODE/; foram considerados válidos os elementos detectados em ao menos duas linhagens celulares distintas).

2.11 ANÁLISES ESTATÍSTICAS

As coortes brasileiras foram analisadas por métodos desenvolvidos para estudos de associação baseados em agrupamentos familiares, que previnem eventuais conflitos de associação genótipo/fenótipo devido ao uso de controles inapropriados ou de estratificação populacional. Foram analisadas distorções na transmissão de alelos dos progenitores para os filhos afetados pela leishmaniose cutânea através do teste de desequilíbrio de transmissão (TDT), com o auxílio do programa FBAT (LAIRD *et al.*, 2000). Nos estudos de associação primários (Brasil 1), os valores empíricos de *p* foram obtidos após 10000 permutações de fenótipos. Uma correção para testes múltiplos (correção de Bonferroni; 0.05/número de testes) foi aplicada aos valores empíricos de *p* com o objetivo de limitar o erro do tipo I (resultados falso-positivos). Após estes ajustes nos estudos primários, valores de $p < 0.05$ foram considerados significantes. A associação de variantes genéticas com a leishmaniose cutânea no Irã foi determinada pelo teste χ^2, que comparou a distribuição dos alelos dos polimorfismos analisados em casos e controles. Fatores de risco clássicos para as leishmanioses (como sexo e idade) não foram incluídos como covariáveis nas análises de associação na coorte iraniana, pois as suas distribuições foram similares entre os casos e os controles (Tabela 1). Não foi necessário corrigir o limite de significância nas análises de replicação (Brasil 2) e de extensão (Irã 1) (*p*-valor assintótico < 0.05 considerado significante), visto que os resultados nos estudos secundários foram comparáveis aos dos estudos primários. Os modos de herança genética (aditivo, recessivo ou dominante) que melhor explicam as associações observadas foram determinados por regressão logística (SCHAID, 1996).

Considerando um polimorfismo bialélico cujos alelos são representados por A e B, os modelos de herança genética foram definidos pelas comparações representadas abaixo:

Aditivo:	AA *versus* AB ou BB *versus* AB
Recessivo:	AA *versus* AB, BB
Dominante:	AA, AB *versus* BB

Polimorfismos para os quais foram obtidas evidências de associação com a LC foram analisados por regressão logística condicional (KOELEMAN *et al.*, 2000; DUDBRIDGE, 2008), com o objetivo de avaliar as relações entre os efeitos capturados por diferentes marcadores.

Uma metanálise de efeito fixo (assumindo a premissa de homogeneidade estatística entre resultados) foi conduzida no Estudo 1 com o auxílio do programa PLINK. Comparações entre grupos (Mann-Whitney) e análises de regressão linear foram realizadas com o uso do programa SPSS (IBM Corporation).

O método da probabilidade binomial mista (MLB), implementado pelo programa MLBGH-I (KRUGLYAK *et al.*, 1996, ABEL e MULLER-MYHSOK, 1998), foi utilizado para a análise de ligação genômica apresentada no Estudo 2.

ESTUDO 1

3. Mutações em *IL2RA* aumentam o risco de leishmaniose cutânea em humanos: impacto nas respostas dependentes da sinalização IL-2/IL-2R.

3.1 JUSTIFICATIVA

O desfecho da infecção por *Leishmania* é fortemente influenciado por fatores parasitários e pelo perfil imunológico/genético do hospedeiro (KAYE e SCOTT, 2011). Muitos estudos em humanos e em modelos experimentais mostram o envolvimento da imunidade inata e adaptativa em resposta à infecção por *Leishmania* (BELKAID *et al.*, 2002; ALEXANDER e BRYSON, 2005; BELKAID e TARBELL, 2009; KAYE e SCOTT, 2011; MOUGNEAU *et al.*, 2011). As células T $CD4^+$ são centrais na coordenação da resposta imune contra o parasito e diferentes subpopulações de células T auxiliares já foram implicadas no desenvolvimento das leishmanioses (SACKS e NOBEN-TRAUTH, 2002; SUFFIA *et al*., 2005; ALEXANDER e BRYSON, 2005; GONZALEZ-LOMBANA *et al*., 2013). Em especial as células T_H1, que têm seu efeito dirigido pelo IFN-γ, estimulando a atividade leishmanicida de macrófagos (MOUGNEAU *et al*., 2011). Esses estudos também indicam que o equilíbrio entre as respostas efetoras e regulatórias é necessário para o controle da infecção, limitando os danos teciduais causados pela resposta inflamatória (BELKAID e TARBELL, 2009). Neste contexto, as T_{regs} $CD4^+CD25^+FoxP3^+$ podem ter um papel central na regulação das respostas efetoras, favorecerendo indiretamente a sobrevivência do parasito (BELKAID *et al*., 2002; SUFFIA *et al*., 2005). De fato, as T_{regs} desempenham um importante papel na supressão da resposta T_H1 em modelos murinos de leishmaniose cutânea (BELKAID e TARBELL, 2009). Além disso, as T_{regs} são encontradas nas lesões de pacientes infectados com *L. braziliensis* (CAMPANELLI *et al*., 2006).

A maioria das evidências citadas acima foram obtidas de modelos murinos com o objetivo de definir vias de resistência/suscetibilidade e para desvendar mecanismos patológicos associados às leishmanioses. Porém, nem sempre é possível extrapolar as conclusões obtidas de modelos experimentais para os humanos. Particularmente em relação às infecções causadas por patógenos que infectam humanos naturalmente, mas que não infectam roedores de modo natural, como certas espécies de *Leishmania*. Deste modo, estudos em populações humanas são imprescindíveis. A epidemiologia genética produz resultados valiosos há muito tempo (ABEL e DESSEIN, 1997; CHAPMAN e HILL, 2012). Esses estudos mostram que, em algumas doenças

infecciosas, como no caso da esquistossomose, um pequeno número de genes desempenha um papel preponderante no controle da doença (MARQUET *et al*., 1996). No entanto, a maioria das doenças infecciosas é influenciada por uma grande diversidade de mutações com efeitos moderados (CHAPMAN e HILL, 2012).

Embora as bases genéticas das leishmanioses não sejam completamente compreendidas, muitas mutações que contribuem para o risco de desenvolver as diferentes formas da doença foram identificadas (LIPOLDOVÁ e DEMANT, 2006). Contudo, a replicação destes sinais de associação em populações geneticamente distintas e afetadas por diferentes espécies de *Leishmania* são raros (LEISHGEN CONSORTIUM *et al*., 2013). Em um estudo prévio, nossa equipe evidenciou que a suscetibilidade à leishmaniose visceral (causada por *L. donovani*) no Sudão é controlada principalmente pelo *locus* 22q12 (BUCHETON *et al*. 2003). Foi mostrado também que mutações no gene *IL2RB*, que codifica a subunidade β (IL-2Rβ) do receptor para a IL-2, localizado no *locus* 22q12, poderiam ser responsáveis por este sinal de ligação genética (BUCHETON *et al*., 2007). Estas mutações podem afetar a via de sinalização IL-2, que é fundamental em diversos aspectos da resposta imune. A sinalização IL-2/IL-2R promove a proliferação e favorece a sobrevivência de células T e B, além de participar das respostas imunológicas primária e de memória *in vivo* (SARAPOV *et al*., 1999; WILLIAMS *et al*., 2006). Ela também favorece a expansão e a atividade citolítica de células NK (FEHNIGER *et al*., 2003). Além disso, a sinalização IL-2/IL-2R controla o desenvolvimento e a função de células T efetoras, favorecendo a indução de células T_H2, restringindo a diferenciação de células T_H17 e aumentando a produção de IFN-γ por células T_H1 (LIAO *et al*., 2011). Mais importante, a sinalização IL-2/IL-2R desempenha um papel não redundante no desenvolvimento e função de T_{regs} (MALEK e BAYER, 2004).

Os resultados prévios que associam mutações no receptor da IL-2 com a predisposição à LV (BUCHETON *et al*., 2007) nos levaram a avaliar o possível papel da via de sinalização IL-2 no desenvolvimento da leishmaniose.

3.2 OBJETIVOS DO ESTUDO

- Objetivo geral:

Avaliar o papel da via de sinalização IL-2 na suscetibilidade humana à leishmaniose cutânea.

- Objetivos específicos:

- Determinar os perfis globais de expressão gênica (transcriptomas) de lesões cutâneas causadas por *L. braziliensis* e de amostras de pele normal;

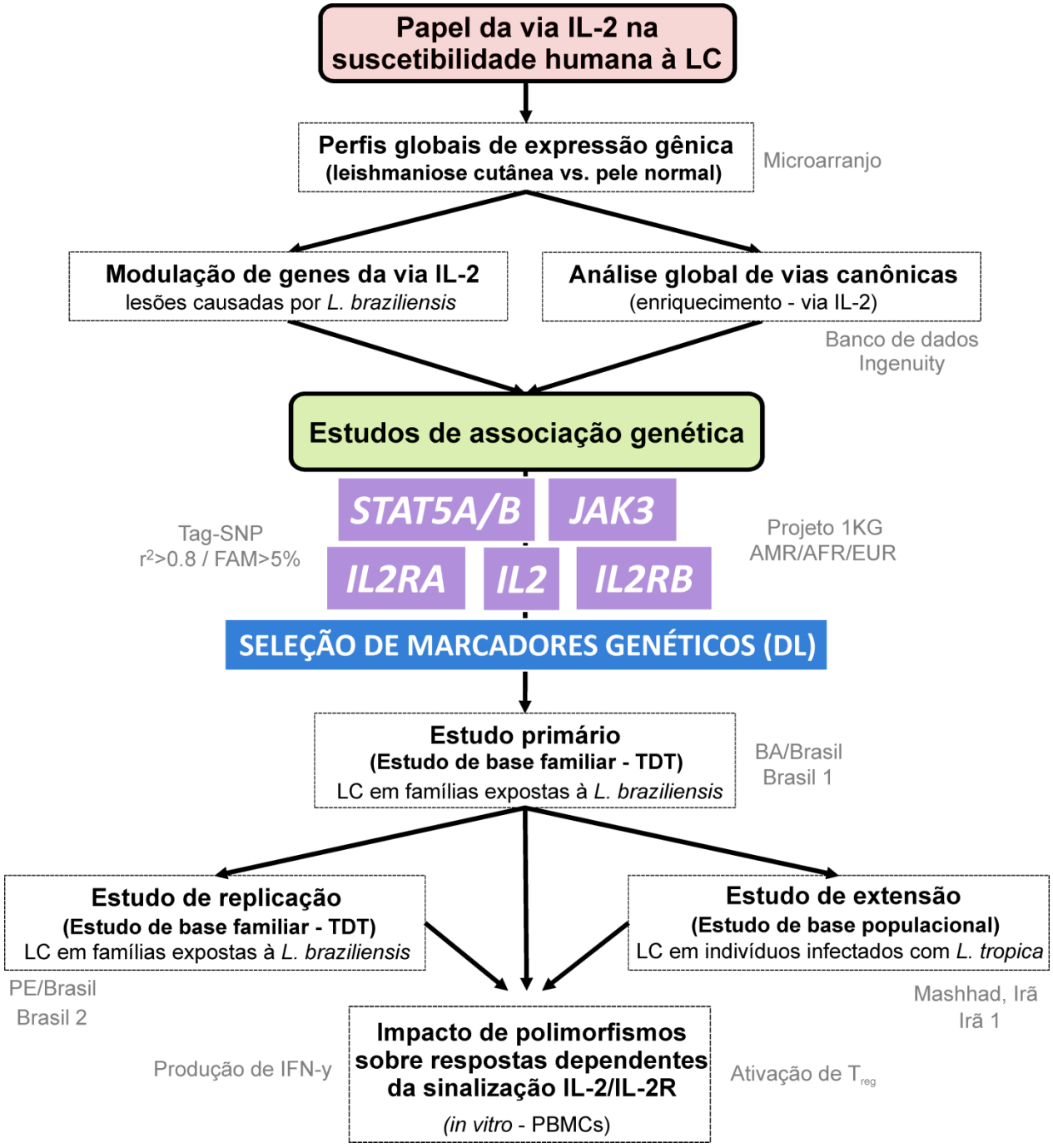
- Comparar a expressão de genes da via de sinalização IL-2 em lesões cutâneas causadas por *L. braziliensis* e em amostras de pele normal;

- Investigar a associação de polimorfismos em genes da via de sinalização IL-2 com a suscetibilidade à leishmaniose cutânea em populações do Brasil e do Irã expostas à *L. braziliensis* e *L. tropica*, respectivamente;

- Avaliar o impacto de mutações em genes da via IL-2 nas respostas dependentes da sinalização IL-2/IL-2R (produção de IFN-γ por células mononucleares e ativação de T_{regs} $FoxP3^{+}$ *in vitro*);

- Realizar análises *in silico* para a determinação de padrões de desequilíbrio de ligação e para a identificação de polimorfismos com potencial regulatório nas regiões dos genes associados à LC.

3.3 DESENHO EXPERIMENTAL



3.4 RESULTADOS

3.4.1 A via de sinalização IL-2 é ativada em lesões cutâneas causadas por *L. braziliensis*

Em um estudo prévio, nosso grupo identificou variantes do gene *IL2RB* que aumentam a predisposição à leishmaniose visceral em indivíduos infectados com *L. donovani* (BUCHETON *et al.*, 2007). Esse resultado nos levou a avaliar o papel da via de sinalização IL-2 no desenvolvimento das leishmanioses. Inicialmente, foram realizados estudos transcriptômicos em 8 lesões cutâneas de pacientes infectados com uma espécie dermotrópica de *Leishmania* e 8 amostras de pele normal de indivíduos sadios (não infectados). As biópsias de pele são menos invasivas do que as biópsias de fígado ou baço em indivíduos com a forma visceral da doença. Os pacientes selecionados para esta análise apresentavam quadro clínico típico de leishmaniose cutânea localizada, causada por *L. braziliensis*, e ainda não haviam recebido qualquer tipo de tratamento.

Após aplicar uma correção estatística rigorosa para limitar a ocorrência de resultados falso-positivos [FDR 90% (taxa de falso-positivos) = 0.001%], foram identificados 2875 genes com expressões induzidas ou inibidas [*fold change* (FC) ≥ 2.0] em lesões cutâneas causadas por *L. braziliensis* quando comparadas a amostras de pele normal (Figura 3A e 3B). O agrupamento hierárquico das amostras foi determinado com base no coeficiente de correlação de Pearson, que identificou dois grupos homogêneos compostos por 8 amostras de pele normal (grupo 1) ou por 8 amostras de lesão cutânea (grupo 2) (Figura 3A). Uma análise de componentes principais (ACP), baseada nos genes diferencialmente expressos, mostrou que os componentes principais 1 e 2 (CP1 e CP2) respondem por mais de 50% da variabilidade dos dados. Esses dois componentes separaram as amostras em dois grupos homogêneos (Figura 3C), confirmando a tendência observada na análise de agrupamento hierárquico. A segregação das amostras em dois grupos homogêneos através de dois componentes principais (que explicam mais da metade da variabilidade dos dados) reforça a confiabilidade estatística na identificação dos genes diferencialmente expressos.

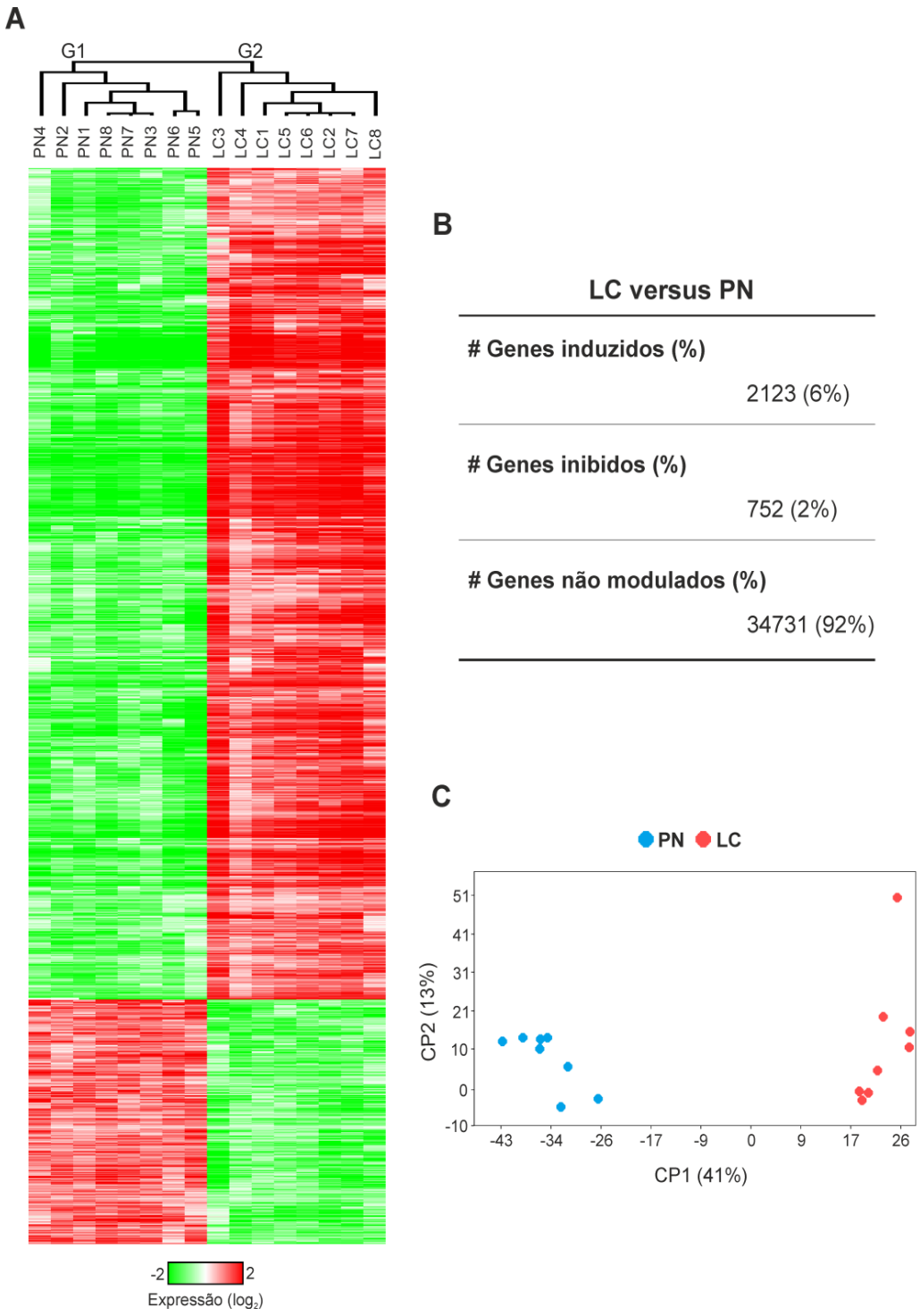


Figura 3. Análises transcriptômicas de lesões cutâneas causadas por *Leishmania braziliensis*. A, *Heat map* mostrando genes com expressões moduladas em 8 biópsias de lesões cutâneas (LC) e 8 amostras de pele normal de indivíduos não infectados (PN). O coeficiente de correlação de Pearson foi usado para definir a relação hierárquica entre as amostras (G1, grupo 1; G2, grupo 2). **B**, Número de genes induzidos ou inibidos em lesões cutâneas. **C**, Análise de componentes principais baseada nos genes diferencialmente expressos (CP1, primeiro componente; CP2, segundo componente). Círculos representam os dados obtidos para cada amostra.

Uma análise global de vias canônicas (banco de dados Ingenuity) utilizando os perfis de expressão gênica revelou o enriquecimento da via de sinalização IL-2 (p = 0.009) em lesões cutâneas (Figura 4A). Efetivamente, a transcrição de genes-chave da via IL-2, especialmente de *IL2RA* (FC = 65.6), foi fortemente induzida em lesões cutâneas (Figura 4B). Além de *IL2RA*, *IL2RB* e *IL2RG*, outros genes associados ao eixo JAK/STAT, como *JAK3*, *STAT5A* e *STAT5B*, tiveram as suas expressões aumentadas em lesões cutâneas quando comparadas às amostras de pele normal (Figura 4B e 4C).

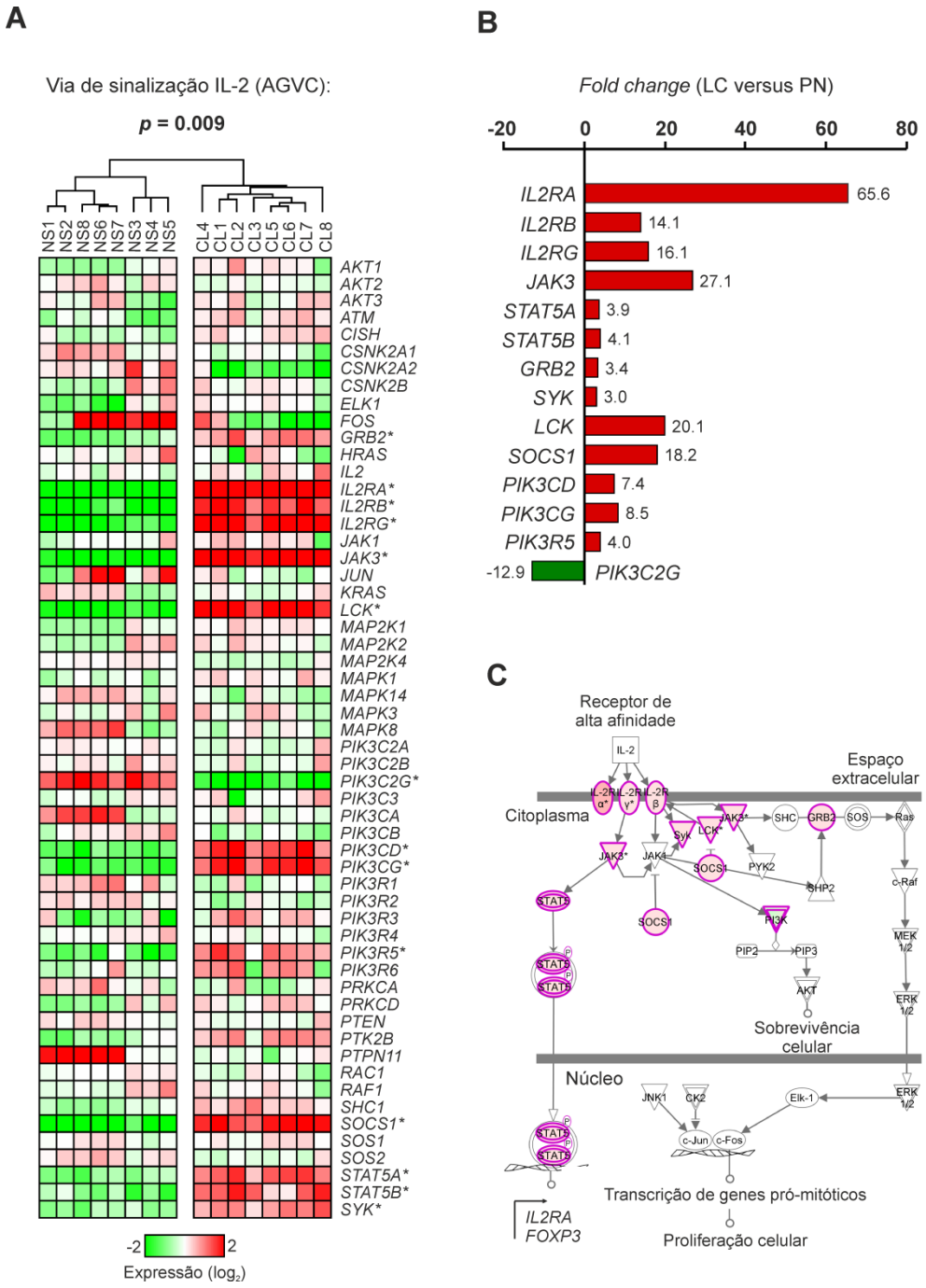


Figura 4. A via de sinalização IL-2 é ativada em lesões cutâneas causadas por *Leishmania braziliensis*. A, *Heat map* mostrando a expressão de 56 genes da via de sinalização IL-2 em 8 biópsias de lesão cutânea (LC) e 8 amostras de pele normal de indivíduos não infectados (PN). Uma análise global de vias canônicas (AGVC; banco de dados Ingenuity) utilizando os perfis de expressão gênica revelou o enriquecimento da via IL-2 em lesões cutâneas. Asteriscos indicam que a expressão do gene foi significativamente modulada entre os grupos. O coeficiente de correlação de Pearson foi usado para definir a relação hierárquica entre as amostras. **B**, Gráfico mostrando valores médios de diferença de expressão (*fold change*) para 14 genes modulados entre LC e PN. **C**, Representação esquemática da via de sinalização IL-2. As cores indicam a indução (vermelho) ou a inibição (verde) de genes em LC.

3.4.2 Estudo de variantes genéticas da via de sinalização IL-2 em famílias brasilieras expostas à *L. braziliensis*

Em seguida, nós avaliamos se polimorfismos em genes-chave da via de sinalização IL-2 (eixo JAK3/STAT5) são fatores de risco para a leishmaniose cutânea em indivíduos expostos à *L. braziliensis*. Cento e trinta e três tag-SNPs (cobrindo os genes *IL2*, *IL2RA*, *IL2RB*, *JAK3*, *STAT5A* e *STAT5B* – Tabela A1, Apêndice) foram avaliados em 754 indivíduos provenientes de 209 famílias nucleares (Amostra primária – Brasil 1; Tabela 1). O gene *IL2RG*, localizado no cromossomo X e que codifica a proteína γc, subunidade comum aos receptores de diferentes citocinas (ROCHMAN *et al.*, 2009), não foi analisado. Considerando a heterogeneidade genética da população brasileira, os marcadores foram selecionados com base em coeficientes de correlação quadrada ($r^2 \geq 0.8$) entre SNPs para populações dos continentes americano, africano e europeu, disponibilizadas pelo projeto 1000 Genomas. Após realizar um controle de qualidade para remover amostras e polimorfismos com baixa qualidade de genotipagem, um total de 128 SNPs foi analisado em 738 indivíduos (205 famílias nucleares). Além de 2 SNPs que apresentaram desvio significante do equilíbrio de Hardy-Weinberg ($p < 10^{-4}$; baseado nos fundadores), outros 3 SNPs e 16 indivíduos apresentaram mais de 10% de genótipos não determinados e foram excluídos das análises posteriores.

Tabela 1. Características das populações de estudo

	Brasil 1 Amostra primária[a]	**Brasil 2 Amostra de replicação[a]**	**Irã 1 Amostra de extensão[b]**		
			Caso	**Controle**	***p***
Número de indivíduos	754	325	114	120	
Sexo (masculino:feminino)	452:302	181:144	73:41	71:49	NS
Mediana da idade em anos (IQR)	33 (22-45)	27 (19-42)	14 (10-18)	13 (12-14)	NS
Número de famílias nucleares	209	80	-	-	
Número de trios	323	130	-	-	

IQR: Intervalo interquartil; *p*: *p*-valor; NS: não significante.
[a]Estudo de base familiar; [b]Estudo de base populacional.

Testes de desequilíbrio de transmissão de alelos (baseados em agrupamentos familiares) revelaram sinais de associação robustos ($p \leq 4x10^{-4}$) com a leishmaniose cutânea capturados por dois tag-SNPs localizados no íntron 1 do gene *IL2RA* [rs10905669 ($p = 3x10^{-4}$) e rs706778 ($p = 3x10^{-4}$)] (Figura 5 e Tabela 2). Este estudo identificou também outros 21 marcadores, nas regiões dos genes *IL2*, *IL2RA*, *IL2RB* e *JAK3*, que foram sugestivamente associados ($p < 0.05$) ao desenvolvimento da LC no Brasil. Após a condução de 10000 permutações e da correção para testes múltiplos (correção de Bonferroni), apenas os marcadores rs10905669 e rs706778 (*IL2RA*) tiveram as suas associações confirmadas (ambos os SNPs apresentaram *p*-valor empírico corrigido < 0.05).

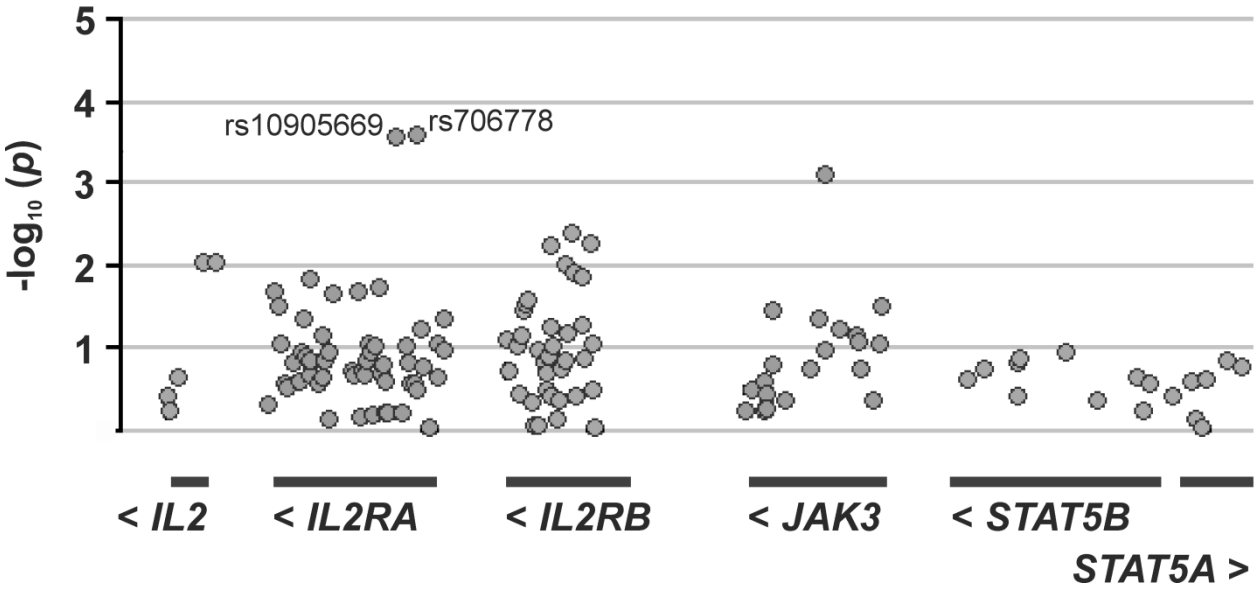


Figura 5. Polimorfismos no gene *IL2RA* são associados ao desenvolvimento de úlceras cutâneas em famílias brasileiras expostas à *Leishmania braziliensis*. Cento e vinte e oito tag-SNPs nas regiões dos genes *IL2*, *IL2RA*, *IL2RB*, *JAK3*, *STAT5A* e *STAT5B* foram analisados em 738 indivíduos (317 trios provenientes de 205 famílias nucleares). Os estudos de associação foram conduzidos através de testes de desequilíbrio de transmissão de alelos. Os círculos representam o valor assintótico de *p* para cada SNP testado.

Tabela 2. Polimorfismos em *IL2RA* são associados ao desenvolvimento de lesões cutâneas em famílias brasileiras expostas à *Leishmania braziliensis* (amostra primária Brasil 1)

Tag-SNP	Gene	Alelo	Freq	Fam	O_t	E_t	*p*	p_c
rs2069762	*IL2*$^{\text{intergênico}}$	A	0.79	76	183	167	0.009^{a}	NS
rs4833248	*IL2*$^{\text{intergênico}}$	G	0.79	76	183	167	0.009^{a}	NS
rs7069976	*IL2RA*$^{\text{intrônico}}$	A	0.97	19	58	51	0.04^{a}	NS
rs942200	*IL2RA*$^{\text{intrônico}}$	C	0.89	9	16	12	0.01^{d}	NS
rs4749920	*IL2RA*$^{\text{intrônico}}$	T	0.86	11	18	14	0.02^{d}	NS
rs7072398	*IL2RA*$^{\text{intrônico}}$	A	0.63	114	258	241	0.02^{a}	NS
rs942201	*IL2RA*$^{\text{intrônico}}$	G	0.88	9	14	10	0.02^{d}	NS
rs10905669	*IL2RA*$^{\text{intrônico}}$	T	0.18	80	84	62	0.0003^{a}	0.03
rs706778	*IL2RA*$^{\text{intrônico}}$	T	0.41	75	76	53	0.0003^{r}	0.01
rs4147359	*IL2RA*$^{\text{intergênico}}$	G	0.70	88	193	180	0.04^{a}	NS
rs84459	*IL2RB*$^{\text{intrônico}}$	T	0.69	79	65	54	0.04^{r}	NS
rs228945	*IL2RB*$^{\text{intrônico}}$	A	0.59	69	62	51	0.03^{r}	NS
rs228947	*IL2RB*$^{\text{intrônico}}$	C	0.73	80	76	64	0.03^{r}	NS
rs3218294	*IL2RB*$^{\text{intrônico}}$	G	0.98	14	34	28	0.005^{a}	NS
rs228965	*IL2RB*$^{\text{intrônico}}$	C	0.40	53	44	33	0.01^{r}	NS
rs1003694	*IL2RB*$^{\text{intrônico}}$	G	0.68	97	227	207	0.004^{a}	NS
rs2235330	*IL2RB*$^{\text{intrônico}}$	T	0.82	64	151	138	0.01^{a}	NS
rs228973	*IL2RB*$^{\text{intrônico}}$	T	0.65	43	51	42	0.01^{d}	NS
rs3218258	*IL2RB*$^{\text{intrônico}}$	T	0.18	11	13	7	0.006^{r}	NS
rs7255931	*JAK3*$^{\text{intrônico}}$	C	0.78	76	178	166	0.04^{a}	NS
rs3212760	*JAK3*$^{\text{intrônico}}$	T	0.66	46	55	47	0.04^{d}	NS
rs3212752	*JAK3*$^{\text{intrônico}}$	A	0.86	56	61	49	0.009^{r}	NS
rs13345965	*JAK3*$^{\text{intergênico}}$	C	0.84	11	14	10	0.03^{d}	NS

Alelo: alelo de referência; Freq: frequência do alelo de referência; Fam: número de famílias informativas; O_t: transmissões observadas; E_t: transmissões esperadas; *p*: *p*-valor assintótico; p_c: *p*-valor empírico (10000 permutações) após correção de Bonferroni; NS: não significante.
^{a}Aditivo, ^{d}dominante ou ^{r}recessivo (modelos genéticos).

Em seguida, os SNPs rs10905669 e rs706778 foram avaliados em uma amostra brasileira independente (Amostra de replicação – Brasil 2; Tabela 1), composta por 325 indivíduos (130 trios provenientes de 80 famílias nucleares). Um controle de qualidade com critérios similares aos utilizados no estudo primário foi coduzido nesta segunda

análise, sendo que 6 indivíduos foram excluídos por apresentar mais de 10% de genótipos não determinados. Testes de desequilíbrio de transmissão de alelos mostraram que, além de sua forte associação no estudo primário, o SNP rs706778 também foi associado ao desenvolvimento de lesões cutâneas nesta segunda amostra brasileira (p = 0.04), com o alelo T aumentando a predisposição à doença (Estudo de replicação – Brasil 2; Tabela 3). Além disso, foi identificada uma tendência de associação para o SNP rs10905669 (p = 0.08).

Tabela 3. Polimorfismos no íntron 1 do gene *IL2RA* são associados ao desenvolvimento de úlceras cutâneas causadas por *Leishmania braziliensis* e *Leishmania tropica*

Estudo de replicação – Brasil 2 (*Leishmania braziliensis*)						
SNP	Alelo de risco	Freq	Fam	O_t	E_t	*p*
rs10905669	T	0.16	20	18	13	0.08[a]
rs706778	T	0.42	43	58	51	0.04[a]
Estudo de extensão – Irã 1 (*Leishmania tropica*)						
SNP	Alelo de risco	Freq (caso:controle)		OR	IC 95%	*p*
rs10905669	T	0.31:0.20		1.5	1.0-3.0	0.03[a]
rs706778	T	0.54:0.44		1.5	1.0-2.5	0.04[a]

Freq: frequência do alelo de risco; Fam: número de famílias informativas; O_t: transmissões observadas; E_t: transmissões esperadas; *p*: *p*-valor assintótico; OR: odds ratio; IC 95%: intervalo de confiança 95%.
[a]Modelo genético aditivo.

3.4.3 SNPs em *IL2RA* também são associados ao desenvolvimento de lesões cutâneas em iranianos infectados com *L. tropica*

Também foi investigado se variantes do gene *IL2RA* são associadas com o desenvolvimento de lesões cutâneas em uma população geneticamente distinta da brasileira, infectada com uma outra espécie dermotrópica de *Leishmania*. Para isto, uma coorte de base populacional, composta por 116 casos e 120 controles, foi recrutada em uma zona endêmica para a leishmaniose cutânea no Irã (cidade de

Mashhad) (Amostra de extensão – Irã 1; Tabela 1). A *L. tropica* é o principal agente etiológico da LC nesta região e pode causar úlceras que persistem durante meses. Na coorte iraniana, nenhuma amostra ou SNP foi excluído pelo controle qualidade pós-genotipagem. Os testes de associação mostram que ambos os polimorfismos testados, rs10905669 (p = 0.03) e rs706778 (p = 0.04), são associados à LC nesta população (Estudo de extensão – Irã 1; Tabela 3). É importante salientar que os alelos associados a uma maior predisposição à LC foram os mesmo em todas as coortes avaliadas (duas amostras brasileiras e uma amostra iraniana).

Subsequentemente, uma metanálise (modelo de efeito fixo) dos resultados dos três estudos confirmou a robustez dos sinais de associação para ambos os polimorfismos avaliados, rs10905669 ($p = 6x10^{-7}$) e rs706778 ($p = 2x10^{-9}$) (Tabela 4). A heterogeneidade entre os estudos, calculada pelo teste Q de Cochran, foi insignificante para ambos os SNPs [rs10905669 (p = 0.50) e rs706778 (p = 0.77)]. Em conjunto, esses resultados suportam a hipótese de que polimorfismos na região do gene *IL2RA* são fatores de risco para a LC.

Tabela 4. Metanálise dos SNPs rs10905669 e rs706778

SNP	Alelo de risco	Amostra primária Brasil 1		Amostra de replicação Brasil 2		Amostra de extensão Irã 1		Metanálise			
		EP	OR	EP	OR	EP	OR	N	Q	OR	*p*
rs10905669	T	0.15	2.1	-	-	0.27	1.5	2	0.50	2.0	$6x10^{-7}$
rs706778	T	0.13	1.8	0.18	2.0	0.24	1.5	3	0.77	1.8	$2x10^{-9}$

Na amostra de replicação Brasil 1, o SNP rs10905669 não atingiu o grau de significância estatística estabelecido para o estudo ($p < 0.05$) e não foi incluído na análise. EP: erro padrão do odds ratio (OR); N: número de estudo válidos; Q: *p*-valor para o teste de heterogeneidade estatística de Cochran; *p*: *p*-valor para a metanálise de efeito fixo.

3.4.4 Os sinais de associação dos SNPs rs10905669 e rs706778 são distintos e independentes

Em seguida, foi investigado se os polimorfismos rs10905669 e rs706778 capturam um único sinal ou se são independentemente associados à doença cutânea

em indivíduos infectados por *Leishmania*. Primeiro, foram avaliados os valores de desequilíbrio de ligação (r^2) entre os dois SNPs. Utilizando a coorte primária Brasil 1 como referência, foi evidenciado que o par de SNPs rs10905669 e rs706778 apresenta fraco DL (r^2 = 0.2). No entanto, estes SNPs poderiam apresentar DL com a mesma mutação causal. Para avaliar esta hipótese, testes condicionais foram realizados com estes SNPs e ambos apresentaram $p < 0.05$ após a regressão logística, indicando que rs10905669 e rs706778 estão capturando efeitos ao menos parcialmente independentes.

3.4.5 O alelo T do SNP rs706778, que aumenta a predisposição à leishmaniose cutânea, é associado à redução de respostas dependentes da sinalização IL-2/IL-2R

Os resultados anteriores nos levaram a avaliar se os polimorfismos no gene *IL2RA* que alteram o risco de LC afetam respostas dependentes da sinalização IL-2/IL-2R. Apesar do efeito pleiotrópico da sinalização IL-2/IL-2R no sistema imune, as análises descritas a seguir foram focadas apenas na produção de IFN-γ e na ativação de T_{regs}, pois: (i) o papel protetor do IFN-γ é bem estabelecido nas leishmanioses; (ii) a via de sinalização IL-2 desempenha um papel crucial (não redundante) na biologia de T_{regs}; e (iii) a LC em humanos (especialmente por *L. braziliensis*) é frequentemente associada a uma resposta inflamatória intensa (imunopatologia), que poderia ser limitada pela ação das células T regulatórias.

Inicialmente, a produção de IFN-γ foi avaliada nos sobrenadantes de culturas de PBMCs de indivíduos infectados com *L. braziliensis*. Quando estimuladas com o extrato de *L. braziliensis* (p = 0.04) ou com PHA (p = 0.07, tendência para significância), as células dos indivíduos portadores do genótipo rs706778 TT produziram menos IFN-γ do que as células dos portadores dos demais genótipos (CT e CC) (Figura 6A).

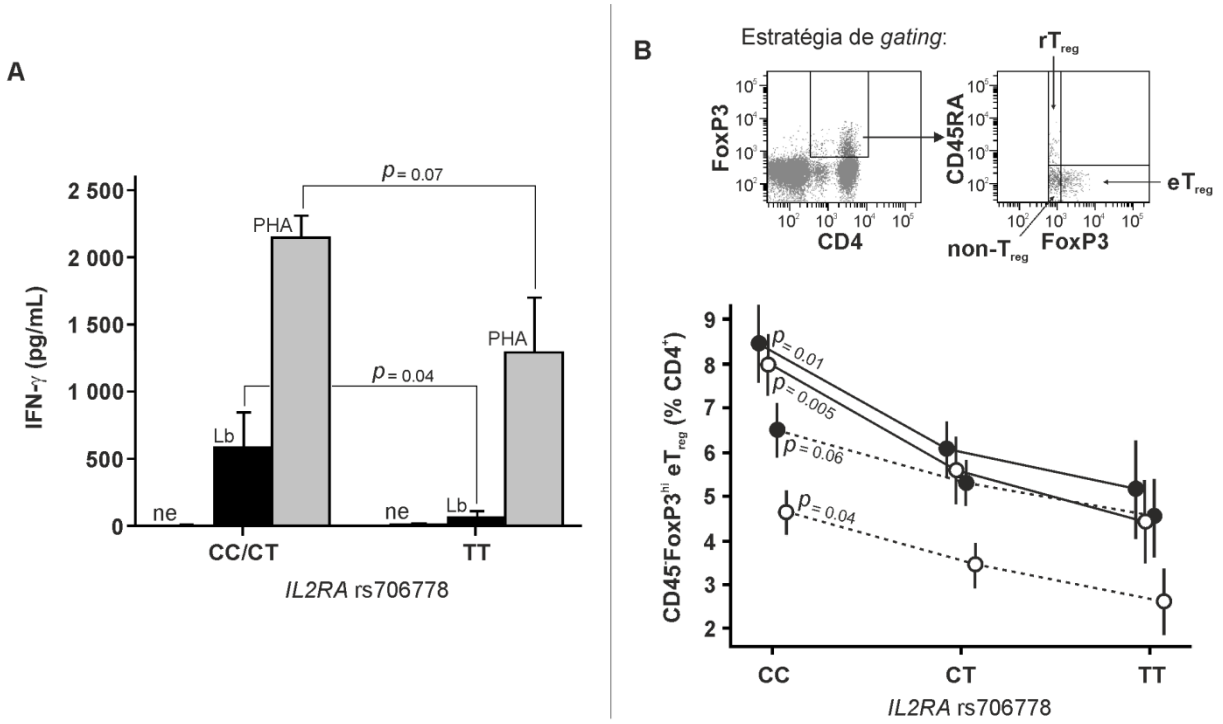


Figura 6. O alelo T do SNP rs706778 é associado a uma menor produção de IFN-γ e a uma menor ativação de células T regulatórias *in vitro*. A, Células mononucleares do sangue periférico (PBMCs) de indivíduos infectados com *Leishmania braziliensis* (*n* = 51) foram cultivadas em meio RPMI (ne, não estimuladas) e estimuladas com o extrato do parasito (Lb) ou com fitoemaglutinina (PHA), como descrito na seção 'Material e Métodos'. Os indivíduos foram classificados de acordo com os seus respectivos genótipos no *locus* rs706778 (CC, CT ou TT) e a concentração de IFN-γ nos sobrenadantes das culturas foi determinada por ELISA. **B**, Parte superior – estratégia de *gating* utilizada para avaliar as subpopulações de células $CD4^+FoxP3^+$ [um anticorpo fluorescente anti-CD45RA foi utilizado para discriminar as células T regulatórias efetoras (eT_{reg}) $CD4^+CD45^-FoxP3^{hi}$ das células T regulatórias não ativadas (rT_{reg}) $CD4^+CD45^+FoxP3^{lo}$]. Parte inferior – PBMCs de indivíduos sadios (*n* = 86) foram estimuladas com anticorpos anti-CD3/CD28 [com uma razão 1(esfera):20(célula) (linha contínua) ou com uma razão 1(esfera):200(célula) (linha pontilhada)] e cultivadas na presença de TGF-β1 (círculos abertos) ou na presença de TGF-β1 combinado à IL-2 (círculos fechados). Análises de regressão linear mostram que a proporção de eT_{regs} varia significativamente de acordo com os genótipos de rs706778. Os dados são representados pelas medianas e erros padrões.

Quando estimuladas na presença de IL-2, as T_{regs} não ativadas (rT_{regs}) aumentam e estabilizam a expressão de *FOXP3* e sofrem uma conversão para um estado ativado, conhecido como T_{reg} efetora (eT_{reg}) (MIYARA *et al*., 2009). Com base nessas informações, é possível supor que mutações em *IL2RA* comprometem a sinalização IL-2/IL-2R, alterando a capacidade de ativação de T_{regs} $FoxP3^+$ em portadores dos diferentes alelos do polimorfismo rs706778. Para testar essa hipótese, PBMCs de indivíduos sadios foram ativadas com anticorpos anti-CD3/CD28 e

cultivadas na presença de TGF-β1 ou de TGF-β1 combinado à IL-2. Após 72 horas, as frequências de eT_{regs} $CD4^{+}CD45RA^{-}FoxP3^{hi}$ foram maiores em portadores do genótipo CC, intermediárias em doadores heterozigotos e menores em indivíduos TT (Figura 6B). Estes resultados foram obtidos nas culturas de células estimuladas com a maior concentração de anticorpos anti-CD3/CD28 (razão esfera:célula = 1:20), com (p = 0.01) ou sem (p = 0.005) a adição de IL-2. A mesma tendência foi observada em culturas estimuladas com uma menor concentração de anticorpos anti-CD3/CD28 (razão esfera:célula = 1:200), na presença (p = 0.06) ou na ausência (p = 0.04) de IL-2 exógena. Este efeito observado em células não estimuladas com IL-2 recombinante deve-se, provavelmente, à produção endógena desta citocina nas culturas.

Não foi possível realizar as mesmas análises com o SNP rs10905669, devido a baixa frequência de seu alelo menor em nossas amostras (frequência do alelo T = 0.17).

3.4.6 rs10905669 e rs706778 não capturam polimorfismos em outros genes próximos ao *IL2RA*

Um marcador genético associado a um determinado traço não é necessariamente a mutação causal. Em vez disso, ele pode estar em desequilíbrio de ligação com uma variante funcional. Diante disso, a localização dos polimorfismos que apresentam moderado a forte DL ($r^2 \geq 0.6$, em uma janela de 1 Mb) com os SNPs rs10905669 (Figura 7A) e rs706778 (Figura 7B) foi avaliada para excluir a possibilidade de que variantes de outros genes (próximos ao *IL2RA*) possam explicar as associações observadas. Os mapas regionais de desequilíbrio de ligação foram construídos a partir de painéis de referência de populações dos continentes americano, africano e europeu, disponibilizadas pelo projeto 1000 genomas. Uma análise detalhada da região indica que todos os polimorfismos capturados pelos marcadores rs10905669 e/ou rs706778 estão localizados em uma zona que se estende a partir de 4 kb do sítio de iniciação de *IL2RA* até aproximadamente 18 kb dentro do íntron 1 do gene.

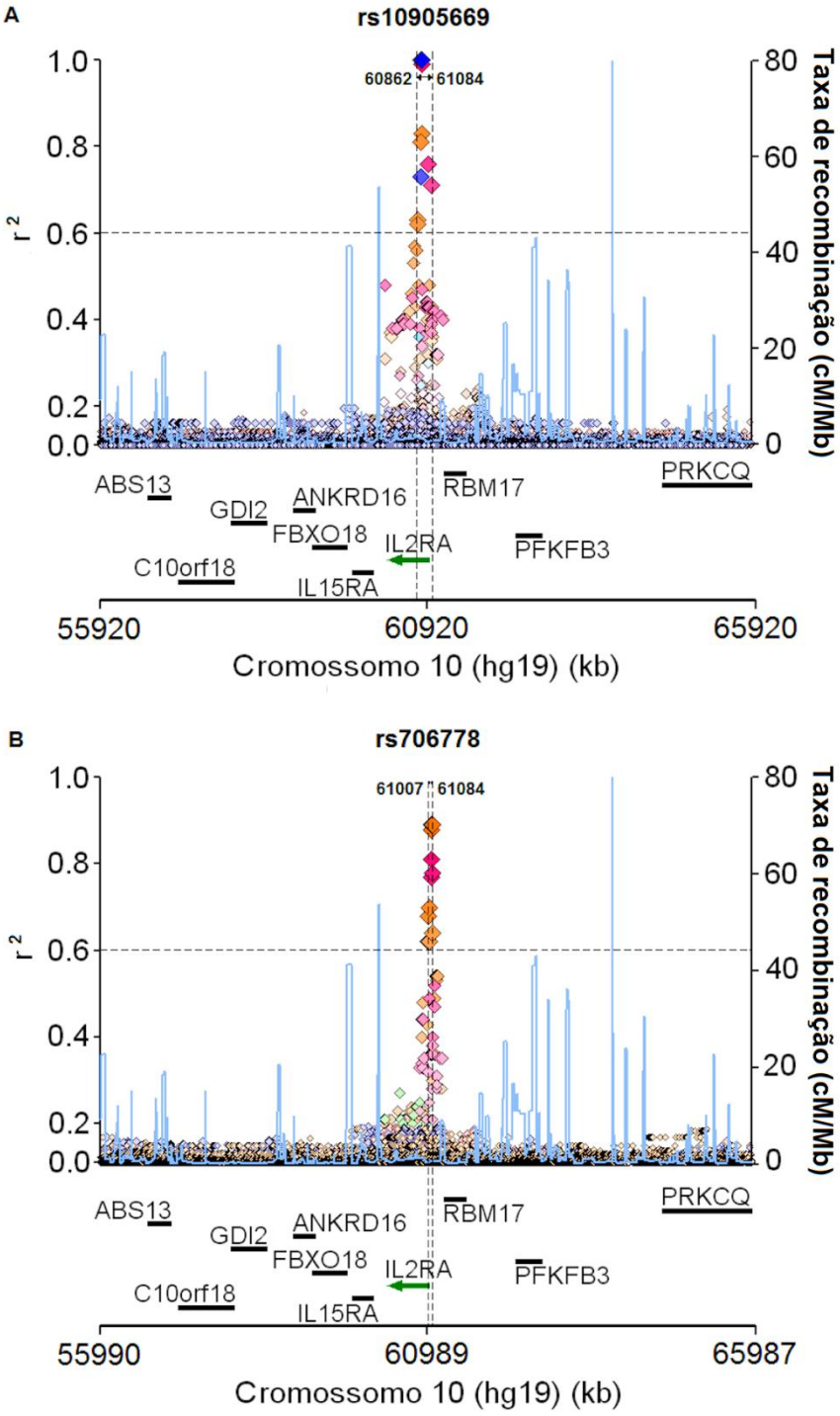


Figura 7. Os marcadores rs10905669 e rs706778 não capturam polimorfismos em outros genes próximos ao *IL2RA*. Mapas regionais de desequilíbrio de ligação (DL) mostrando os polimorfismos capturados por (**A**) rs10905669 ou (**B**) rs706778 ($r^2 \geq 0.6$, em uma janela de 1 Mb). Valores de DL entre SNPs foram calculados separadamente para populações dos continentes americano (lilás), africano (azul) e europeu (laranja), disponibilizadas pelo projeto 1000 Genomas. A região que contém variantes capturadas pelos marcadores testados foi delimitada entre duas linhas verticais pontilhadas. Taxas de recombinação (linhas azuis) foram plotadas para fornecer informações sobre a estrutura de DL da região.

3.4.7 Identificação de variantes com potencial regulatório na região do gene *IL2RA*

Por fim, as posições dos polimorfismos capturados por rs10905669 ou rs706778 [($r^2 \geq 0.6$); populações dos continentes americano, africano e europeu (projeto 1000 genomas)] foram cruzadas com anotações genômicas e epigenômicas, obtidas dos bancos de dados Ensembl e UCSC e do projeto ENCODE. Diversas informações foram avaliadas nos conjuntos de polimorfismos, incluindo conservação de sequências, elementos regulatórios (marcas de histonas para promotores ou *enhancers*) e hipersensibilidade à DNAse I (indicativo de acessibilidade da cromatina) (Figura 8). No bloco de desequilíbrio de ligação cujo marcador é rs10905669, os polimorfismos rs942201, rs1107345, rs10905668 e rs10905669 foram identificados como possíveis candidatos a variantes funcionais, visto que eles estão localizados dentro de um elemento regulatório acessível à ação eventual de fatores de transcrição. No grupo cujo tag-SNP é rs706778, esta mesma análise apontou o SNP rs3134883 como o melhor candidato a mutação regulatória. Este polimorfismo está localizado dentro de um elemento conservado em mamíferos placentários, que é sensível à ação da DNAse I (acessível) e que apresenta indícios de atividade promotora (marca de histona H3K4Me3).

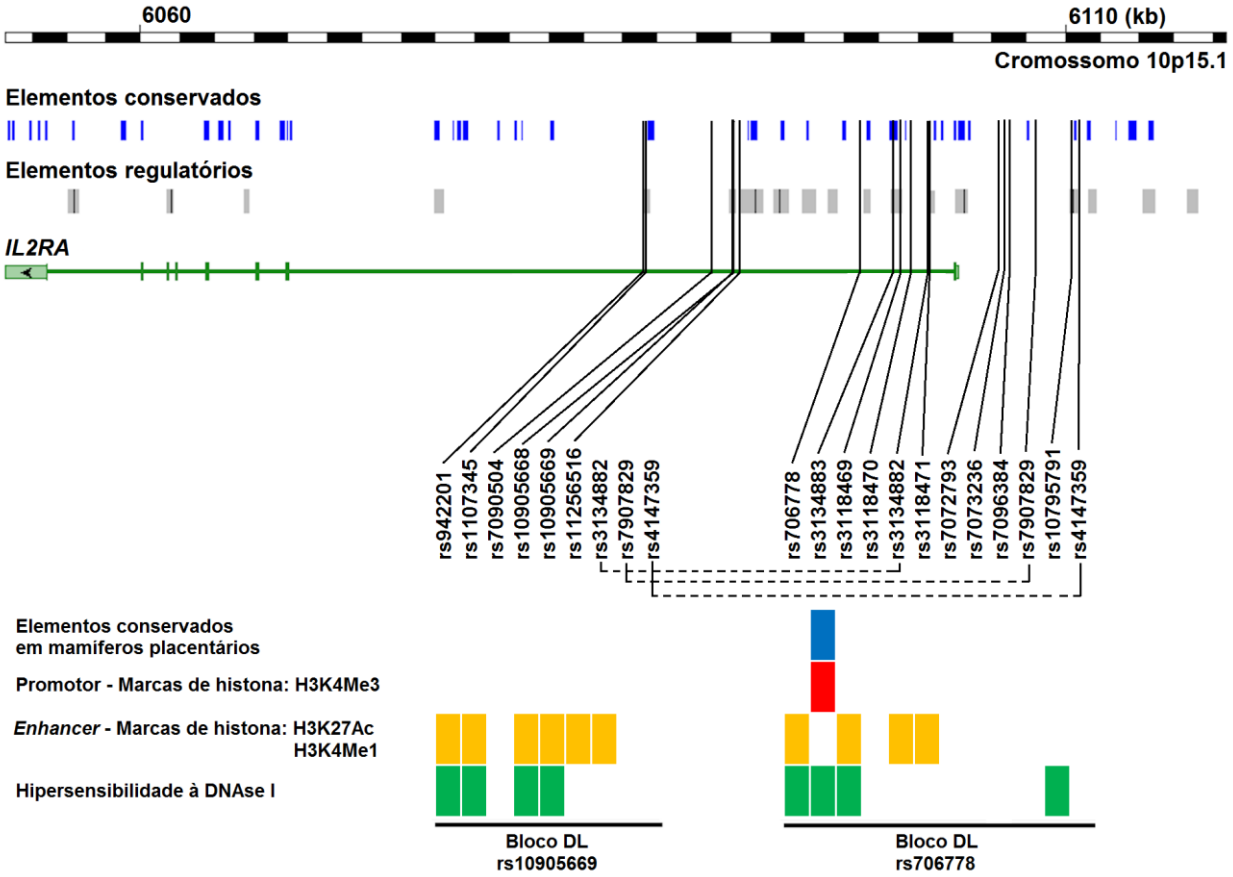


Figura 8. Análise *in silico* dos polimorfismos em desequilíbrio de ligação (DL) com rs10905669 e/ou rs706778. Os blocos de DL foram construídos com base nos polimorfismos capturados ($r^2 \geq 0.6$) por rs10905669 e/ou rs706778, de acordo com painéis de referência para populações dos continentes americano, africano e europeu (projeto 1000 genomas). A posição dos polimorfismos foi cruzada com anotações de sequência de DNA, incluindo elementos conservados em mamíferos placentários, marcas epigenéticas para regiões promotoras e *enhancers* e hipersensibilidade à DNAse I (MultiCell – ENCODE). As análises foram realizadas com base em informações disponibilizadas pelos bancos de dados Ensembl e UCSC. As linhas pontilhadas indicam que um determinado polimorfismo é capturado por ambos os marcadores avaliados (rs10905669 e rs706778). Sequência de referência GRCh37/hg19.

3.5 DISCUSSÃO

No presente estudo, foi mostrado que diversos genes da via de sinalização IL-2 (particularmente aqueles do eixo JAK3-STAT5) são fortemente induzidos em úlceras cutâneas causadas por *L. braziliensis*, indicando que esta via imunológica participa do desenvolvimento da LC. No entanto, a maioria das amostras utilizadas no estudo de expressão apresentou quantidade muito baixa ou indetectável de transcritos do gene *IL2* (Figura 4A). Esta informação é muito relevante, visto que as células T efetoras e regulatórias requerem diferentes quantidades de IL-2, com um baixo sinal sendo suficiente para manter as funções biológicas das T_{regs} FoxP3^{+} (MALEK e CASTRO, 2010). Além disso, a abundância de IL-2RA (CD25) na superfície de T_{regs} FoxP3^{+} faz com elas sejam capazes de responder a baixas concentrações de IL-2, que é rapidamente consumida *in vivo* (BOYMAN e SPRENT, 2012). Desta maneira, a regulação das respostas efetoras nos sítios de infecção por *Leishmania* pode ser favorecida com quantidades limitadas de IL-2. Interessantemente, a sinalização IL-2/IL-2R em T_{regs} resulta em um padrão distinto de sinalização intracelular, com a ativação do eixo JAK3/STAT5, aumentando as expressões de *FOXP3* e de *IL2RA* (BENSINGER *et al*., 2004).

A fim de avaliar o ambiente imune em lesões cutâneas causadas por *L. braziliensis*, as assinaturas de subpopulações de células T auxiliares previamente implicadas no desenvolvimento da LC (T_H1 e T_H2, SACKS e NOBEN-TRAUTH, 2002; T_{reg}, BELKAID *et al*., 2002; T_H17, GONZALEZ-LOMBANA *et al*., 2013) foram determinadas através dos perfis de expressão de genes que regulam a diferenciação, migração e função destas células (Figura A1, Apêndice). Como esperado, a maioria dos genes associados à resposta T_H1 foi fortemente expresso nas lesões cutâneas, incluindo o principal fator de transcrição envolvido na diferenciação das células T_H1 (*TBX21* ou T-bet), sua principal citocina efetora (*IFNG*), além de diversos fatores associados à sua migração (*CXCR3*, *CXCL9*, *CXCL10* e *CXCL11*). É importante salientar que estas quimiocinas e receptor de quimiocinas são importantes para a migração e retenção de eT_{regs} FoxP3^{+} nos sítios com infiltração de células T_H1 (LIM *et al*., 2006). De fato, foi possível detectar nas lesões cutâneas uma assinatura robusta de

células T regulatórias, determinada pela abundância de transcritos dos genes *FOXP3*, *TGFB1*, *IL10* e *CTLA4*. Por outro lado, *GATA3* e *RORC* (RORγT), que são os principais reguladores transcricionais de células T_H2 e T_H17, respectivamente, foram inibidos em lesões, além de outros componentes-chave dessas células cujas expressões não sofreram alteração entre amostras de lesão e de pele normal.

Em seguida, estudos de associação genética identificaram pelo menos dois SNPs no gene *IL2RA* que foram independentemente associados à LC em famílias brasileiras expostas à *L. braziliensis*. Essas associações foram replicadas em uma segunda amostra brasileira e em iranianos infectados com *L. tropica*, que assim como a *L. braziliensis*, causa lesões cutâneas crônicas. Portanto, SNPs em *IL2RA* são associados à LC em populações geneticamente distintas e infectadas com diferentes espécies de *Leishmania*. A heterogeneidade entre os estudos (calculada pelo teste Q de Cochran) foi insignificante para ambos os SNPs: rs10905669 (p = 0.50) e rs706778 (p = 0.77) (Tabela 4). Os sinais consistentes desses dois polimorfismos em populações da América do Sul e do Oriente Médio, que foram submetidas a diferentes processos de adaptação e de seleção natural, sugerem que eles exercem efeitos funcionais ou que estão em forte desequilíbrio de ligação com polimorfismo(s) causal(is) ainda não identificado(s). Os dados apresentados na Figura 7 indicam que os sinais de associação não estão ligados a polimorfismos em outros genes próximos ao *IL2RA*, incluindo genes que não codificam componentes da via de sinalização IL-2.

Como citado anteriormente, a sinalização IL-2/IL-2R controla muitos aspectos das respostas imunes, regulando inclusive a diferenciação e função de células T $CD4^+$ (LIAO *et al.*, 2011). Além disso, a IL-2 desempenha um papel não-redundante no desenvolvimento e função de T_{regs} $FoxP3^+$ (MALEK e CASTRO, 2010). Portanto, mutações em *IL2RA*, que codifica uma molécula (CD25) que aumenta consideravelmente a afinidade entre IL-2 e IL-2R, podem afetar o desenvolvimento da LC de diversas maneiras. Foi mostrado que o genótipo TT do SNP rs706778 é associado a uma menor produção de IFN-γ por células do sangue periférico de indivíduos que vivem em uma área endêmica para a LC. Além disso, foi mostrado que os portadores deste mesmo genótipo apresentam frequências reduzidas de T_{regs} efetoras $CD4^+CD45RA^-FoxP3^{hi}$ em culturas de PBMCs. Diferentes linhas de evidência

mostram que o IFN-γ (produzido principalmente por células T_H1 e células NK) é um dos principais fatores envolvidos no controle da infecção por *Leishmania* (MOUGNEAU *et al.*, 2011). Portanto, mutações no gene *IL2RA* que afetam a produção de IFN-γ podem comprometer o controle do parasito em indivíduos infectados. Os dados apresentados neste estudo também indicam que a maior predisposição à LC pode ser associada com uma menor capacidade de ativação de T_{regs}. Contudo, a análise isolada desses dados não determina conclusivamente que as T_{regs} $FoxP3^+$ têm um papel protetor na leishmaniose cutânea, já que os efeitos de mutações em *IL2RA* na suscetibilidade à LC podem estar associados aos efeitos sobre a produção de IFN-γ ou a outros fatores não avaliados neste trabalho. Além disso, estudos prévios sugerem que as T_{regs} podem ter um papel agravante na LC causada por *L. braziliensis* (COSTA *et al.*, 2013; RODRIGUES *et al.*, 2014). Em contrapartida, durante infecções com outros patógenos, como *Trypanosoma cruzi* (DE ARAUJO *et al*., 2011) e *Toxoplasma gondii* (MORAMPUDI *et al*., 2011), e em doenças autoimunes (GARG *et al.*, 2012), a diminuição da frequência de células T regulatórias circulantes é associada com a exacerbação das respostas imunes efetoras que causam danos teciduais. Ademais, nossos dados são consistentes com estudos realizados recentemente, mostrando que o IFN-γ participa da eliminação da *L. braziliensis* e tem pouca influência sobre a imunopatologia (SILVA *et al.*, 2013). Por outro lado, a destruição tecidual é principalmente causada pela atividade citotóxica de células T $CD8^+$ (SILVA *et al.*, 2013; NOVAIS *et al.*, 2013). Desta maneira, a deficiência na ativação de T_{regs} pode resultar na exacerbação da resposta citotóxica e a redução na produção de IFN-γ pode afetar o controle do parasito. Em conjunto, essas observações indicam que variantes do gene *IL2RA* podem alterar o risco de leishmaniose cutânea através de diferentes efeitos sobre as respostas dependentes da sinalização IL-2/IL-2R. Apesar disso, experimentos adicionais são necessários para determinar o impacto exato destas mutações na resposta imune durante as leishmanioses.

No presente estudo, mutações no gene *IL2RB* foram sugestivamente associadas com a LC causada por *L. braziliensis* (Tabela 2). Contudo, os SNPs que alteram o risco de LV no Sudão (BUCHETON *et al.*, 2007) não foram associados à doença cutânea no Brasil. Outros estudos são, portanto, necessários para determinar se mecanismos

específicos destas formas clínicas e/ou diferenças nas estruturas de desequilíbrio de ligação entre as populações brasileira e sudanesa podem ser responsáveis por tais resultados. Ainda assim, nossos resultados acerca de infecções com *L. braziliensis* e *L. tropica* e os resultados prévios sobre indivíduos infectados com *L. donovani* (BUCHETON *et al.*, 2007) mostram que polimorfismos em genes do receptor da IL-2 alteram a suscetibilidade humana às leishmanioses causadas por diferentes espécies de *Leishmania*.

Outros estudos identificaram polimorfismos no *locus IL2RA* que aumentam o risco de doenças autoimunes (INTERNATIONAL MULTIPLE SCLEROSIS GENETICS CONSORTIUM, 2007; LOWE *et al.*, 2007; WELLCOME TRUST CASE CONTROL CONSORTIUM *et al.*, 2008). É provável que esta maior predisposição à autoimunidade esteja ligada a uma deficiência na resposta de células T regulatórias (GARG *et al.*, 2012). Outros grupos também descreveram mutações nas regiões dos genes *IL2* e *IL2RB* que são associadas com alterações na homeostase do sistema imunológico (ZHERNAKOVA *et al.*, 2007; VAN HEEL *et al.*, 2007; WELLCOME TRUST CASE CONTROL CONSORTIUM, 2007). É importante salientar que os dados do presente estudo são a primeira demonstração de que polimorfismos no gene *IL2RA* alteram a suscetibilidade a uma doença infecciosa. Visto que estas mutações podem afetar, de modo geral, as respostas dependentes da sinalização IL-2/IL-2R, sendo mais restritivas em algumas condições patológicas, esses resultados devem motivar a avaliação de variantes de *IL2RA* em outras doenças infecciosas que afetam populações humanas, particularmente aquelas causadas por patógenos intracelulares.

ESTUDO 2

4. Polimorfismos em *SRGN* (Serglicina), localizado na região de ligação 10q21-q23, são fatores de risco para a leishmaniose cutânea no Brasil.

4.1 JUSTIFICATIVA

A LC é a forma clínica mais comum entre as manifestações associadas às leishmanioses e pode ser causada por diversas espécies dermotrópicas de *Leishmania*, algumas das quais induzem lesões cutâneas brandas, enquanto outras, como a *L. braziliensis*, podem causar úlceras crônicas graves na pele dos indivíduos infectados (REITHINGER *et al.*, 2007; DE OLIVEIRA e BRODSKYN, 2012). De modo geral, camundongos controlam rapidamente a infecção experimental com *L. braziliensis*, produzindo uma forma benigna da doença, que é resolvida em poucas semanas (DEKREY *et al*., 1998). Desta forma, a maior parte do conhecimento acerca da imunobiologia da leishmaniose cutânea é inferida de estudos experimentais com outras espécies de *Leishmania*, tal como *L. major* (SACKS e NOBEN-TRAUTH, 2002). Diversas linhas de evidência mostram que a resposta imune celular é crucial para o controle da infecção por *Leishmania*. A resposta protetora é associada à ativação de células apresentadoras de antígenos, ao predomínio da resposta T_H1 e às atividades citolítica e regulatória de células T $CD8^+$ e NK (SACKS e NOBEN-TRAUTH, 2002; UZONNA *et al.*, 2004; ALEXANDRE e BRYSON, 2005; MOUGNEAU *et al.*, 2011; BOGDAN, 2012).

Em humanos, a doença tegumentar causada por *L. braziliensis* (incluindo as formas cutânea e mucocutânea) é geralmente relacionada a uma resposta inflamatória intensa, que contribui amplamente para o dano tecidual e a formação da lesão. Neste contexto, foi mostrado que a frequência de células T citotóxicas apresenta correlação positiva com a extensão da lesão em indivíduos infectados com *L. braziliensis* (FARIA *et al.*, 2009; SILVA *et al.*, 2013). Além disso, foi mostrado recentemente que a ação citotóxica de células T $CD8^+$ é o principal fator envolvido na destruição tecidual em lesões cutâneas causadas por *L. braziliensis* (NOVAIS *et al.*, 2013).

Interessantemente, alguns indivíduos infectados com *L. braziliensis* não controlam a infecção, que culmina no desenvolvimento de uma ou mais úlceras cutâneas. No entanto, cerca de 70% dos indivíduos infectados são capazes de destruir o parasito e não desenvolvem lesões ou quaisquer manifestações patológicas detectáveis (FOLLADOR *et al.*, 2002). As razões que determinam a maior

suscetibilidade de alguns indivíduos a este patógeno ainda são pouco compreendidas. No entando, é provável que fatores genéticos do hospedeiro tenham papéis importantes na predisposição à doença. As evidências de que esses fatores influenciam a suscetibilidade às leishmanioses são substanciais e incluem agregação familiar de fenótipos clínicos (CASTELLUCCI *et al*., 2005; CABELLO *et al*., 1995), ampla variabilidade clínica entre indivíduos infectados com a mesma espécie/cepa de *Leishmania* (ZIJLSTRA *et al*., 2003) e risco aumentado entre irmãos (PEACOCK *et al*., 2001).

Diversos estudos de ligação ou associação genômica foram conduzidos com o objetivo de desvendar a arquitetura genética da leishmaniose visceral (BUCHETON *et al.*, 2003; MILLER *et al.*, 2007; JAMIESON *et al.*, 2007; JERONIMO *et al.*, 2007; LEISHGEN CONSORTIUM *et al.*, 2013). No entanto, a busca por fatores genéticos que alteram a suscetibilidade à LC em populações humanas expostas à espécies dermotrópicas de *Leishmania* foi, até o momento, baseada na análise de alguns genes candidatos (OLIVO-DIAZ *et al.*, 2004; SALHI *et al.*, 2008; CASTELLUCCI *et al.*, 2010). Desta forma, a arquitetura genética da LC permanece amplamente desconhecida.

4.2 OBJETIVOS DO ESTUDO

- Objetivo geral:

Identificar regiões genômicas (e genes) que influenciam a suscetibilidade à leishmaniose cutânea em famílias brasilieiras expostas à *L. braziliensis*.

- Objetivos específicos:

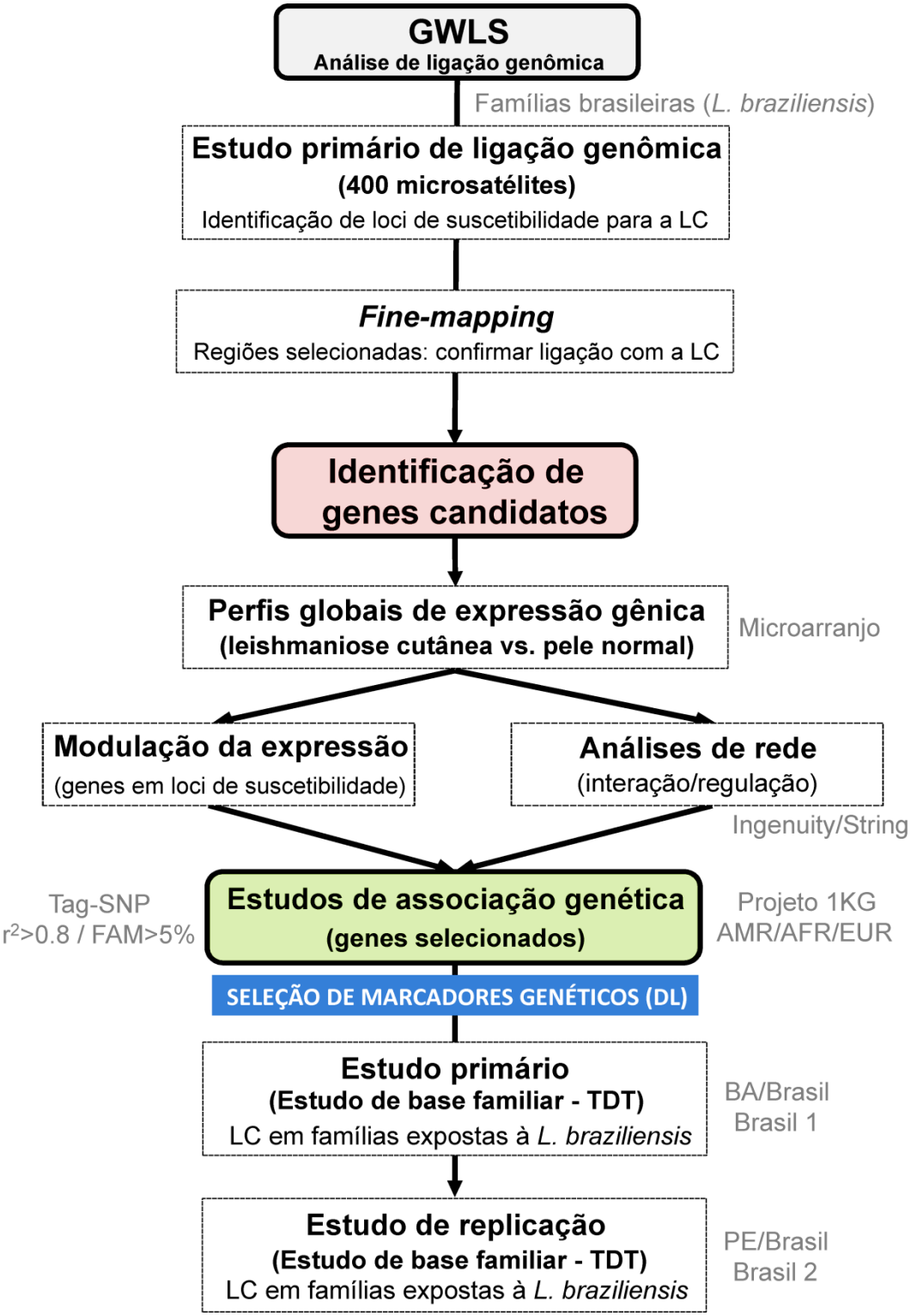
- Realizar uma análise de ligação genômica para identificar os principais *loci* de suscetibilidade à LC no Brasil;

- Identificar genes-alvo nas regiões de suscetibilidade à LC com o auxílio das análises transcriptômicas de lesões cutâneas causadas por *L. braziliensis* (perfis de expressão gênica e análise de redes de interação entre moléculas);

- Investigar a associação de polimorfismos em genes-alvo (nos *loci* de suscetibilidade) com o risco de desenvolver lesões cutâneas em famílias brasileiras exposta à *L. braziliensis*;

- Conduzir análises *in silico* para determinar padrões de desequilíbrio de ligação e identificar polimorfismos com potencial regulatório nos *loci* associados à LC.

4.3 DESENHO EXPERIMENTAL



4.4 RESULTADOS

4.4.1 Identificação de um novo *locus* de suscetibilidade para a leishmaniose cutânea no Brasil

No presente estudo, uma análise de ligação genômica (GWL) foi conduzida em famílias expostas à *L. braziliensis* para identificar os principais *loci* envolvidos na suscetibilidade à leishmaniose cutânea no Brasil. Este estudo de ligação foi realizado em duas etapas. Inicialmente, 32 famílias multicaso (Tabela 5 – Análise primária) foram genotipadas para 400 marcadores microssatélites abrangendo todos os cromossomos autossômicos. Os dados foram analisados através do método da probabilidade binomial mista (MLB), cujos algoritmos são indicados para análises de ligação em doenças complexas (KRUGLYAK *et al*., 1996; ABEL e MULLER-MYHSOK, 1998). Este estudo identificou marcadores em três regiões cromossômicas distintas apresentando valores de LOD-multiponto sugestivos de ligação com a LC (acima de 1.50): 1q31-q32, 10q21-q23 e 18q22-q23 (Figura 9). A análise multiponto é útil para localizar sinais de ligação entre dois marcadores e para maximizar a obtenção de informação de um conjunto de marcadores (HALPERN e WHITTEMORE, 1999). Os picos de LOD (marcador único) em cada região foram 2.20, 1.85 e 3.25, para D1S413 (1q31-q32), D10S1652 (10q21-q23) e D18S462 (18q22-q23), respectivamente. Não foram encontradas evidências de ligação com *loci* previamente ligados ou associados à leishmaniose visceral no Brasil, na Índia ou no Sudão (BUCHETON *et al.*, 2003; MILLER *et al.*, 2007; JAMIESON *et al.*, 2007; JERONIMO *et al.*, 2007; LEISHGEN CONSORTIUM *et al.*, 2013).

Tabela 5. Número de filhos afetados pela leishmaniose cutânea (estudo de ligação genômica)

	Número de famílias com					
	2 filhos afetados	**3 filhos afetados**	**4 filhos afetados**	**5 filhos afetados**	**6 filhos afetados**	**Total**
Análise primária	16	11	3	1	1	32
Análise detalhada	13 (29)	5 (16)	3 (6)	-	-	21 (53)

Os números entre parênteses representam o total de famílias brasileiras avaliadas na análise detalhada (*fine-mapping*).

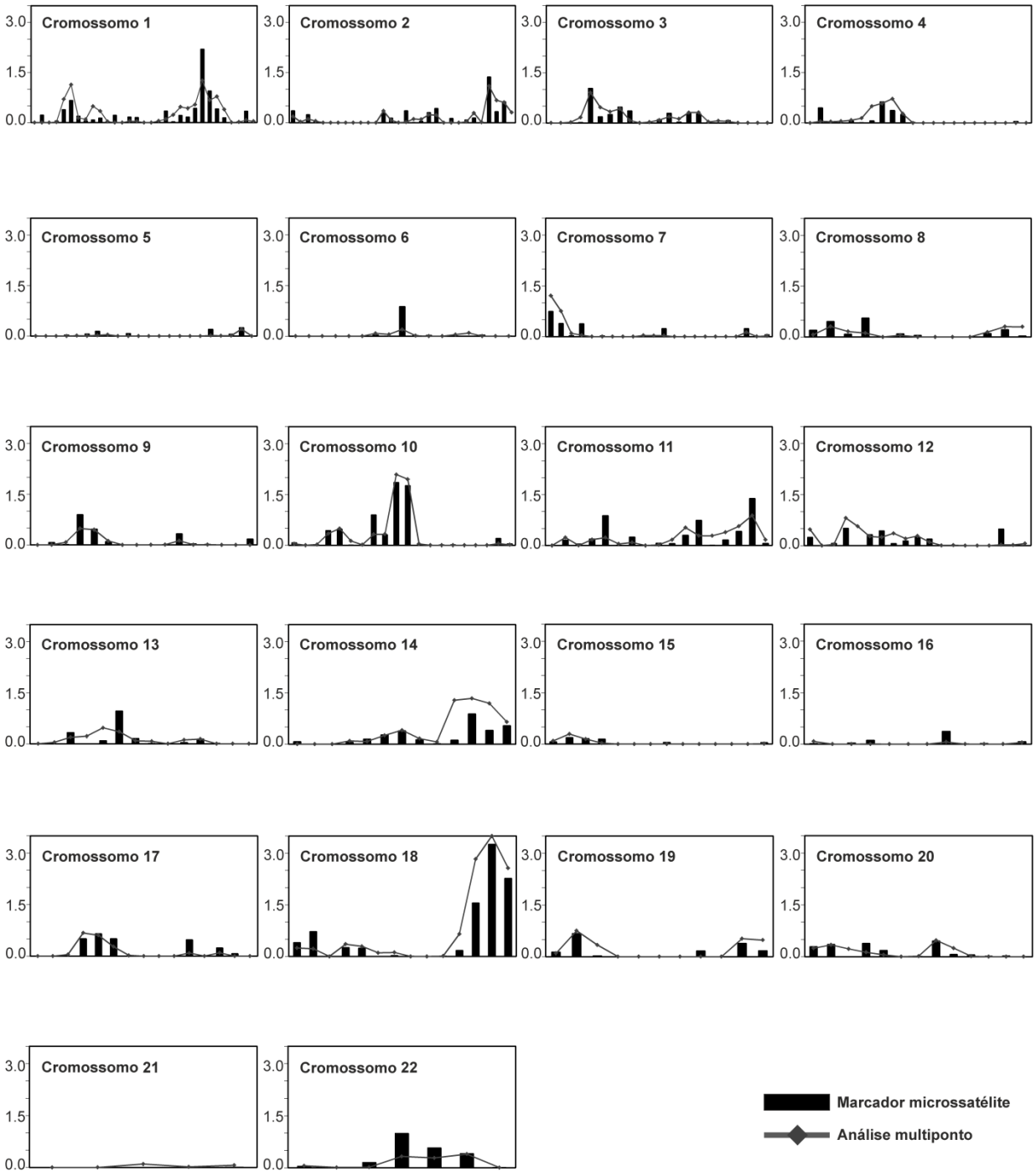


Figura 9. Estudo primário de ligação genômica em 32 famílias multicaso afetadas pela leishmaniose cutânea no Brasil. Os resultados de cada um dos 400 marcadores microssatélites testados nos 22 cromossomos autossômicos são mostrados no eixo da abscissas. No eixo das ordenadas, os valores de LOD para cada marcador (individualmente) são representados por barras e os valores para a análise multiponto são representados por linhas.

Em seguida, os *loci* apresentando sinais de ligação sugestivos com a LC (1q31-q32, 10q21-q23 e 18q22-q23) foram avaliados de modo mais detalhado (*fine-mapping*), através da adição de 12 a 10 marcadores microssatélites em cada uma das regiões. Além disso, o número de famílias multicaso foi ampliado de 32 para 53 e o número de filhos afetados de 88 para 141 (Tabela 5 – Análise detalhada). A análise individual dos novos marcadores confirmou os sinais de ligação com diversos microssatélites nas regiões 10q21-q23 e 18q22-q23, mas não em 1q31-q32 (Tabela 6). Na região 18q22-q23, diversos marcadores apresentaram valores de LOD acima de 2.0, ou até mesmo acima de 3.0. Contudo, a análise multiponto reduziu drasticamente o sinal de ligação para quase todos os marcadores desta região (abaixo de 1.50), exceto para D18S1091 (LOD multiponto = 2.08). A análise individual dos marcadores revelou um sinal de ligação contíguo sugestivo na região 10q21-q23, delimitado por D10S581 e D10S210. Nesta mesma região, quase todos os marcadores avaliados tiveram as suas significâncias melhoradas pela análise multiponto, com D10S1647 (LOD multiponto = 1.68) sendo incluído nesta zona de ligação contígua (Tabela 6 e Figura 10). O valor máximo de LOD na região 10q21-q23 foi obtido pelo marcador D10S522 (LOD multiponto = 2.39).

Tabela 6. Análise detalhada das regiões apresentando ligação sugestiva com a LC no Brasil

Região e marcador		LOD escore em Análise primária		LOD escore em Análise detalhada	
		Individual	Multiponto	Individual	Multiponto
1q31-1q32	D1S238	0.42	0.54	0.11	0.05
	D1S384	-	-	0.001	0.007
	D1S492	-	-	0.02	0.07
	D1S2877	-	-	0.73	0.37
	D1S2625	-	-	0.47	0.15
	D1S412	-	-	0.30	0.10
	D1S413	2.20	1.26	1.49	0.08
	D1S2745	-	-	0.00003	0.002
	D1S1723	-	-	0.04	0.006
	D1S2683	-	-	0.73	0.29
	D1S510	-	-	0.13	0.10
	D1S2773	-	-	0.34	0.15
	D1S249	0.94	0.67	0.56	0.18
	D1SIL10G	-	-	0.54	0.58
	D1SIL10R	-	-	0.58	0.68
10q21-q23	D10S609	-	-	0.93	0.76
	D10S1652	1.85	2.08	0.67	0.92
	Gata151f09	-	-	1.24	0.94
	D10S581	-	-	1.81	1.84
	D10S522	-	-	1.90	2.39
	D10S1670	-	-	1.75	1.83
	D10S210	-	-	1.99	1.68
	D10S1647	-	-	0.97	1.68
	D10S676	-	-	0.73	0.89
	D10S1688	-	-	1.47	1.36
	D10S537	1.70	1.94	1.63	1.36
	D10S1650	-	-	1.64	1.09
18q22-18q23	D18S1091	-	-	3.10	2.08
	D18S469	-	-	0.63	1.37
	D18S58	-	-	2.24	1.34
	D18S880	-	-	0.31	0.94
	D18S1161	1.55	2.83	0.72	1.16
	D18S1112	-	-	2.42	1.22
	D18S1009	-	-	2.07	1.17
	D18S1097	-	-	1.66	1.06
	D18S462	3.25	3.50	1.81	1.32
	D18S461	-	-	2.19	1.41
	D18S1122	-	-	2.77	0.90
	D18S1141	-	-	1.10	1.33

A análise primária foi realizada com 32 famílias multicaso (88 filhos afetados). Além das 32 famílias iniciais, outras 21 famílias (com outros 53 casos) foram empregadas nas análises detalhadas.

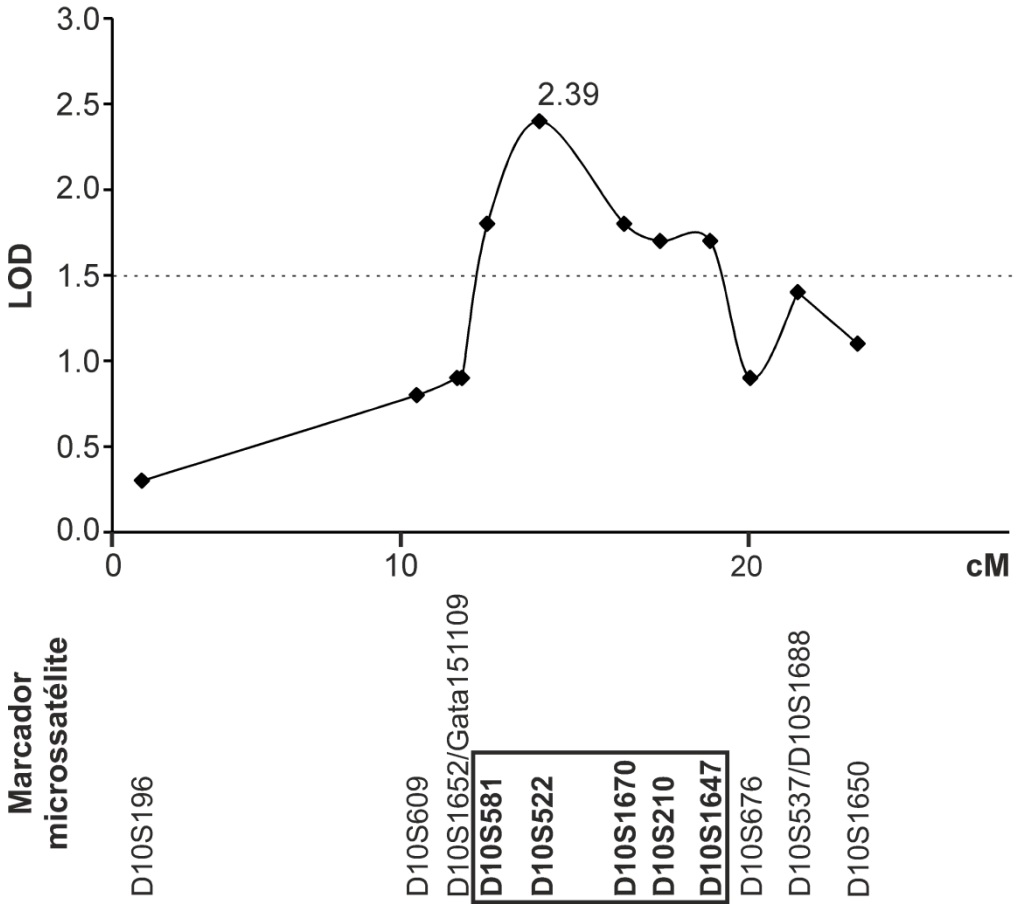


Figura 10. Análise detalhada da região 10q21-q23. Foram avaliadas 53 famílias multicaso afetadas pela leishmaniose cutânea (*L. braziliensis*) no Brasil. A linha contínua corresponde aos valores de LOD (multiponto) encontrados na região 10q21-q23. A linha pontilhada representa o limite sugestivo de ligação genética (LOD > 1.50). O quadrado delimita a zona na qual foi observado um sinal de ligação contíguo. O valor máximo de LOD na região 10q21-q23 foi obtido pelo marcador D10S522 (LOD multiponto = 2.39). cM: centimorgan.

4.4.2 A expressão dos genes *PRF1* e *SRGN*, na região 10q21-q23, é fortemente induzida em lesões cutâneas causadas por *L. braziliensis*

Como a análise anterior evidenciou um sinal de ligação sugestivo na região 10q21-q23 com a suscetibilidade à leishmaniose cutânea, nós partimos para avaliar os genes presentes nesta região cujas variantes poderiam influenciar a predisposição à doença. Inicialmente, nossos dados sobre a expressão global de genes em lesões cutâneas causadas por *L. braziliensis* foram reavaliados (Figura 3, Estudo 1). Quando

as análises foram limitadas aos perfis de expressão de 96 genes (que codificam proteína) localizados na região 10q21-q23 (GENCODE, versão 20), foi observado que, em todo esse intervalo no cromossomo 10, somente 7 genes foram modulados entre amostras de lesão cutânea e de pele normal. Dentre estes, 5 foram induzidos (*SRGN*, *SAR1A*, *PRF1*, *SLC29A3* e *PSAP*) e 2 foram inibidos (*TTC18* e *USP54*) em úlceras cutâneas (Figura 11A). Notavelmente, os genes *PRF1* (FC = 49.3) e *SRGN* (FC = 21.8) foram fortemente expressos em lesões cutâneas. *PRF1* codifica a perforina 1, uma proteína que forma poros na membrana de células-alvo dos linfócitos citotóxicos (DE SAINT BASILE *et al*., 2010). Por sua vez, o gene *SRGN* codifica um proteoglicano conhecido como serglicina, envolvido na formação de grânulos citoplasmáticos (RAJA *et al*., 2002). Ambos, perforina 1 e serglicina, são essenciais para a atividade citotóxica de células T $CD8^+$ e NK, que participam da eliminação de células infectadas com *Leishmania* e, possivelmente, da imunopatologia associada à LC (JORDAN e HUNTER, 2010; BOGDAN, 2012; NOVAIS *et al*., 2013).

Em seguida, uma análise foi realizada para avaliar a expressão de moléculas relacionadas aos 7 genes modulados na região 10q21-q23. As redes de interação para *SRGN*/*PRF1* (que interagem fisicamente), *SAR1A*, *SLC29A3*, *PSAP*, *TTC18* e *USP54* foram construídas com base nos dados de expressão (análises transcriptômicas) e em informações sobre interação direta (física) ou indireta (funcional) entre moléculas, obtidas dos bancos de dados Ingenuity e String (Tabela A3, Apêndice). Interessantemente, foi observado um enriquecimento da rede *SRGN*/*PRF1*, que tem quase a metade dos seus genes induzidos em lesões causadas por *L. braziliensis* (*p*-valor relativo = 6.4×10^{-5}) (Figura 11B e 11C). Por outro lado, as demais redes de interação não foram associadas à leishmaniose cutânea (*p*-valores relativos ≥ 0.38).

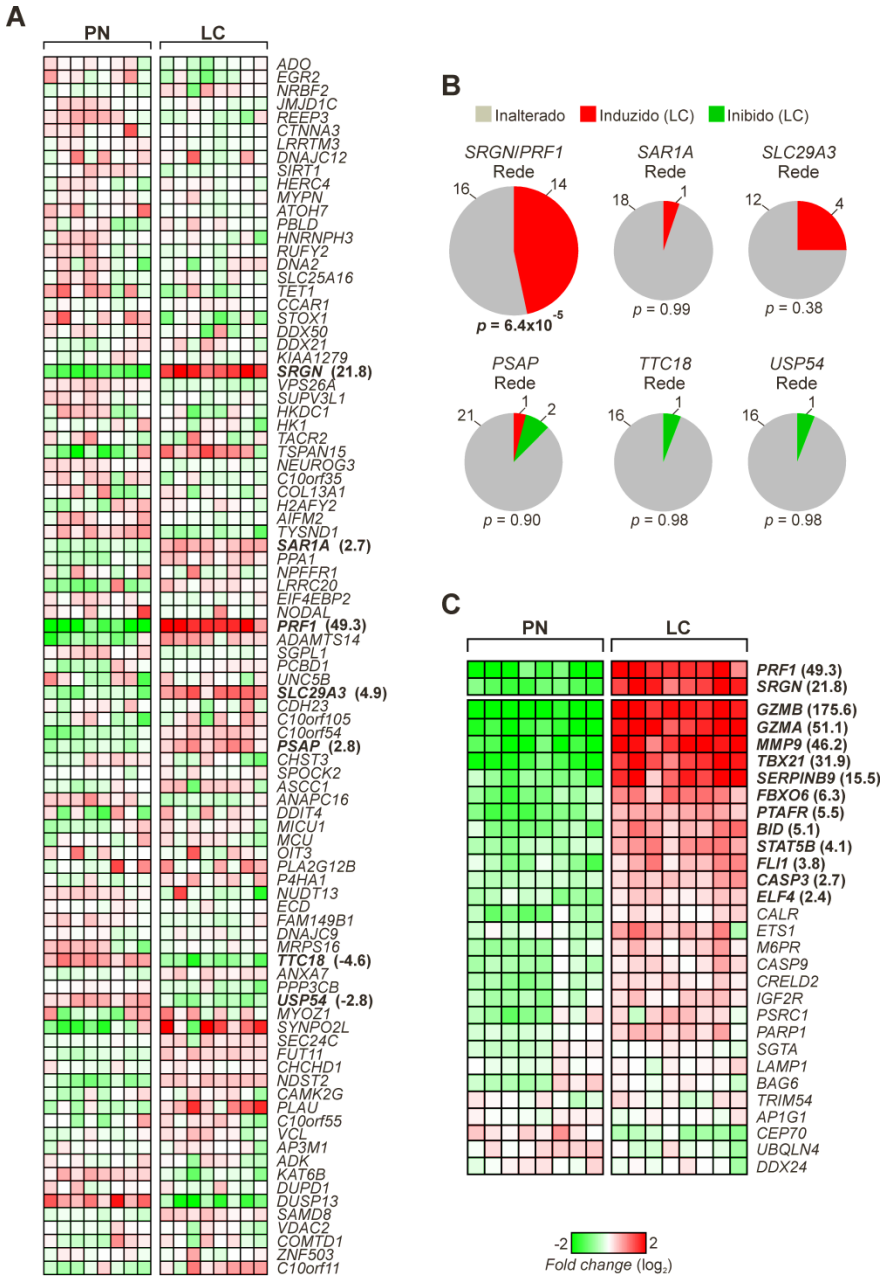


Figura 11. A rede de interação *SRGN*/*PRF1* é ativada em lesões cutâneas causadas por *Leishmania braziliensis*. *A*, *Heat map* mostrando os perfis de expressão de 96 genes da região 10q21-q23 em 8 biópsias de lesões cutâneas (LC) e 8 amostras de pele normal de indivíduos não infectados (PN). A análise de significância para microarranjos (SAM) foi utilizada para a identificação de genes diferencialmente expressos entre LC e PN (FDR < 0.001%; *fold change* ≥ 2.0). Genes significativamente modulados são mostrados em negrito. Os valores entre parênteses representam a diferença média de expressão (*fold change*) entre os grupos (fenótipo de referência: lesão cutânea). ***B***, As redes de interação para *SRGN/PRF1*, *SAR1A*, *SLC29A3*, *PSAP*, *TTC18* e *USP54* foram construídas baseadas em informações sobre interação/regulação entre moléculas, disponibilizadas pelos bancos de dados Ingenuity e String. O *p*-valor relativo para uma rede de interação foi calculado pelo teste exato de Fisher (cauda direita), comparando a proporção de genes modulados em uma determinada rede com a proporção de genes modulados nas demais redes avaliadas. ***C***, *Heat map* mostrando os perfis de expressão de componentes da rede *SRGN*/*PRF1*.

4.4.3 Polimorfismos na região do gene *SRGN*, e não em *PRF1*, são associados à suscetibilidade à leishmaniose cutânea no Brasil

Posteriormente, foi conduzido um estudo de associação genética em 209 famílias brasileiras expostas à *L. braziliensis* (amostra primária Brasil 1), para determinar se variantes dos genes *PRF1* e/ou *SRGN* podem explicar o sinal de ligação da região 10q21-q23 com a leishmaniose cutânea (Tabela 7; Brasil 1 – Amostra primária).

Tabela 7. Características das amostras populacionais utilizadas nos estudos de associação genética (agrupamentos familiares)

	Brasil 1 **Amostra primária**	**Brasil 2** **Amostra de replicação**
Tamanho da amostra	754	325
Sexo (masculino:feminino)	452:302	181:144
Mediana da idade em anos (IQR)	33 (22-45)	27 (19-42)
Número de famílias nucleares	209	80
Número de trios	323	130

IQR: : Intervalo interquartil.

Ambos os genes avaliados, *PRF1* (Chr10:70597348-70602775) e *SRGN* (Chr10:69088106-69104811), encontram-se no *locus* 10q22.1. Utilizando a mesma estratégia adotada nas análises de associação genética do Estudo 1, 45 tag-SNPs cobrindo os genes *PRF1* e *SRGN* (incluindo regiões regulatórias; HUMPHRIES *et al*., 1992; PIPKIN *et al*., 2007) foram selecionados com base em coeficientes de correlação quadrada ($r^2 \geq 0.8$) entre SNPs para populações dos continentes americano, africano e europeu, disponibilizadas pelo projeto 1000 Genomas (Tabela A2, Apêndice). Cinco SNPs e 19 indivíduos foram excluídos das análises posteriores por apresentarem mais de 10% de genótipos não determinados. Através do teste de desequilíbrio de transmissão de alelos foi possível identificar 3 marcadores em regiões não codificantes

próximas ao gene *SRGN* [rs10998538 (*p* = 0.001), rs10998564 (*p* = 0.002) e rs12437 (*p* = 0.003)] que são associados (*p*-valor empírico corrigido < 0.05) à LC nessa amostra populacional (Tabela 8, Brasil 1 – Estudo primário). Para confirmar esses resultados, um estudo de replicação foi conduzido em uma amostra brasileira distinta, composta por 80 famílias expostas à *L. braziliensis* (Tabela 7; Brasil 2 – Amostra de replicação). Os SNPs rs10998538, rs10998564 e rs12437 foram genotipados nesta segunda coorte e 6 indivíduos foram eliminados após a realização do controle de qualidade (todos com base no percentual de genótipos não determinados). Ao contrário do SNP rs10998564, que não foi associado à predisposição à LC nesta análise secundária, as associações dos SNPs rs10998538 (*p* = 0.01) e rs12437 (*p* = 0.007) foram replicadas nesta amostra brasileira independente, com o alelo C de ambos os SNPs aumentado o risco de desenvolvimento de lesões cutâneas em indivíduos infectados com *L. braziliensis* (Tabela 8, Brasil 2 – Estudo de replicação).

Tabela 8. Mutações na região do gene *SRGN* (10q22.1) são associadas com o desenvolvimento de lesões cutâneas em famílias brasileiras expostas à *Leishmania braziliensis*

Tag-SNP	Gene	Alelo	Freq	Fam	O_t	E_t	*p*	p_c
Brasil 1 – Estudo primário								
rs10998538	*SRGN*$^{\text{intergênico}}$	C	0.88	45	49	36	0.001^{r}	0.03
rs12267089	*SRGN*$^{\text{intergênico}}$	G	0.92	40	125	114	0.02^{a}	NS
rs17557564	*SRGN*$^{\text{ntergênico}}$	C	0.93	28	80	72	0.02^{a}	NS
rs6480379	*SRGN*$^{\text{ntergênico}}$	T	0.51	90	157	142	0.02^{a}	NS
rs10998564	*SRGN*$^{\text{ntergênico}}$	G	0.69	88	194	175	0.002^{a}	0.04
rs2805915	*SRGN*$^{\text{intrônico}}$	G	0.50	100	101	88	0.04^{a}	NS
rs12437	*SRGN*$^{\text{3'-UTR}}$	C	0.78	83	199	184	0.003^{a}	0.04
rs7915836	*SRGN*$^{\text{ntergênico}}$	G	0.87	52	126	117	0.04^{a}	NS
rs885822	*PRF1*$^{\text{éxon}}$	A	0.72	93	230	216	0.04^{a}	NS
Brasil 2 – Estudo de replicação								
rs10998538	*SRGN*$^{\text{ntergênico}}$	C	0.88	17	61	52	0.01^{r}	-
rs10998564	*SRGN*$^{\text{ntergênico}}$	G	0.64	45	73	75	NS	-
rs12437	*SRGN*$^{\text{3'-UTR}}$	C	0.77	32	100	88	0.007^{a}	-

Alelo: alelo de referência; Freq: frequência do alelo de referência; Fam: número de famílias informativas; O_t: transmissões observadas; E_t: transmissões esperadas; *p*: *p*-valor assintótico; p_c: *p*-value empírico (10000 permutações) após correção de Bonferroni; NS: não significante.
No estudo de replicação, *p*-valores assintóticos < 0.05 foram considerados significantes.
^{a}Aditivo e ^{r}recessivo (modelos genéticos).

4.4.4 Os sinais de associação dos SNPs rs10998538 e rs12437 são distintos e independentes

Na etapa seguinte, foi avaliado se rs10998538 e rs12437 capturam o mesmo sinal na região do gene *SRGN* ou se são independentemente associados ao risco de desenvolver lesões cutâneas em indivíduos infectados com *L. braziliensis*. Primeiro, foi evidenciado que este par de polimorfismos não está em desequilíbrio de ligação (r^2 < 0.002) nas amostras brasileiras. Apesar disso, eles podem estar correlacionados (graus intermediários de DL) com com a mesma mutação causal. Análises de regressão logística sugerem que rs10998538 e rs12437 capturam sinais independentes de associação com a LC (ambos os SNPs conservaram *p*-valores < 0.05 após as análises condicionais).

4.4.5 Busca por variantes regulatórias na região do gene *SRGN*

Como citado anteriormente, um marcador genético pode capturar o efeito de um polimorfismo funcional. Diante disso, análises *in silico* das variantes em DL ($r^2 \geq 0.6$, em uma janela de 1 Mb) com rs10998538 ou rs12437 foram realizadas com o objetivo de identificar polimorfismos com potencial regulatório e de investigar se variantes em outros genes próximos ao *SRGN* poderiam estar envolvidas nas associações observadas. Valores de DL entre SNPs foram obtidos do projeto 1000 genomas (painéis de referência para populações dos continentes americano, africano e europeu). Como ilustrado na Figura 12A, o tag-SNP rs10998538 captura polimorfismos localizados em uma região relativamente curta (aproximadamente 10 kb), a 38 kb 5' do sítio de iniciação do gene *SRGN*. Interessantemente, esta região apresenta elementos regulatórios com indícios de atividade promotora (marcas de histona H3K4Me3, projeto ENCODE–MultiCell). O SNP rs10998538 está localizado dentro de um destes sítios regulatórios potenciais (Figura 13). Por outro lado, o tag-SNP rs12437 captura outros 51 polimorfismos no *locus* 10q22.1, distribuídos ao longo de uma região de aproximadamente 126 kb (Figura 12B). Além do gene *SRGN*, diversas mutações em desequilíbrio de ligação com rs12347 sobrepõem outros dois genes (*VPS26A* e

SUPV3L1) e estão localizadas dentro de elementos regulatórios (rs4745976, rs2491024, rs2008553, rs2008541, rs4746817, rs4746818, rs4284308, rs10823301 e rs6480384) ou, como no caso do SNP rs10823298, dentro de uma região sensível à ação da DNAse I (indicativo de acessibilidade da cromatina) (Figura 13).

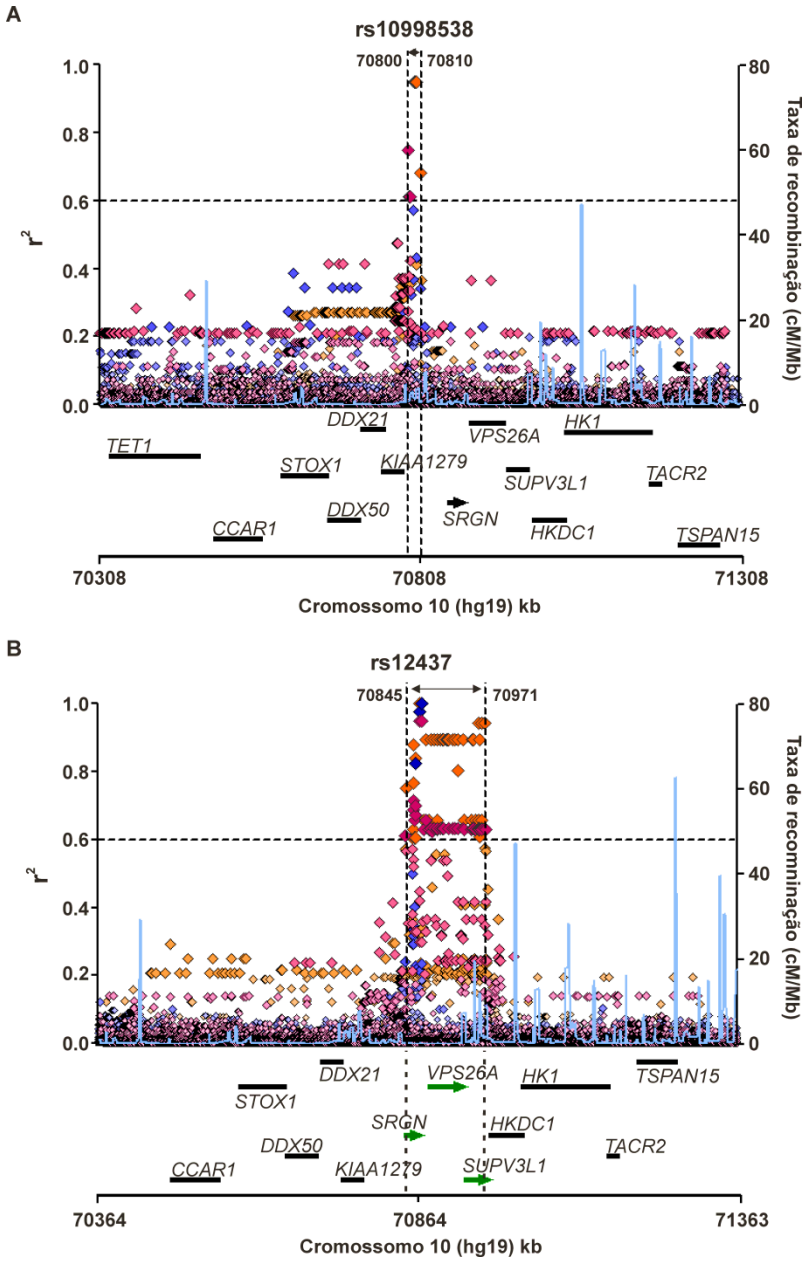


Figura 12. Mapas regionais de desequilíbrio de ligação (DL) para os tag-SNPs rs10998538 e rs12437. Blocos de DL capturados por (**A**) rs10998538 ou (**B**) rs12437. Valores de DL entre SNPs foram calculados separadamente para populações dos continentes americano (lilás), africano (azul) e europeu (laranja), disponibilizadas pelo projeto 1000 Genomas. A região que contém variantes capturadas pelos marcadores testados foi delimitada entre duas linhas verticais pontilhadas. Taxas de recombinação (linhas azuis) foram plotadas para fornecer informações sobre a estrutura de DL da região.

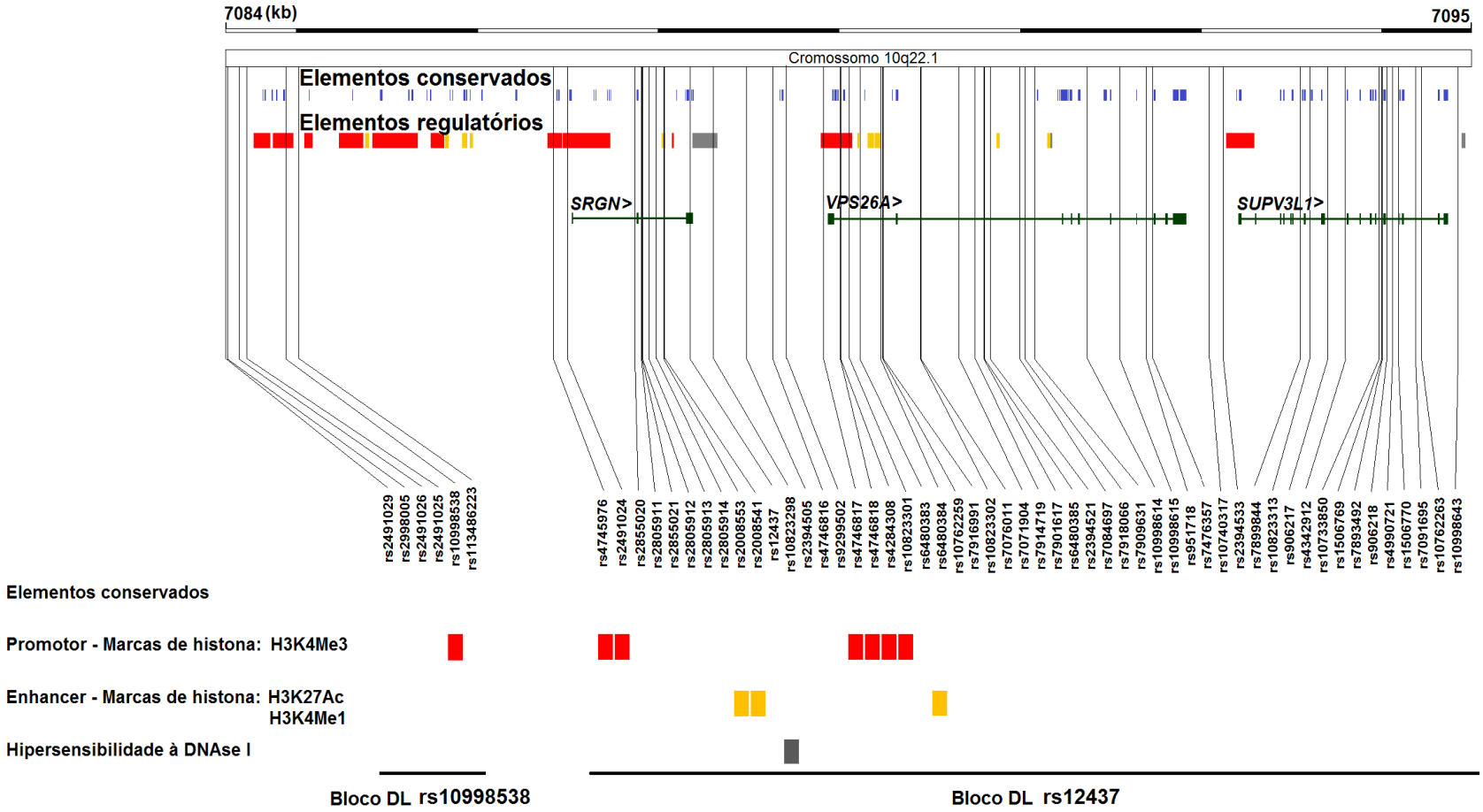


Figura 13. Identificação de variantes com potencial regulatório na região do gene *SRGN* (10q22.1). Blocos de desequilíbrio de ligação (DL) foram construídos com base nos polimorfismos capturados ($r^2 \geq 0.6$) por rs10998538 ou rs12437, de acordo com painéis de referência para populações dos continentes americano, africano e europeu (projeto 1000 genomas). A posição dos polimorfismos foi cruzada com anotações de sequência de DNA, incluindo elementos conservados em mamíferos placentários, marcas epigenéticas para regiões promotoras e *enhancers* e hipersensibilidade à DNAse I (MultiCell – ENCODE). As análises foram realizadas com base em informações disponibilizadas pelos bancos de dados Ensembl e UCSC. Sequência de referência GRCh37/hg19.

4.5 DISCUSSÃO

Espécies distintas de *Leishmania* podem induzir diferentes doenças em humanos. Porém, diversas evidências indicam que pacientes infectados com a mesma espécie/cepa do parasito podem desenvolver manifestações clínicas distintas (BUCHETON *et al.*, 2002, ZIJLSTRA *et al.*, 2003) e podem também diferir em relação à resposta ao tratamento (BERMAN, 1997). Além disso, indivíduos com infecção sub-clínica são capazes de controlar a carga parasitária (*Leishmania*) e não desenvolvem sintomas associados à infecção (FOLLADOR *et al*., 2002). As bases desta variação ainda são pouco conhecidas, embora diversos genes envolvidos na suscetibilidade às leishmanioses já tenham sido identificados (LIPOLDOVÁ e DEMANT, 2006). A arquitetura genética da leishmaniose cutânea em humanos vem sendo lentamente desvendada através de análises de genes candidatos, selecionados com base em estudos imunológicos (OLIVO-DIAZ *et al.*, 2004; SALHI *et al.*, 2008; CASTELLUCCI *et al.*, 2010).

No presente estudo, foi conduzida uma análise de ligação genômica em famílias brasileiras expostas à *L. braziliensis*, cujos resultados iniciais evidenciaram três regiões (1q31-q32, 10q21-q23 e 18q22-q23) com valores de LOD sugestivos de ligação com a LC. Em um segundo momento, essas regiões cromossômicas foram analisadas de modo mais detalhado (*fine-mapping*) para excluir eventuais resultados falso-positivos (Tabela 6). Diferentemente da região 10q21-q23, na qual diversos marcadores microssatélites mantiveram valores de LOD elevados (valor máximo de LOD multiponto = 2.39), os sinais de ligação dentro das regiões 1q31-q32 e 18q22-q23 não foram confirmados após o *fine-mapping*. Estas perdas de sinal podem indicar a ocorrência de resultados falso-positivos no estudo de ligação primário. Outra explicação plausível para este fenômeno é a de que os valores elevados de LOD encontrados na análise inicial tenham sido influenciados por poucas famílias com um grande número de casos. O número de famílias avaliadas no estudo primário não foi grande (38 famílias nucleares com até 6 filhos afetados) e algumas famílias numerosas podem ter inflado os resultados obtidos nas regiões 1q31-q32 e 18q22-q23. Além disso, é possível que as duas amostras utilizadas nestas análises apresentem diferenças étnicas. A região na

qual as famílias brasileiras foram recrutadas (sul do estado da Bahia) é habitada por indivíduos com diferentes origens étnicas (miscigenação entre nativos americanos, africanos e europeus), que são algumas vezes difíceis de determinar. A região 1q31-q32 chamou a nossa atenção pois ela contém o gene *IL10* e, em um estudo prévio, nosso grupo identificou uma variante de *IL10* que aumenta a predisposição à LC nesta mesma população (SALHI *et al.*, 2008). Embora os marcadores na região 1q31-q32 não apresentaram sinais de ligação significantes com a LC, análises mais detalhadas deste *loci* podem produzir resultados interessantes. A ausência de ligação significante e a demonstração de um sinal de associação não são incompatíveis. De fato, os estudos de associação têm maior poder estatístico para detectar relações fenótipo-genótipo do que as análises de ligação, especialmente para variantes com frequências baixas e com efeitos fracos ou moderados.

Os resultados descritos neste estudo indicam que a região 10q21-23 pode conter um ou mais genes cujas variantes podem afetar o desenvolvimento de lesões cutâneas em pacientes infectados com *L. braziliensis*. Diversos estudos prévios identificaram que variantes genéticas nesta região do cromossomo 10 são associadas ao risco de doenças crônicas não-infecciosas (ABNET *et al.*, 2010; HOU *et al.*, 2014; ZHANG *et al.*, 2014). Contudo, nenhum sinal de ligação ou associação com fenótipos relacionados a doenças parasitárias ou infecciosas foi previamente descrito na região 10q21-q23. Como a nossa análise de ligação não permitiu a localização precisa dos genes envolvidos no sinal da região 10q21-q23, nós decidimos avaliar uma área de aproximadamente 6 cM entre os microssatélites D10S581 e D10S1647 (Figura 10). Com o objetivo de identificar os genes cujas variantes poderiam influenciar a suscetibilidade à LC, os perfis de expressão de todos os genes descritos nesta região foram comparados entre amostras de lesão cutânea (causada por *L. braziliensis*) e de pele normal. Além de 2 genes inibidos, os transcritos de 5 genes [notavelmente *PRF1* (FC = 49.3) e *SRGN* (FC = 21.8)] foram fortemente expressos durante a doença. Apesar de não ser possível excluir definitivamente o envolvimento de outros genes na região 10q21-q23, mutações em *PRF1* e *SRGN* podem afetar o desenvolvimento da LC. A perforina 1 (codificada por *PRF1*) e a serglicina (codificada por *SRGN*) formam um complexo com a granzima B (RAJA *et al.*, 2002), que é essencial para a atividade

citotóxica das células T CD8$^+$ e NK, envolvidas na eliminação de células infectadas com *Leishmania* (JORDAN e HUNTER, 2010; BOGDAN, 2012). Notavelmente, nossa análise de redes de interação baseada nos dados dos transcriptomas evidenciou que diversas moléculas associadas à resposta citotóxica são fortemente induzidas durante a LC. De fato, como previamente descrito por Novais e colaboradores (2014), *GZMB*, que codifica a granzima B, foi um dos genes mais expressos em lesões cutâneas causadas por *L. braziliensis* (FC = 175.6). O complexo perforina 1/granzima B/serglicina fica retido nos grânulos citoplasmáticos de linfócitos citotóxicos (DE SAINT BASILE *et al*., 2010). Esses grânulos são secretados por exocitose e induzem a apoptose das células-alvo. Diversas linhas de evidência sugerem que as células citotóxicas (NK e T CD8$^+$) têm papéis protetores durante as infecções por *Leishmania* (MULLER *et al.*, 1991; UZONNA *et al.*, 2004; BOGDAN, 2012). Esses efeitos protetores podem ser exercidos através de dois mecanismos: (i) pela secreção de citocinas efetoras como IFN-γ e TNF, que auxiliam no desenvolvimento da resposta T_H1 e aumentam a capacidade leishmanicida de macrófagos infectados (KAYE e SCOTT, 2011); e (ii) pela atividade citotóxica, auxiliando na eliminação das células infectadas com *Leishmania* (JORDAN e HUNTER, 2010; BOGDAN, 2012). No entanto, a gravidade da doença cutânea em pacientes infectados com *L. braziliensis* parece estar diretamente associada ao número de células T CD8$^+$ citotóxicas (FARIA *et al.*, 2009; SILVA *et al.*, 2013). De fato, um estudo recente evidenciou que a imunopatologia associada à infecção com *L. braziliensis* é principalmente causada pela atividade citolítica (dependente de perforina) de células T CD8$^+$ (NOVAIS *et al.*, 2013).

Visto isso, nós avaliamos se polimorfismos nos genes *PRF1* e *SRGN* são fatores de risco para a leishmaniose cutânea no Brasil. Nossa busca por mutações em *PRF1* (incluindo a sua região regulatória distal) não resultou em nenhum sinal de associação significante com a LC. Por outro lado, foi evidenciado dois polimorfismos na região do gene *SRGN* (rs10998538 e rs12437) associados à doença em duas coortes brasileiras independentes. Como mostrado na Figura 12A, o SNP rs10998538 captura polimorfismos em uma região a aproximadamente 38 kb 5’ do sítio de iniciação do gene *SRGN*. Além disso, rs12437 pode estar capturando sinais de variantes que afetam os genes *VPS26A* e *SUPV3L1* (Figura 12B). VPS26A pertence a um complexo de

proteínas vacuolares, que formam uma estrutura conhecida como retrômero, envolvido na maturação do endossomo (em células fagocíticas) e no tráfego (reverso) de proteínas do endossomo para o trans-Golgi (HAFT *et al*., 2000; BONIFACINO e HURLEY, 2008). Por outro lado, *SUPV3L1* codifica um helicase dependente de ATP, que pode atuar como sentinela na detecção de moléculas de RNAs estranhas (*nonself*) em vertebrados (RANJI e BORIS-LAWRIE, 2010; STEIMER e KLOSTERMEIER, 2012). Desta forma, variantes dos genes *VPS26A* e *SUPV3L1* podem ter influência sobre o desenvolvimento da LC. Mesmo não sendo possível excluir a participação desses genes com base nas análises de desequilíbrio de ligação, é importante salientar que *VPS26A* e *SUPV3L1* não foram incluídos nos estudos de associação genética, pois as suas expressões não foram moduladas entre amostras de lesão cutânea e de pele normal (Figura 11A). Além disso, o estado de expressão inalterado das moléculas que interagem fisicamente ou que regulam as funções de *VPS26A* ou *SUPV3L1* sustentam a hipótese de que as suas respectivas redes não são ativadas durante a leishmaniose cutânea (Tabela A5, Apêndice).

Estudos utilizando camundongos *srgn*$^{-/-}$ forneceram informações relevantes sobre as funções da serglicina, indicando que esta molécula está envolvida na formação de grânulos intracelulares, armazenamento e secreção de moléculas efetoras em diversas células hematopoiéticas, incluindo neutrófilos, linfócitos, monócitos, macrófagos, megacariócitos, plaquetas e mastócitos (SCULLY *et al*., 2012). De fato, os mastócitos de camundongos *srgn*$^{-/-}$ exibem capacidade anormal de degranulação, caracterizada pela diminuição da secreção de proteases e de histamina (ABRINK *et al*., 2004). Perturbações também foram observadas na composição de grânulos azurofílicos de neutrófilos (GULLBERG *et al*., 1997). A ausência de serglicina também diminui a capacidade de secreção de TNF e de moléculas antimicrobianas por macrófagos (ZERNICHOW *et al*., 2006; KOLSET e ZERNICHOW, 2008). Além disso, foi evidenciado que as células T CD8^{+} deficientes em serglicina exibem maturação incompleta de grânulos secretórios (GRUJIC *et al.*, 2005) e, embora a transcrição de *gzmb* não seja afetada nestas células, a granzima B não é estocada nos grânulos citotóxicos.

Por fim, como as células citotóxicas agem principalmente via secreção de perforina/granzima, nós hipotetizamos que rs10998538 e rs12437 ou outros polimorfismos em desequilíbrio de ligação com eles alteram a disponibilidade de serglicina em células citotóxicas, o que teria impactos na eliminação das células infectadas e/ou no controle da magnitude da imunopatologia nos sítios de infecção por *L. braziliensis*. A degranulação anormal de mastócitos e neutrófilos, além da modulação da secreção de moléculas efetoras por macrófagos, também poderiam afetar o controle do parasito e a progressão da doença. Outros experimentos são necessários para avaliar os impactos destas mutações na região 10q22.1 e para determinar o papel exato da serglicina na suscetibilidade humana à leishmaniose cutânea.

5 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

No Estudo 1 desta tese, foram apresentadas evidências sobre a ativação da via de sinalização IL-2 em lesões cutâneas causadas por *L. braziliensis* e sobre a associação de variantes do gene *IL2RA* com a suscetibilidade à LC em famílias brasileiras. Estas associações genéticas foram confirmadas em uma coorte brasileiria independente e foram extendidas para a LC em iranianos infectados com *L. tropica* (afastando a possibilidade de associações espúrias devido a fatores como acaso ou erros de genotipagem). As análises de desequilíbrio de ligação realizadas no *locus* 10p15.1 indicam que os sinais de associação com a leishmaniose cutânea são relacionados exclusivamente ao gene *IL2RA* (e não a outros genes na região). Ademais, foi demonstrado que uma das variantes de *IL2RA* que aumenta a predisposição à doença cutânea também é associada à redução de respostas dependentes da sinalização IL-2/IL-2R (diminuição da produção de IFN-γ por células mononucleares e diminuição da capacidade de ativação de T_{regs} *in vitro*). Conjuntamente, os nossos resultados indicam que a via de sinalização IL-2 é um fator central na imunidade contra espécies dermotrópicas de *Leishmania*, podendo estar envolvida no controle da carga parasitária e na regulação da imunopatologia associada à infecção. Todavia, experimentos adicionais são necessários para determinar o efeito exato das modulações de IFN-γ e de T_{regs} (além da modulação de outros fatores não avaliados no presente estudo) no desenvolvimento da leishmaniose cutânea. Um análise inicial mostrou que todos os SNPs capturados por rs10905669 e/ou rs706778 encontram-se em regiões não codificantes do *locus* 10p15.1. Selecionar um polimorfismo causal em um região não codificante é bastante difícil, visto que, de modo geral, seus efeitos são associados a modulações sutis na expressão de genes ou na estabilidade de transcritos. Desta forma, é importante a realização de estudos mais aprofundandos visando a identificação dos polimorfismos funcionais e a determinação de seus efeitos sobre o gene *IL2RA*.

No Estudo 2 desta tese, foram apresentados os resultados da primeira análise de ligação genômica (GWL) para a leishmaniose cutânea. Esse estudo revelou um novo *locus* de suscetibilidade (10q21-q23) para a doença em famílias brasileiras expostas à *L. braziliensis*. A análise dos perfis de expressão dos genes da região 10q21-q23 identificou somente 7 genes com expressões moduladas após o desenvolvimento da LC. Interessantemente, as expressões dos genes *PRF1* e *SRGN*, que codificam proteínas cruciais para a resposta citotóxica, foram fortemente induzidas em lesões cutâneas. Por fim, foram identificados dois tag-SNPs (rs10998538 e rs12437) próximos ao gene *SRGN* que são independentemente associados à leishmaniose cutânea no Brasil. Apesar disso, não foi possível excluir (através de análises de DL) a possibilidade de que estes polimorfismos estejam capturando sinais de variantes que afetam outros genes nesta mesma região (como *VPS26A* e *SUPV3L1*). É importante salientar que os resultados obtidos na análise de ligação multiponto podem indicar que os marcadores microssatélites estejam capturando mais de um sinal na região 10q21-q23. Por se tratar de um intervalo relativamente curto, é improvável que este fenômeno seja explicado pelas associações capturadas independentemente por rs10998538 e rs12437. Desta forma, é possível que mutações funcionais (comuns ou raras) que afetam outros genes (que não passaram nos critérios de seleção do presente estudo) também estejam contribuindo para o sinal de ligação da região 10q21-q23. Além disso, não é possível excluir a contribuição de mutações que afetam genes que não codificam proteínas (não avaliados em nosso estudo). Assim, o sequenciamento da região 10q21-q23 (capturando também os polimorfismos raros) nos pacientes afetados pela LC será importante para a identificação de outras variantes genéticas que influenciam a predisposição à doença.

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APÊNDICE

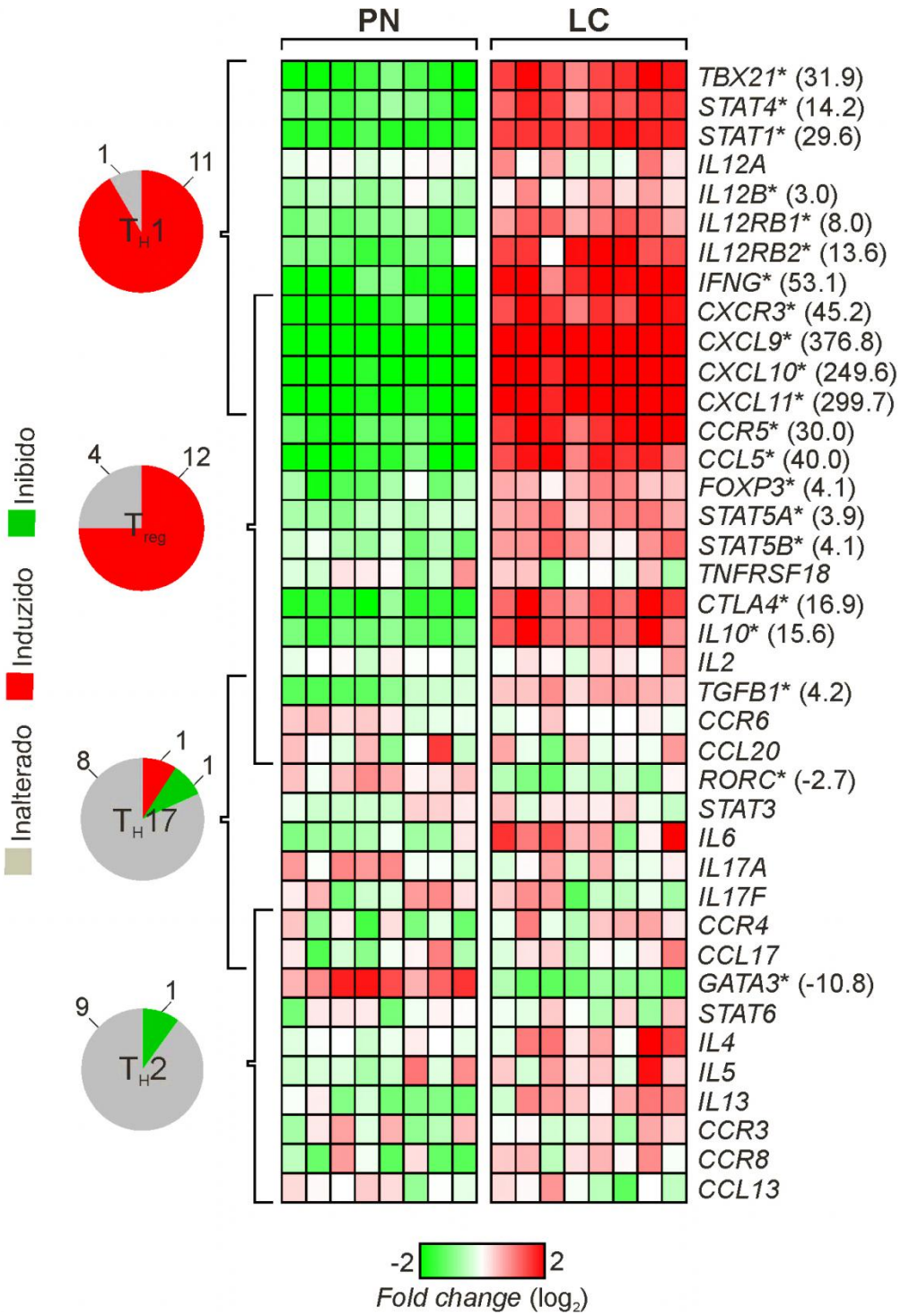

Figura A1. *Heat map* mostrando os perfis de expressão de fatores-chave envolvidos na diferenciação, migração e função de subclasses de células T CD4⁺. Os valores entre parênteses representam a diferença média de expressão (*fold change*) entre lesões cutâneas (LC; *n* = 8) e amostras de pele normal (PN; *n* = 8). Os asteriscos indicam que a expressão do gene foi significativamente modulada entre os grupos.

Tabela A1. Número de filhos afetados pela leismaniose cutânea (estudos de associação)

Número de filhos (LC) por família	Coortes brasileiras – Estudos de associação		TOTAL
	Primária (Brasil 1)[a]	Replicação (Brasil 2)[b]	
1	125	34	159
2	51	20	71
3	26	17	43
4	4	5	9
5	3	4	7
TOTAL	209	80	289

LC: leishmaniose cutânea causada por *Leishmania braziliensis*.
[a]Coorte recrutada no estado da Bahia; [b]Coorte recrutada no estado de Pernambuco.

Tabela A2. Tag-SNPs em genes da via de sinalização IL-2 avaliados no Estudo 1 (análise primária)

	Tag-SNP	Coordenada	Alelos		Frequência[Alt]			Localização
			Ref	Alt	AMR	AFR	EUR	
IL2								
	rs11575812	123371049	A	G	0.17	0.13	0.27	1.6kb 3' *IL2*
	rs11575810	123371292	G	C	0.05	0.00	0.08	1.3kb 3' *IL2*
	rs2069772	123373133	T	C	0.17	0.03	0.30	Íntron 3 *IL2*
	rs2069762	123377980	A	C	0.30	0.05	0.30	99bp 5' *IL2*
	rs4833248	123380405	G	A	0.30	0.05	0.30	2.5kb 5' *IL2*
IL2RA								
	rs10795737	6049344	C	T	0.22	0.23	0.22	3.3kb 3' *IL2RA*
	rs12359875	6051107	C	T	0.16	0.02	0.28	1.5kb 3' *IL2RA*
	rs12722605	6053163	T	A	0.10	0.01	0.19	3'-UTR *IL2RA*
	rs12244380	6053374	A	G	0.45	0.31	0.43	3'-UTR *IL2RA*
	rs12722710	6055280	T	C	0.02	0.15	0.00	Íntron 7 *IL2RA*
	rs9663421	6055604	C	T	0.35	0.09	0.31	Íntron 7 *IL2RA*
	rs2386841	6057732	G	T	0.33	0.19	0.17	Íntron 7 *IL2RA*
	rs4749894*	6058323	A	G	0.22	0.02	0.29	Íntron 7 *IL2RA*
	rs7899538	6059898	C	A	0.08	0.04	0.11	Íntron 7 *IL2RA*
	rs12722588	6060433	C	T	0.11	0.21	0.17	Íntron 6 *IL2RA*
	rs7069976	6061277	G	A	0.99	0.87	1.00	Íntron 6 *IL2RA*
	rs2274037	6062108	G	A	0.18	0.06	0.04	Íntron 4 *IL2RA*
	rs2076846	6063253	A	G	0.20	0.14	0.35	Íntron 4 *IL2RA*
	rs7093069	6063319	C	T	0.11	0.21	0.17	Íntron 4 *IL2RA*
	rs942200	6063674	T	C	0.96	0.65	0.98	Íntron 3 *IL2RA*
	rs12722574	6066462	G	A	0.15	0.45	0.19	Íntron 2 *IL2RA*
	rs2025345	6067688	A	G	0.49	0.12	0.39	Íntron 2 *IL2RA*
	rs2228150	6067969	C	T	0.04	0.24	0.02	Íntron 1 *IL2RA*
	rs7078547	6068547	G	C	1.00	0.91	1.00	Íntron 1 *IL2RA*
	rs12722563	6069561	G	A	0.05	0.00	0.10	Íntron 1 *IL2RA*
	rs12722561	6069893	C	T	0.07	0.12	0.13	Íntron 1 *IL2RA*
	rs4749920	6071453	T	C	0.15	0.13	0.25	Íntron 1 *IL2RA*
	rs7910961	6077796	T	C	0.55	0.81	0.64	Íntron 1 *IL2RA*
	rs6602392	6078079	C	A	0.14	0.23	0.12	Íntron 1 *IL2RA*
	rs11256448*	6079479	A	G	0.18	0.16	0.27	Íntron 1 *IL2RA*
	rs7072398	6079846	G	A	0.59	0.67	0.49	Íntron 1 *IL2RA*
	rs12722518	6080637	A	C	0.04	0.09	0.05	Íntron 1 *IL2RA*
	rs12722515	6081230	C	A	0.08	0.07	0.14	Íntron 1 *IL2RA*
	rs791591	6081924	A	C	0.96	0.84	0.95	Íntron 1 *IL2RA*

(continua)

Tabela A2. Continuação

rs4749924	6082396	A	C	0.28	0.03	0.33	Íntron 1 *IL2RA*
rs6602398	6082953	G	T	0.28	0.03	0.33	Íntron 1 *IL2RA*
rs11598648	6084025	G	A	0.31	0.56	0.43	Íntron 1 *IL2RA*
rs12243643	6084608	C	T	0.01	0.08	0.00	Íntron 1 *IL2RA*
rs4749926	6085312	G	A	0.52	0.30	0.40	Íntron 1 *IL2RA*
rs706780	6087026	A	C	0.91	0.88	0.95	Íntron 1 *IL2RA*
rs11256497	6087794	G	A	0.33	0.09	0.41	Íntron 1 *IL2RA*
rs791587	6088699	A	G	0.54	0.37	0.49	Íntron 1 *IL2RA*
rs12722641	6088733	T	C	0.00	0.07	0.00	Íntron 1 *IL2RA*
rs791589	6089571	G	A	0.69	0.30	0.87	Íntron 1 *IL2RA*
rs10905669	6092093	C	T	0.33	0.06	0.24	Íntron 1 *IL2RA*
rs1323658	6094354	A	C	0.06	0.22	0.06	Íntron 1 *IL2RA*
rs12722498	6095836	A	G	0.17	0.03	0.03	Íntron 1 *IL2RA*
rs2256867	6096512	T	G	0.99	0.85	1.00	Íntron 1 *IL2RA*
rs2256774	6097165	T	C	0.49	0.43	0.38	Íntron 1 *IL2RA*
rs706779	6098824	T	C	0.58	0.49	0.49	Íntron 1 *IL2RA*
rs706778	6098949	C	T	0.52	0.48	0.41	Íntron 1 *IL2RA*
rs2104286	6099045	T	C	0.13	0.03	0.22	Íntron 1 *IL2RA*
rs3118470	6101713	G	A	0.17	0.04	0.30	Íntron 1 *IL2RA*
rs12722486	6103762	C	T	0.06	0.03	0.05	Íntron 1 *IL2RA*
rs7072793	6106266	C	T	0.52	0.33	0.42	1.9kb 5' *IL2RA*
rs7073236	6106552	T	C	0.53	0.45	0.42	2.2kb 5' *IL2RA*
rs10795791	6108340	A	G	0.51	0.28	0.42	4kb 5' *IL2RA*
rs4147359	6108439	G	A	0.36	0.28	0.35	4.1kb 5' *IL2RA*
IL2RB							
rs3218343	37520757	A	C	0.00	0.06	0.00	1.1kb 3' *IL2RB*
rs3218339	37521211	C	T	0.09	0.07	0.12	660bp 3' *IL2RB*
rs3218329	37523692	T	G	0.01	0.30	0.00	3'-UTR *IL2RB*
rs3218327	37524044	G	A	0.02	0.17	0.00	3'-UTR *IL2RB*
rs228942	37524619	G	T	0.30	0.08	0.19	Éxon 10^{mis} *IL2RB*
rs84459	37525320	T	C	0.42	0.32	0.30	Íntron 9 *IL2RB*
rs228945	37525880	T	C	0.43	0.57	0.30	Íntron 9 *IL2RB*
rs228947	37526529	G	A	0.37	0.23	0.26	Íntron 9 *IL2RB*
rs3218322	37528098	C	T	0.20	0.13	0.28	Íntron 9 *IL2RB*
rs3218318	37528576	A	G	0.22	0.32	0.30	Íntron 8 *IL2RB*
rs3218316	37529348	C	T	0.05	0.15	0.09	Íntron 8 *IL2RB*
rs3218315	37529724	G	A	0.39	0.22	0.33	Íntron 8 *IL2RB*
rs228953	37531436	G	A	0.43	0.38	0.42	Éxon 8^{syn} *IL2RB*
rs3218303	37531968	C	T	0.01	0.09	0.00	Íntron 7 *IL2RB*
rs228954	37532665	T	C	0.44	0.48	0.42	Íntron 6 *IL2RB*

(continua)

Tabela A2. Continuação

rs3218295	37533068	C	T	0.08	0.36	0.13	Íntron 6 *IL2RB*
rs228956	37533143	T	C	0.01	0.14	0.00	Íntron 6 *IL2RB*
rs3218294	37533286	C	G	0.00	0.05	0.00	Íntron 6 *IL2RB*
rs2284033	37534034	G	A	0.43	0.37	0.42	Íntron 5 *IL2RB*
rs228959	37534734	G	A	0.00	0.09	0.00	Íntron 5 *IL2RB*
rs3218286	37535779	G	A	0.00	0.12	0.00	Íntron 4 *IL2RB*
rs228963	37535948	A	G	0.44	0.71	0.41	Íntron 4 *IL2RB*
rs228965	37537058	C	G	0.45	0.83	0.41	Íntron 4 *IL2RB*
rs228966	37537514	T	C	0.45	0.73	0.41	Íntron 4 *IL2RB*
rs3218276	37538140	G	A	0.01	0.06	0.00	Íntron 4 *IL2RB*
rs1003694	37539128	C	T	0.30	0.33	0.28	Íntron 3 *IL2RB*
rs2235330	37539713	T	C	0.28	0.09	0.22	Íntron 2 *IL2RB*
rs228971	37540122	C	T	0.01	0.12	0.00	Íntron 2 *IL2RB*
rs228973	37541812	A	G	0.44	0.31	0.35	Íntron 1 *IL2RB*
rs228975	37542201	G	A	0.46	0.41	0.37	Íntron 1 *IL2RB*
rs2284034	37542762	G	A	0.42	0.13	0.34	Íntron 1 *IL2RB*
rs3218258	37544245	G	A	0.16	0.14	0.28	Íntron 1 *IL2RB*
rs3218252	37544917	C	T	0.57	0.46	0.45	Íntron 1 *IL2RB*
rs228979	37544931	T	G	0.76	0.96	0.72	Íntron 1 *IL2RB*
rs228980	37545797	T	C	0.02	0.12	0.00	Íntron 1 *IL2RB*
rs229498*	37567030	T	G	0.94	0.61	0.97	4.1kb 5' *IL2RB*
JAK3							
rs2382987	17934018	G	A	0.36	0.40	0.27	1.6kb 3' *JAK3*
rs3761049	17935253	G	A	0.15	0.23	0.17	335bp 3' *JAK3*
rs3008	17937429	A	G	0.54	0.70	0.46	3'-UTR *JAK3*
rs3212799	17937516	C	T	0.01	0.05	0.00	3'-UTR *JAK3*
rs3212798	17937758	C	A	0.14	0.45	0.15	Íntron 23 *JAK3*
rs3212797	17937786	C	T	0.12	0.45	0.11	Íntron 23 *JAK3*
rs7255931	17938844	C	G	0.18	0.21	0.20	Íntron 23 *JAK3*
rs10419991	17938891	A	G	0.45	0.70	0.43	Íntron 23 *JAK3*
rs2302600	17941143	A	C	0.37	0.28	0.31	Íntron 22 *JAK3*
rs2072496	17946054	G	A	0.07	0.08	0.11	Íntron 14 *JAK3*
rs3212760	17947546	A	G	0.34	0.33	0.38	Íntron 13 *JAK3*
rs3212753	17948658	T	C	0.03	0.23	0.00	Íntron 12 *JAK3*
rs3212752	17948732	T	C	0.05	0.25	0.09	Íntron 12 *JAK3*
rs3212741	17951178	G	A	0.18	0.01	0.24	Íntron 8 *JAK3*
rs3212722	17954389	C	T	0.01	0.12	0.00	Íntron 3 *JAK3*
rs3212714	17954947	G	A	0.37	0.44	0.30	Íntron 2 *JAK3*
rs3212713	17955001	C	T	0.38	0.52	0.30	Íntron 2 *JAK3*
rs3212701	17957024	C	T	0.30	0.55	0.25	Íntron 1 *JAK3*

(continua)

Tabela A2. Continuação

rs2110586*	17957309	T	C	0.93	0.91	0.83	Íntron 1 *JAK3*
rs7250423	17958617	C	T	0.14	0.57	0.07	Íntron 1 *JAK3*
rs13345965	17959012	C	T	0.07	0.23	0.06	131bp 5' *JAK3*
STAT5B* e *STAT5A							
rs17500235	40356360	T	G	0.04	0.01	0.07	Íntron 17 *STAT5B*
rs9897531	40362708	A	T	0.01	0.06	0.00	Íntron 15 *STAT5B*
rs7209095	40375205	C	G	0.04	0.46	0.00	Íntron 5 *STAT5B*
rs7213630*	40375658	C	T	0.01	0.06	0.00	Íntron 4 *STAT5B*
rs9900213	40375881	G	T	0.13	0.61	0.14	Íntron 4 *STAT5B*
rs6503691	40394090	C	T	0.11	0.65	0.10	Íntron 1 *STAT5B*
rs8081929	40405571	A	C	0.01	0.10	0.00	Íntron 1 *STAT5B*
rs6503692	40421513	G	A	0.19	0.56	0.29	Íntron 1 *STAT5B*
rs7212173	40423496	T	C	0.02	0.21	0.00	Íntron 1 *STAT5B*
rs9907247	40425876	G	A	0.16	0.21	0.29	Íntron 1 *STAT5B*
rs7220367	40435113	C	G	0.03	0.37	0.00	4.5kb 5' *STAT5A*
rs8067443	40441819	A	G	0.03	0.36	0.00	Íntron 3 *STAT5A*
rs8077372	40443461	A	G	0.02	0.19	0.00	Íntron 4 *STAT5A*
rs16967637	40446422	C	A	0.17	0.40	0.29	Íntron 5 *STAT5A*
rs7217728	40447401	T	C	0.19	0.63	0.29	Íntron 5 *STAT5A*
rs9906989	40455846	G	T	0.10	0.18	0.19	Íntron 10 *STAT5A*
rs2293155	40460989	A	G	0.10	0.27	0.19	Íntron 17 *STAT5A*
rs12601982	40461674	A	G	0.10	0.28	0.19	Íntron 19 *STAT5A*

Coordenada: pares de base – GRCh37 (hg19); Ref: Alelo de referência; Alt: Alelo alterado; AMR: americanos; AFR: africanos; EUR: europeus – projeto 1000 genomas (*phase 1*).
*SNPs excluídos após controle de qualidade (como descrito em Pacientes, material e métodos).
Mutação: ^{mis}missense; ^{syn}sileciosa (*synonimous*).

Tabela A3. Tag-SNPs nas regiões dos genes *PRF1* e *SRGN* avaliados no Estudo 2 (análise primária)

	Tag-SNP	Coordenada	Alelos		Frequência[Alt]			Localização
			Ref	Alt	AMR	AFR	EUR	
PRF1								
	rs1413710	72346471	A	C	0.49	0.56	0.37	11kb 3' *PRF1*
	rs12765134	72351827	C	T	0.10	0.03	0.15	5kb 3' *PRF1*
	rs885822	72358577	G	A	0.63	0.83	0.60	Éxon 3[syn] *PRF1*
	rs885821	72358655	G	A	0.11	0.05	0.21	Éxon 3[syn] *PRF1*
	rs1641661	72365130	G	A	0.33	0.25	0.37	3kb 5' *PRF1*
	rs74233701*	72370044	G	T	0.06	0.00	0.00	8kb 5' *PRF1*
	rs10823592	72412121	T	G	0.15	0.07	0.25	50kb 5' *PRF1*[reg]
	rs10999456	72413827	C	T	0.17	0.40	0.23	52kb 5' *PRF1*[reg]
	rs12359330	72414845	C	T	0.16	0.22	0.23	53kb 5' *PRF1*[reg]
	rs2184389	72415016	A	G	0.35	0.31	0.49	53kb 5' *PRF1*[reg]
SRGN								
	rs12412173	70808484	A	G	0.15	0.14	0.07	39kb 5' *SRGN*
	rs10998538	70808691	C	G	0.13	0.08	0.14	39kb 5' *SRGN*
	rs7896490*	70811554	T	C	0.96	0.76	0.99	36kb 5' *SRGN*
	rs10998540	70811779	C	T	0.13	0.02	0.13	36kb 5' *SRGN*
	rs10823282	70811852	G	A	0.22	0.20	0.30	36kb 5' *SRGN*
	rs2394525	70812408	C	T	0.77	0.69	0.70	35kb 5' *SRGN*
	rs10823283	70813238	C	T	0.30	0.24	0.38	34kb 5' *SRGN*
	rs10998544	70813802	G	A	0.23	0.14	0.30	33kb 5' *SRGN*
	rs12267089	70816882	G	A	0.02	0.14	0.00	30kb 5' *SRGN*
	rs2001758	70817975	C	T	0.98	0.86	1.00	29kb 5' *SRGN*
	rs10823287	70822574	A	T	0.35	0.47	0.42	24kb 5' *SRGN*
	rs17557564	70823662	C	T	0.04	0.02	0.09	23kb 5' *SRGN*
	rs7909789	70823831	A	G	0.32	0.34	0.41	23kb 5' *SRGN*
	rs11816061*	70823953	A	G	0.15	0.12	0.24	23kb 5' *SRGN*
	rs2394527	70825489	G	A	0.18	0.15	0.25	21kb 5' *SRGN*
	rs10509309	70827281	T	C	0.34	0.42	0.32	19kb 5' *SRGN*
	rs6480379	70827582	C	T	0.55	0.64	0.37	19kb 5' *SRGN*
	rs12412740*	70827782	G	A	0.10	0.02	0.08	18kb 5' *SRGN*
	rs2001760	70835346	G	A	0.23	0.16	0.34	10kb 5' *SRGN*
	rs10998564	70843891	G	A	0.35	0.30	0.34	2kb 5' *SRGN*
	rs10823297	70845091	T	C	0.05	0.17	0.03	1kb 5' *SRGN*
	rs4745976	70845181	A	G	0.74	0.75	0.88	1kb 5' *SRGN*
	rs2855022	70849959	G	A	0.49	0.21	0.58	Íntron 1 *SRGN*

(continua)

Tabela A3. Continuação

rs2855024	70851106	G	A	0.12	0.00	0.19	Íntron 1 *SRGN*
rs2805907	70856455	G	A	0.49	0.19	0.58	Íntron 1 *SRGN*
rs2805910	70856852	G	A	0.65	0.31	0.85	Éxon 2^{mis} *SRGN*
rs17558323	70857810	C	G	0.12	0.02	0.27	Íntron 2 *SRGN*
rs2008541	70860599	A	G	0.75	0.64	0.89	Íntron 2 *SRGN*
rs2805915	70861976	A	G	0.47	0.21	0.54	Íntron 2 *SRGN*
rs12437	70864065	T	C	0.73	0.58	0.89	3'-UTR *SRGN*
rs10998577	70867173	G	A	0.47	0.21	0.55	3kb 3' *SRGN*
rs7915836	70870281	G	T	0.06	0.22	0.00	6kb 3' *SRGN*
rs10998579	70871082	A	G	0.07	0.04	0.07	7kb 3' *SRGN*
rs9299502*	70876934	C	T	0.72	0.58	0.87	13kb 3' *SRGN*
rs12774307	70878347	G	A	0.14	0.08	0.26	14kb 3' *SRGN*

Coordenada: pares de base – GRCh37 (hg19); Ref: alelo de referência; Alt: alelo alterado; AMR: americanos; AFR: africanos; EUR: europeus – projeto 1000 genomas (*phase 1*).
*SNPs excluídos após controle de qualidade (como descrito em Pacientes, material e métodos).
Mutação: ^{mis}missense; ^{syn}sileciosa (*synonimous*).
^{reg}Região regulatória distal.

Tabela A4. Expressão de moléculas associadas aos genes modulados no *locus* 10q21-q23

SRGN/PRF1		***SAR1A***		***PSAP***		***USP54***		***SLC29A3***		***TTC18***	
Gene	**FC**	**Gene**	**FC**	**Gene**	**FC**	**Gene**	**FC**	**Gene**	**FC**	**Gene**	**FC**
*GZMB**	175.6	*FN1*	3.6	*MRC1*	3.0	*LATS2*	1.7	*SLC6A12**	17.8	*CCDC65*	1.8
*GZMA**	51.1	*SAR1A**	2.7	*PSAP**	2.8	*CHMP5*	1.4	*TIMP1**	11.9	*TTC21A*	1.7
*PRF1**	49.3	*TRAF3*	1.6	*SGK223*	1.9	*CHMP4B*	1.2	*SLC29A3**	4.9	*TMEM200A*	1.3
*MMP9**	46.2	*GNPTAB*	1.3	*CTSD*	1.7	*CHMP6*	1.2	*CD63**	2.6	*WDR66*	1.2
*TBX21**	31.9	*TRAF6*	1.3	*LRP1*	1.3	*YWHAB*	1.1	*SLC6A14*	2.3	*MRPS15*	1.1
*SRGN**	21.8	*IKBKE*	1.3	*GBA*	1.3	*CHMP4A*	1.0	*SELP*	1.8	*GFM1*	1.1
*SERPINB9**	15.5	*ELAVL1*	1.3	*USP4*	1.3	*ATXN1*	1.0	*MBNL1*	1.6	*TUFM*	1.1
*FBXO6**	6.3	*UBC*	1.1	*COPS6*	1.1	*CHMP2A*	-1.1	*PHF1*	1.6	*MRPS22*	-1.1
*PTAFR**	5.5	*ATXN1*	1.0	*ZBED1*	1.0	*MYC*	-1.1	*SLC6A16*	1.3	*CCDC39*	-1.1
*BID**	5.1	*METTL21B*	-1.0	*MAFF*	1.0	*ATXN1L*	-1.2	*SLC28A2*	1.1	*RPS20*	-1.2
*STAT5B**	4.1	*EIF1B*	-1.1	*CD1D*	-1.1	*ATN1*	-1.2	*HNRNPH1*	-1.0	*MRPS9*	-1.3
*FLI1**	3.8	*DCTN1*	-1.3	*GPR37*	-1.1	*NR3C1*	-1.3	*HMGN3*	-1.1	*MRPS16*	-1.4
*CASP3**	2.7	*VHL*	-1.3	*TFEB*	-1.2	*CHMP2B*	-1.3	*SLC6A13*	-1.1	*ZMYND17*	-1.4
CALR	2.5	*APP*	-1.4	*BACE1*	-1.3	*CHMP1A*	-1.6	*SLC6A15*	-1.5	*C6orf48*	-1.5
ETS1	2.5	*EPB41*	-1.5	*SMAD2*	-1.5	*CHMP1B*	-2.0	*LAMP2*	-1.8	*DDR1*	-2.6
*ELF4**	2.4	*PEX3*	-1.8	*CELSR1*	-1.7	*CHMP4C*	-2.5	*SLC6A9*	-2.8	*CCDC113*	-4.1
M6PR	2.4	*MCC*	-2.0	*UBE3A*	-1.7	*USP54**	-2.8			*TTC18**	-4.6
CASP9	2.3	*KCNH2*	-2.9	*CFTR*	-1.7						
CRELD2	2.2	*TTR*	-3.9	*SMAD9*	-1.9						
IGF2R	2.2			*GPR37L1*	-2.1						
PSRC1	2.0			*GHR*	-4.0						
PARP1	1.9			*CHAT*	-5.3						
SGTA	1.2			*SGCG**	-14.2						
LAMP1	1.2			*PSAPL1**	-32.8						
BAG6	1.2										
TRIM54	1.1										

(continua)

Tabela A4. Continuação

AP1G1	1.0
DDX24	-1.1
UBQLN4	-1.4
CEP70	-2.3

As redes de interação dos genes *SRGN*/*PRF1*, *SAR1A*, *SLC29A3*, *PSAP*, *TTC18* e *USP54* (sublinhados) foram construídas com base em informações sobre interação/regulação entre genes/proteínas, disponibilizadas pelos bancos de dados Ingenuity e String. A análise de significância para microarranjos (SAM) foi utilizada para a identificação de genes diferencialmente expressos entre lesões cutâneas causadas por *Leishmania braziliensis* (*n* = 8) e amostras de pele normal de doadores não infectados (*n* = 8) [FDR < 0.001%; *fold change* (FC) ≥ 2.0].
*Genes diferencialmente expressos entre os grupos (fenótipo de referência: lesão cutânea).

Tabela A5. Expressão de moléculas associadas aos genes *VPS26A* ou *SUPV3L1*

Rede *VPS26A*			Rede *SUPV3L1*		
Gene	**FC**	**SAM (FDR < 0.001%)**	**Gene**	**FC**	**SAM (FDR < 0.001%)**
SORL1	2.4	NS	*HNF4A*	1.6	NS
IGF2R	2.2	NS	*XRN1*	1.5	NS
SNX2	1.4	NS	*LSM7*	1.5	NS
GUSB	1.3	NS	*EXOSC4*	1.4	NS
VPS29	1.2	NS	*EXOSC10*	1.3	NS
UBC	1.1	NS	*EXOSC3*	1.3	NS
SNX27	1.1	NS	*EXOSC1*	1.3	NS
MUL1	1.0	NS	*ELAVL1*	1.3	NS
RAB5A	-1.0	NS	*EXOSC9*	1.2	NS
RAB7A	-1.1	NS	*EXOSC8*	1.2	NS
VPS35	-1.2	NS	*C1D*	1.1	NS
HP1BP3	-1.2	NS	*ICT1*	1.1	NS
BACE1	-1.3	NS	*PNPT1*	-1.1	NS
TBC1D5	-1.3	NS	*DIS3L*	-1.2	NS
APP	-1.4	NS	*SUPV3L1*	-1.2	NS
SLC11A2	-1.4	NS	*EXOSC6*	-1.2	NS
KIAA0368	-1.4	NS	*HNF1A*	-1.4	NS
HECTD1	-1.6	NS	*EXOSC2*	-1.4	NS
VPS26B	-1.7	NS	*EXOSC5*	-1.5	NS
EIF3L	-1.7	NS	*UPF2*	-1.5	NS
VPS26A	-1.7	NS	*CUL3*	-1.8	NS
SNX1	-1.8	NS	*EXOSC7*	-2.1	NS
RYK	-2.0	NS	*DIS3*	-2.3	NS
SLC2A1	-2.1	NS			

As redes de interação dos genes *VPS26A* e *SUPV3L1* (sublinhados) foram construídas com base em informações sobre interação/regulação entre genes/proteínas, disponibilizadas pelos bancos de dados Ingenuity e String. A análise de significância para microarranjos (SAM) foi utilizada para a identificação de genes diferencialmente expressos entre lesões cutâneas causadas por *Leishmania. braziliensis* (*n* = 8) e amostras de pele normal de doadores não infectados (*n* = 8) [FDR < 0.001%; *fold change* (FC) ≥ 2.0]. NS: não significante.

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Instituições participantes

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Fundação Oswaldo Cruz, Centro de Pesquisas Aggeu Magalhães (CPqAM). Av. Professor Moraes Rego, s/n - Campus da UFPE - Cidade Universitária, Recife/PE – Brasil, CEP: 50.740-465.

Faculdade de Medicina, Universidade Aix-Marseille, INSERM UMR 906. 27 Bd Jean Moulin, Marseille. 1385 CEDEX 05, França.

Título do projeto: Genética da leishmaniose cutânea em humanos.

Pesquisadores responsáveis:

Dr. Lain Carlos Pontes de Carvalho (CPqGM)
Dr.ª Valéria Pereira (CPqAM)
Dr. Alain Dessein (INSERM UMR 906)

Como voluntário, o Sr.(a) está sendo convidado para participar de uma pesquisa da Fundação Oswaldo Cruz (FIOCRUZ) e do Instituto Nacional de Pesquisa Médica e da Saúde da França (INSERM), que tem como objetivo identificar os fatores genéticos que agravam a doença causada por *Leishmania*. A *Leishmania* é um parasita do homem que pode causar lesões na pele, conhecida como leishmaniose cutânea. O Sr.(a) está sendo convidado para participar deste estudo por se encontrar em uma das situações a seguir:

A) Tem a leishmaniose cutânea;
B) Teve a leishmaniose cutânea;
C) É pai ou mãe de uma pessoal que tem ou teve a leishmaniose cutânea; ou
D) Vive há mais 5 anos em uma região onde muitas pessoas ao seu redor têm ou tiveram a leishmaniose cutânea.

Se concordar em participar deste estudo, serão solicitadas algumas informações a respeito da sua saúde e a doação de 5 mL de sangue (equivalente ao volume de uma colher de chá). Em alguns casos, médicos poderão coletar biópsias (pequenos fragmentos) das lesões. O risco associado à retirada de sangue ou da biópsia da lesão limita-se a um pequeno desconforto no local da coleta. O material será utilizado para estudar os genes que podem aumentar o risco de desenvolver a leishmaniose cutânea.

A sua participação neste estudo não trará benefícios a curto prazo para o seu problema. Entretanto, o conhecimento obtido a partir destes testes poderá contribuir para um melhor conhecimento da resistência humana à *Leishmania*. A sua identidade será preservada e nenhum resultado obtido com esta pesquisa informará o seu nome ou qualquer outra informação pessoal. Os resultados desta pesquisa serão publicados em revistas científicas. Os pesquisadores responsáveis estarão à sua disposição para esclarecer qualquer dúvida que o Sr.(a) tenha em relação a este projeto. O Sr.(a) poderá recusar-se a participar do estudo agora, ou em qualquer momento, sem que isto lhe traga qualquer constrangimento ou penalidade.

Os pesquisadores responsáveis declaram ainda que a amostra de sangue poderá ser analisada na França. A amostra de sangue será armazenada por até cinco anos após a coleta e não poderá ser utilizada para qualquer outro fim, ao não ser para os objetivos citados acima. Para utilizar a amostra de sangue por um período maior do que cinco anos ou para outros estudos, os pesquisadores responsáveis deverão solicitar autorização junto às comissões de ética no Brasil e na França.

Se o Sr.(a) concorda em participar deste estudo, por favor, assine abaixo.

Declaro que li este termo de consentimento e que concordei, por livre e espontânea vontade, em participar desta pesquisa.

Nome: _____ RG: _____

Assinatura: _____

Local: _____ Data: _____

ARTIGOS PUBLICADOS OU ACEITOS PARA PUBLICAÇÃO

Artigo 1: Oliveira, P., H. Dessein, A. Romano, S. Cabantous, M. E. F. Brito, F. Santoro, M. G. R. Pitta, Pereira, V., L. C. Pontes-de-Carvalho, V. Rodrigues Jr, S. Rafati, L. Argiro and D. Alain. *IL2RA* genetic variants reduce IL-2-dependent responses and aggravate human cutaneous leishmaniasis. (2015) *The Journal of Immunology*. [Epub ahead of print] doi: 10.4049/jimmunol.1402047.

Artigo 2: Sertorio, M., X. Hou, R. F. Carmo, H. Dessein, S. Cabantous, M. Abdelwahed, A. Romano, F. Albuquerque, L. Vasconcelos, T. Carmo, J. Li, A. Varoquaux, V. Arnaud, P. Oliveira, A. Hamdoun, H. He, S. Adbelmaboud, A. Mergani, J. Zhou, A. Monis, L. B. Pereira, P. Halfon, M. Bourlière, R. Parana, M. Dos Reis, D. Gonnelli, P. Moura, N. E. Elwali, L. Argiro, Y. Li, and A. Dessein. Interleukin-22 and IL-22 binding protein (IL-22BP) regulate fibrosis and cirrhosis in hepatitis C virus and schistosome infections. (2014) *Hepatology*. [Epub ahead of print] doi: 10.1002/hep.27629.

Artigo 3: Cronemberger-Andrade, A., L. Aragão-França, C. F. de Araujo, V. J. Rocha, C. M. Borges-Silva, C. P. Figueiras, P. R. Oliveira, L. A. de Freitas, P. S. Veras, and L. Pontes-de-Carvalho. Extracellular vesicles from *Leishmania*-infected macrophages confer an anti-infection cytokine-production profile to naïve macrophages. (2014) *PLoS Neglected Tropical Diseases*. 8(9): e3161. doi: 10.1371/journal.pntd.0003161.

Artigo 4: Romano, A., X. Hou, M. Sertorio, H. Dessein, S. Cabantous, P. Oliveira, J. Li, S. Oyegué, V. Arnaud, X. Luo, M. Chavanieu, O. Mariani, X. Sastre, A. M. Dombey, H. He, Y. Li, and A. Dessein. FOXP3^{+} regulatory T cells in hepatic fibrosis and splenomegaly caused by *Schistosoma japonicum*. The spleen may be a major source of T_{regs}. (2015) *The Journal of Immunology*. Submetido.

Artigo 5: Cabral-Miranda, G., J. R. de Jesus, P. R. Oliveira, G. S. Britto, L. C. Pontes-de-Carvalho, R. F. Dutra, and N. M. Alcântara-Neves. Detection of parasite antigens in *Leishmania infantum*-infected spleen tissue by monoclonal antibody-, piezoelectric-based immunosensors. (2014) *Journal of Parasitology*. 100(1): 73–78. doi: 10.1645/GE-3052.1.

Artigo 6: Silva, V. M. G., C. F. De-Araújo, I. C. Navarro, P. R. S. Oliveira, and L. Pontes-de-Carvalho. Antibody markers of infection susceptibility in *Leishmania* extract-injected mice with experimental cutaneous leishmaniasis. (2015) *BMC Research Notes*. Aceito para publicação.

The Journal of Immunology

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

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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. Pitta,*,† 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* rs10905669 T ($P_{combined} = 6 \times 10^{-7}$) and *IL2RA* rs706778 T ($P_{combined} = 2 \times 10^{-9}$) 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). *Leishmania* is transmitted to humans by phlebotomine sandflies and causes a wide spectrum of clinical manifestations, from severe visceral disease (kala-azar [KA]) to cutaneous lesions (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

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The microarray data in this article have been submitted to the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/ under accession number GSE63931.

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Abbreviations used in this article: CL, cutaneous leishmaniasis; eTreg, effector regulatory T; KA, kala-azar; rTreg, resting regulatory T; SNP, small nucleotide polymorphism; Treg, regulatory T.

(CD122, encoded by *IL2RB*); and γ_c (CD132, encoded by *IL2RG*) (15). IL-2R is abundant only on Treg cells and on T lymphocytes recently activated by Ag (16).

Our results for KA led us to evaluate whether the IL-2 pathway was activated in *Leishmania*-infected human tissues. We performed this analysis on skin biopsies from *L. braziliensis*–infected patients and found that several genes of the IL-2 pathway were highly transcribed in these cutaneous lesions. This observation led us to perform a genetic analysis of key genes of the IL-2 pathway. We found that polymorphisms of the *IL2RA* gene were associated 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 $\geq$ 8.0).

Sample amplification, 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/ under accession no. GSE63931). A two-class unpaired 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 $\geq$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 areas 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 Cortês) 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 $\geq$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 $r^2 \geq 0.8$ 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 Biosciences). A FITC–anti-CD45RA Ab (HI100) was used to discriminate

between effector Treg (eTreg) 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 χ^2 tests (also under additive, dominant, and recessive genetic models). The significance threshold applied in our replication/extension 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 was carried out with PLINK software. Odds ratios and SE values were obtained for the SNPs tested by converting the Brazilian samples into a population-based format (using only founders). Linear regression analysis and group comparisons (two-tailed, nonparametric test) were carried out with SPSS software (IBM).

Results

The IL-2 pathway is activated in skin ulcers caused by L. braziliensis

We previously identified variants of the *IL2RB* gene predisposing individuals infected with *L. donovani* to KA (14). This prompted us to evaluate the expression profiles of the genes of the IL-2 pathway in *Leishmania*-infected tissue. We carried out this analysis on skin ulcers from patients infected with a dermotropic *Leishmania* species. Skin biopsies are less invasive than liver or spleen biopsies on subjects with visceral disease. The patients selected for the gene expression analysis had recent *L. braziliensis* infection that had not yet been treated. Global canonical pathway analysis (from the Ingenuity database) of the entire expression dataset (whole-genome expression profiling) revealed that the genes of the IL-2 pathway were more strongly expressed in lesions than in normal skin samples from uninfected donors ($p = 0.009$) (Fig. 1A). Indeed, the transcripts of many key genes of the IL-2 pathway, especially *IL2RA* (fold change of 65.6), were more strongly expressed in cutaneous ulcers than in uninfected skin (Fig. 1B).

Genetic analysis of key genes of the IL-2 pathway in Brazilians exposed to L. braziliensis

We then evaluated whether polymorphisms of key genes of the IL-2 pathway were risk factors for the development of skin ulcers in subjects living in a region in which *L. braziliensis* was endemic. We tested 133 tag-SNPs (covering the *IL2*, *IL2RA*, *IL2RB*, *JAK3*, *STAT5A*,and *STAT5B* genes) in 754 individuals from 209 nuclear families (described in Table I). The *IL2RG* gene, located on chromosome X and common to the receptor complexes for several different cytokines (28), was not analyzed. Assuming recent genetic admixture in the Brazilian population, we selected tag-SNPs from 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).

We performed a family-based transmission disequilibrium test and the strongest association signals ($p \leq 4 \times 10^{-4}$) were observed for two SNPs in intron 1 of the *IL2RA* gene [rs10905669 ($p = 3 \times 10^{-4}$) and rs706778 ($p = 3 \times 10^{-4}$)] (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 ($p = 6 \times 10^{-7}$) and rs706778 ($p = 2 \times 10^{-9}$) (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 (r^2 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

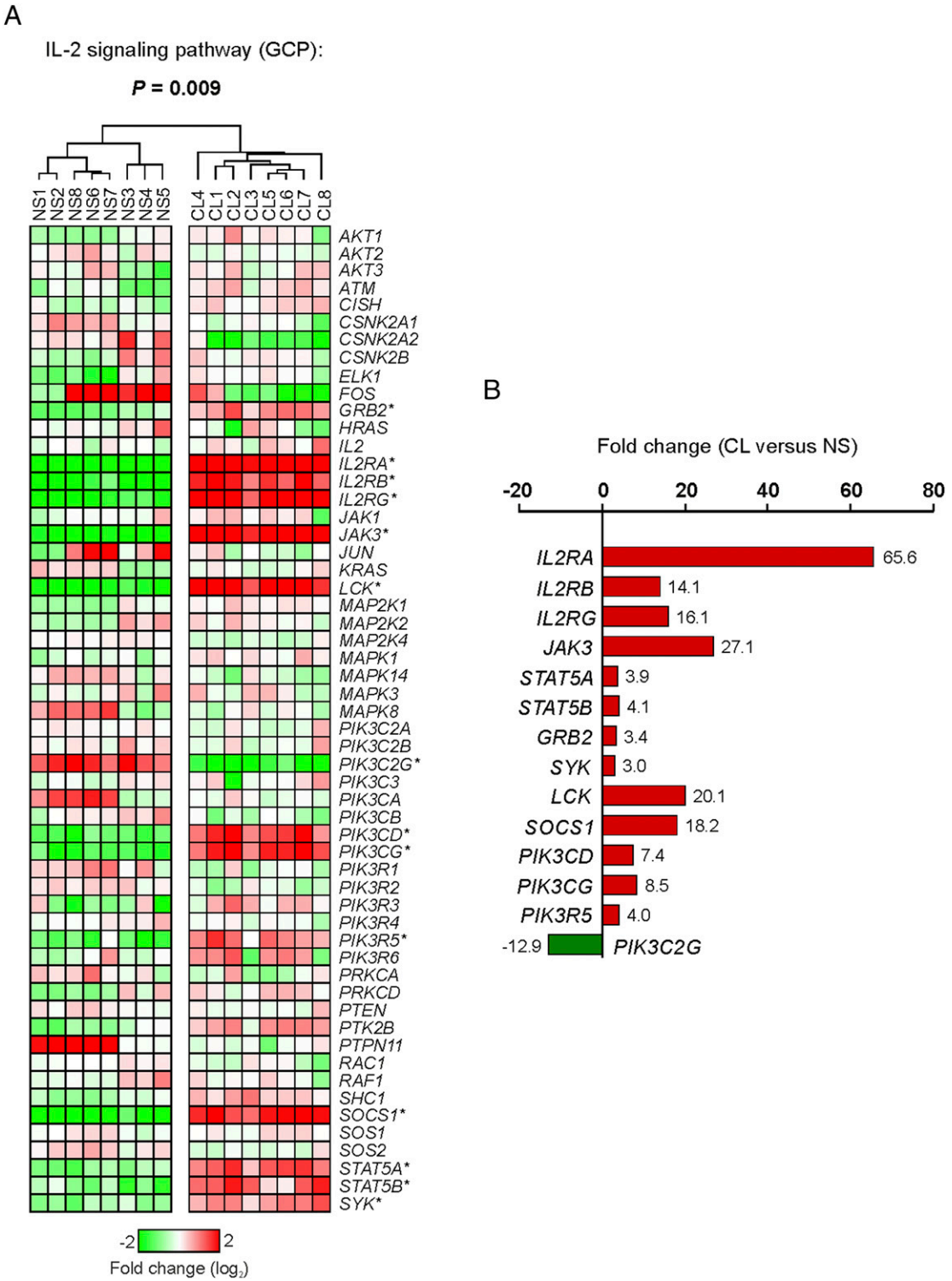

FIGURE 1. Whole-genome expression profiling of cutaneous lesions from patients infected with *L. braziliensis* revealing activation of the IL-2 pathway. Significance analysis of microarray was carried out to evaluate whether gene expression differed significantly between cutaneous lesions (CL) and normal 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.

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.

Table I. Characteristics of the studied populations

Sample Characteristics	Brazilian Discovery Sample[a]	Replication Sample[a]	Iranian Extension Sample[b]		
			Case	Control	*p*
Sample size	754	325	114	120	
Sex (male/female)	452/302	181/144	73/41	71/49	NS
Median age, y (IQR)	33 (22–45)	27 (19–42)	14 (10–18)	13 (12–14)	NS
Nuclear families	209	80	—	—	
Affected offspring trios	323	130	—	—	

The *p* values were determined by a χ^2 test.
[a]Family-based design.
[b]Population-based design.
IQR, interquartile range.

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 nonredundant role in the development/function of Treg cells; and 3) human CL (principally that due to *L. 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. 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. 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). Unstimulated cells produced very low or undetectable IFN-γ. Nonendemic healthy controls produced no detectable IFN-γ after stimulation with *L. braziliensis* extract (data not shown).

When stimulated, rTreg cells can increase and stabilize *FOXP3* expression and convert to an effector status known as eTreg cells (22). In this study, we hypothesized that *IL2RA* mutations may impair the ability of IL-2 to signal through its receptor, decreasing the frequency of eTreg cells. We tested this hypothesis by assessing the proportions of CD4$^+$CD45RA$^-$FOXP3^{hi} eTreg cells in cultures of PBMCs from healthy donors carrying different *IL2RA* rs706778 genotypes. PBMCs from these individuals were activated with anti-CD3/CD28 Abs and cultured in the presence of TGF-β1 or TGF-β1 plus IL-2. The frequencies of eTreg cells after 72 h of culture were highest in CC carriers, intermediate in heterozygous donors (CT), and lowest in TT individuals (Fig. 3B). This was observed in cultures stimulated with the highest concentrations of anti-CD23/CD28 Abs (1 bead/20 cells ratio) either with ($p = 0.01$) or without ($p = 0.005$) IL-2, and a similar trend was observed in the cultures with the lowest concentrations of anti-CD23/CD28 Abs (1 bead/200 cells ratio) either with ($p = 0.06$) or without ($p = 0.04$) IL-2. This effect in the absence of added IL-2 was probably due to the endogenous production of this cytokine. In contrast, the frequencies of CD4$^+$CD45RA$^+$FOXP3^{lo} rTreg cells were similar ($p > 0.5$) between the three genotypes in all conditions tested (data not shown). It was not possible to carry out the same analyses for rs10905669,

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

Tag-SNP	Gene	Allele	Freq	Fam	O_t	E_t	*P*	P_c
rs2069762	*IL2*^{intergenic}	A	0.79	76	183	167	0.009[a]	NS
rs4833248	*IL2*^{intergenic}	G	0.79	76	183	167	0.009[a]	NS
rs7069976	*IL2RA*^{intronic}	A	0.97	19	58	51	0.04[a]	NS
rs942200	*IL2RA*^{intronic}	C	0.89	9	16	12	0.01[d]	NS
rs4749920	*IL2RA*^{intronic}	T	0.86	11	18	14	0.02[d]	NS
rs7072398	*IL2RA*^{intronic}	A	0.63	114	258	241	0.02[a]	NS
rs942201	*IL2RA*^{intronic}	G	0.88	9	14	10	0.02[d]	NS
rs10905669	*IL2RA*^{intronic}	T	0.18	80	84	62	0.0003[a]	0.03
rs706778	*IL2RA*^{intronic}	T	0.41	75	76	53	0.0003[r]	0.01
rs4147359	*IL2RA*^{intergenic}	G	0.70	88	193	180	0.04[a]	NS
rs84459	*IL2RB*^{intronic}	T	0.69	79	65	54	0.04[r]	NS
rs228945	*IL2RB*^{intronic}	A	0.59	69	62	51	0.03[r]	NS
rs228947	*IL2RB*^{intronic}	C	0.73	80	76	64	0.03[r]	NS
rs3218294	*IL2RB*^{intronic}	G	0.98	14	34	28	0.005[a]	NS
rs228965	*IL2RB*^{intronic}	C	0.40	53	44	33	0.01[r]	NS
rs1003694	*IL2RB*^{intronic}	G	0.68	97	227	207	0.004[a]	NS
rs2235330	*IL2RB*^{intronic}	T	0.82	64	151	138	0.01[a]	NS
rs228973	*IL2RB*^{intronic}	T	0.65	43	51	42	0.01[d]	NS
rs3218258	*IL2RB*^{intronic}	T	0.18	11	13	7	0.006[r]	NS
rs7255931	*JAK3*^{intronic}	C	0.78	76	178	166	0.04a	NS
rs3212760	*JAK3*^{intronic}	T	0.66	46	55	47	0.04[d]	NS
rs3212752	*JAK3*^{intronic}	A	0.86	56	61	49	0.009[r]	NS
rs13345965	*JAK3*^{intergenic}	C	0.84	11	14	10	0.03[d]	NS

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_c, empirical *p* value (10,000 permutations), corrected for all tests.

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

Brazilian Replication Study (*L. braziliensis*)						
Marker	Risk Allele	Freq	Fam	O_t	E_t	*P*
rs10905669	T	0.16	20	18	13	0.08[a]
rs706778	T	0.42	43	58	51	0.04[a]
Iranian Extension Study (*L. tropica*)						
Marker	Risk allele	Freq (case/control)		OR	95% CI	*P*
rs10905669	T	0.31/0.20		1.5	1.0–3.0	0.03[a]
rs706778	T	0.54/0.44		1.5	1.0–2.5	0.04[a]

For asymptotic *p* values (*P*), the superscript "a" indicates additive genetic model.

Freq, frequency of the risk allele; Fam, number of informative families; O_t, observed transmissions; E_t, expected transmissions; *P*, asymptotic *p* value; OR, odds ratio; 95% CI, 95% confidence interval.

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 ($r^2 \geq 0.6$) 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 IV. Meta-analysis of the studied population samples from Brazil and Iran

		Brazilian Discovery Study		Brazilian Replication Study		Iranian Extension Study		Meta-analysis			
SNP	Risk Allele	SE	OR	SE	OR	SE	OR	*N*	*Q*	OR	*P*
rs10905669	T	0.15	2.1	—	—	0.27	1.5	2	0.50	2.0	6.10^{-7}
rs706778	T	0.13	1.8	0.18	2.0	0.24	1.5	3	0.77	1.8	2.10^{-9}

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.

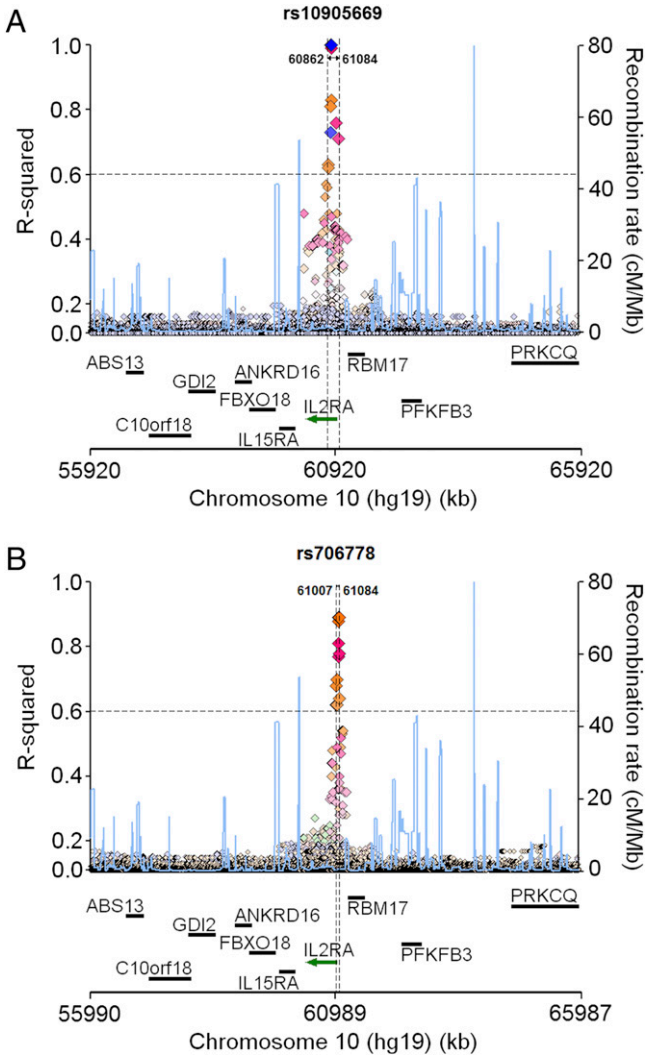

FIGURE 2. No polymorphisms outside the *IL2RA* region could account for the associations of rs10905669 and rs706778 with CL. Regional linkage disequilibrium (LD) plots show the LD blocks tagged by (**A**) rs10905669 and (**B**) rs706778 over a 1 Mb window (centered on each marker). LD was calculated separately for American (violet), African (blue), and European (orange) populations (from the 1000 Genomes Project). The region containing SNPs correlated ($r^2 \geq 0.6$) with the tested markers is shown between two vertical dotted lines. Estimated recombination rates (blue lines) are plotted to provide information about regional LD structure.

Our data are consistent with those of recent studies showing that IFN-γ 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 noncoding 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

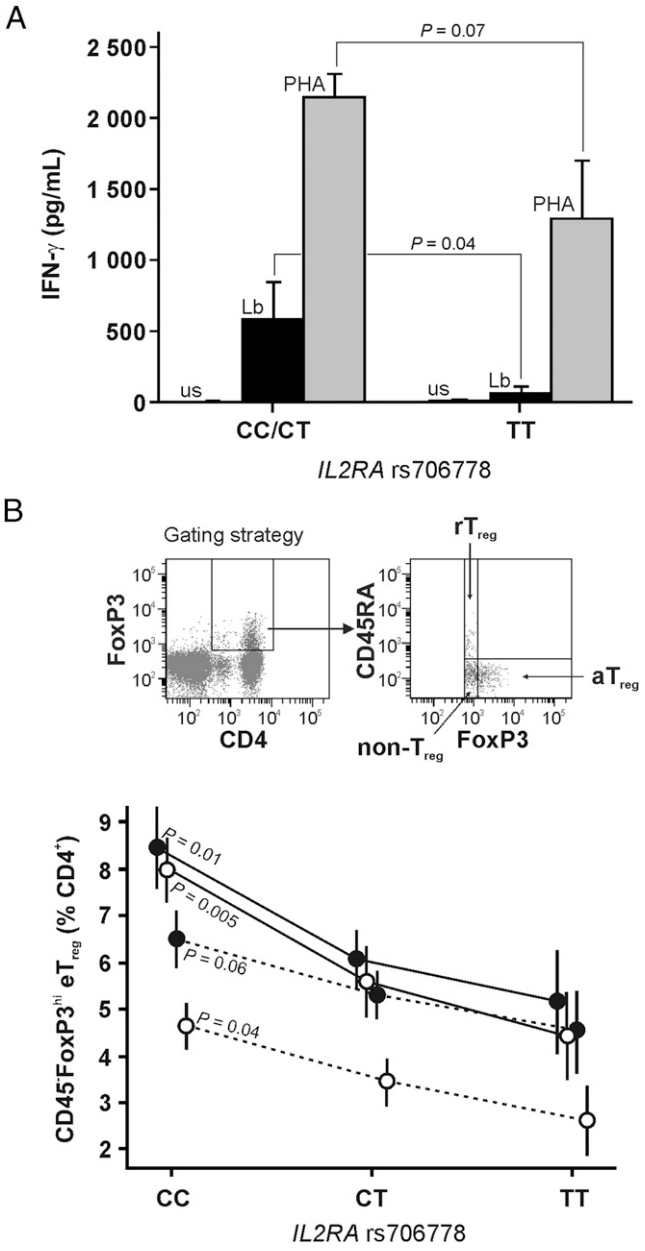

FIGURE 3. *IL2RA* rs706778 is associated with altered IL-2–dependent responses. (**A**) PBMCs from subjects exposed to *L. braziliensis* ($n = 51$) were cultured in RPMI 1640 medium (unstimulated [us]) and stimulated with *L. braziliensis* Ags (Lb) or with PHA, as described in *Materials and Methods*. Individuals were classified according their rs706778 genotypes, and IFN-γ levels in culture supernatants were measured by ELISA. (**B**) *Top*: Gating strategy used to discriminate $CD4^+FOXP3^+$ subpopulations (an anti-CD45RA fluorescent Ab was used to discriminate $CD4^+CD45^-FOXP3^{hi}$ eTreg cells, $CD4^+CD45^+FOXP3^{lo}$ rTreg cells, and $CD4^+CD45^-FOXP3^{lo}$ non-Treg cells). *Bottom*: PBMCs from healthy donors ($n = 86$) were stimulated with anti-CD3/CD28 Abs at a 1:20 (bead/cell) dilution (continuous lines) or at a 1:200 dilution (dotted lines) and cultured in the presence of TGF-β1 only (○) or TGF-β1 plus IL-2 (●). Linear regression showed that the proportion of eTreg cells varied with rs706778 genotype. Data (mean and SD) are representative of two independent experiments.

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 Treg 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.

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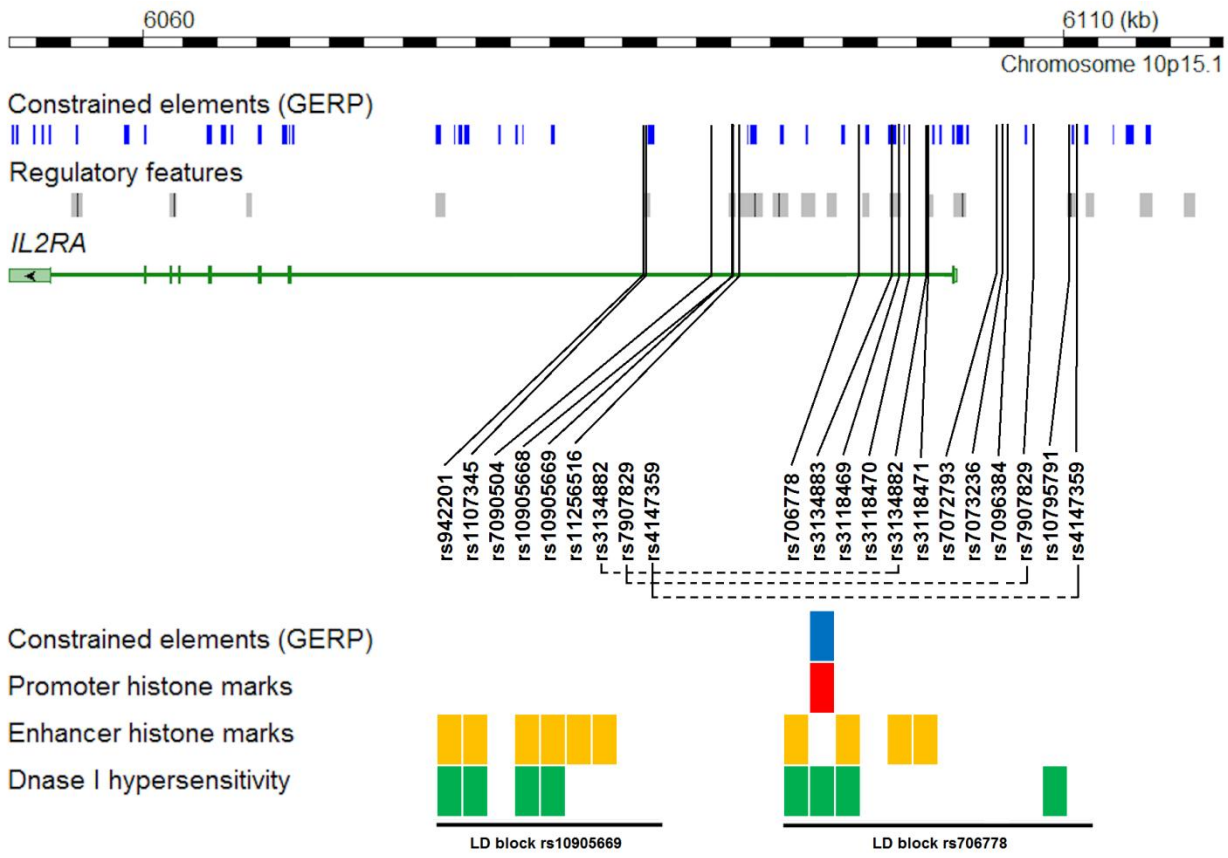

SUPPLEMENTAL FIGURE 1. *In silico* analysis of the properties of *IL2RA* polymorphisms in linkage disequilibrium (LD) with rs10905669 and/or rs706778. LD blocks were constructed considering all SNPs tagged ($r^2 \geq 0.6$) by rs10905669 and/or rs706778 in American, African and European populations from the 1000 Genomes project. The position of SNPs was evaluated in terms of GERP (Genomic Evolutionary Rate Profiling)-constrained elements (36 eutherian mammals), histone promoter/enhancer marks and DNase I hypersensitive sites. Comparative genomic data and regulatory sequence annotations were obtained from both the Ensembl and UCSC databases. Dotted lines indicate SNPs that are common to both rs10905669 and rs706778 LD blocks.

Interleukin-22 and IL-22 binding protein (IL-22BP) regulate fibrosis and cirrhosis in hepatitis C virus and schistosome infections

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[3] Abbreviations: HF hepatic fibrosis, CentF central hepatic fibrosis; NF network fibrosis, LD Linkage disequilibrium. IL-22BP IL-22 binding protein; IL22RA2, IL22RA1 genes encoding IL-22BP and IL-22R1

Summary

IL-22 acts on epithelia, hepatocytes and pancreatic cells and stimulates innate immunity, tissue protection and repair. IL-22 may also cause inflammation and abnormal cell proliferation. The binding of IL-22 to its receptor is competed by IL-22BP, which may limit the deleterious effects of IL-22. The role of IL-22 and IL-22BP in chronic liver diseases is unknown. We addressed this question in individuals chronically infected with schistosomes or HCV. We first demonstrate that schistosome eggs stimulate the production of IL-22 transcripts and inhibit the accumulation of IL22-BP transcripts in schistosome-infected mice and that schistosome eggs selectively stimulate the production of IL-22 in cultures of blood leukocytes from individuals chronically infected with S.japonicum. High IL-22 levels in cultures correlated with protection against hepatic fibrosis and portal hypertension. To test further the implication of IL-22/IL-22BP in hepatic disease, we analyzed common genetic variants of IL22RA2, which encodes IL-22BP, and found that the genotypes AA, GG of rs6570136 (p=0.003; OR=2), and CC, TT of rs2064501 (p=0.01; OR=2), were associated with severe fibrosis in Chinese infected with S.japonicum. We confirmed this result in Sudanese (rs6570136 GG (p=0.0005; OR=8.1), rs2064501 TT (p=0.008, OR=3.6)) and Brazilians (rs6570136 GG (p=0.003; OR=26), rs2064501 TC, TT (p=0.03, OR=11)) infected with *S.mansoni*. The aggravating genotypes were associated with high IL22RA2 transcripts levels. Furthermore, these same variants were also associated with HCV-induced fibrosis and cirrhosis (rs6570136 GG, GA (p=0.007; OR=1.7), rs2064501 TT, TC (p=0.004; OR=2.4)). These results provide strong evidence that IL-22 protects against and IL-22BP aggravates liver fibrosis and cirrhosis in humans with chronic liver infections. Thus, pharmacological modulation of IL-22 BP may be an effective strategy to limit cirrhosis.

Introduction

IL-22 is produced by a variety of hematopoietic cells such as innate lymphoid cells (ILC), lymphoid tissue-inducers (1), T helper cells (2) and γ/δ T cells (3). However, unlike most cytokines, IL-22 does not act on hematopoietic cells but affects epithelia, hepatocytes and pancreatic cells (4, 5), suggesting an important role for this cytokine at epithelial barriers of the intestine, skin and lungs and in the liver and pancreas. Indeed, IL-22 stimulates innate immunity by promoting the production of anti-microbial peptides (2, 6, 7), the secretion of mucus, the release of chemokines (8-10) and by enhancing cell mobility (6, 7, 11). IL-22 also protects tissues from damage and mediates tissue repair (5, 12, 13). Deregulated IL-22 responses may cause pathological inflammation (12), abnormal cell proliferation (14) and enhanced chemokines production as occurs in experimental models of psoriaris (8-10), *T.gondi*-induced ileitis (15) and arthritis (16). Furthermore, the release of both IL-17A and IL-22 in the same inflammatory sites may aggravate pathology since IL-17A enhances the pro-inflammatory effects of IL-22 (9, 16, 17). Few studies have evaluated the role of IL22 / IL-22BP in disease, and with the exception of skin psoriasis, it is not known whether this cytokine protects against or aggravates various human diseases. Here, we evaluated whether IL-22 and its inhibitor IL-22BP (18, 19) influence liver disease in humans infected with HCV or schistosomes. Hepatic fibrosis (HF) and cirrhosis develop during chronic liver inflammation caused by HCV, HBV, schistosomes, steatosis and alcohol. Fibrosis is an excessive deposition of extracellular matrix proteins in healing lesions. HF and cirrhosis

cause varices, ascites, liver failure and death in millions of patients. IL-22 protects against acute hepatitis (20) and stimulates tissue regeneration (21) in experimental models of liver disease, but it aggravates inflammation in a mouse model of HBV infection (17). Furthermore, in a murine model of schistosomiasis, IL-22 was not detected during infection (22). Intestinal ILC produce IL-22 in inflammatory conditions and when damage occurs to the intestinal barrier; therefore, we hypothesized that IL-22 is produced when schistosome eggs perforate the intestine. The consequences of these egg-induced intestinal lesions on the human immune response during schistosomiasis deserve further investigations since most studies have focused on splenomegaly and on periportal fibrosis. Here, we present evidence that IL-22 / IL-22BP play significant roles in hepatic fibrosis and cirrhosis in patients with chronic schistosome and HCV infections.

Results

IL22, IL22RA2, IL22RA1 transcripts in mice infected with *Schistosoma mansoni*. : Schistosome eggs stimulate the production of IL-22 in the intestine.

We measured *Il22*, *Il22ra2* and *Il22ra1* mRNA levels in the intestine, liver and spleen of mice infected with *S. mansoni* to test whether schistosomes induce an IL-22 / IL22BP response (Fig.1a-d). *Il22* mRNA levels were low before infection (Fig.1a); they started to increase in the colon and in Peyer patches, at 4 weeks when egg laying had just begun, and peaked at 6 weeks (Fig.1b). *Il22* mRNA was not detectable in the liver or spleen. *Il22ra2* mRNA levels were high in the intestine and spleen (but not in the liver) before infection (Fig.1a) and were 5-10 fold lower after 11 weeks of infection (Fig.1c). Thus, schistosome eggs promote the production of *Il22* mRNA and inhibit that of *Il22ra2*. *Il22ra1* mRNA levels remained stable in the intestine and liver, and were high in the spleen after infection (Fig.1d), indicating that these tissues can respond to IL-22.

Production of IL-22 by blood mononuclear cells from individuals with chronic schistosome infection. IL-22 is produced by at least two different cell populations.

We evaluated the amount of IL-22 produced by peripheral blood mononuclear cells (PBMCs) from 66 Chinese fishermen infected with *S. japonicum* and 18 non exposed controls (described in methods). IL-22 was detectable in resting cultures of patient PBMCs at 72 and 144 hours (hrs) of cell culture and its production was stimulated by schistosome eggs ($p<10^{-3}$) (Fig.2a). IL-22 was detectable in cultures of control PBMCs only at 144 hrs of culture. and its production was unaffected by eggs. IL-17A was detectable in cultures of PBMCs at 144 hrs of culture and its production was unaffected by eggs (data not shown). Flow cytometry analysis of patient PBMCs showed that both $CD3^{+}CD4^{+}$ and $CD3^{-}CD4^{-}$ cell populations produced IL-22, and neither cell population produced IL-17A (Supporting Fig.1). (1, 23, 24)

IL-22 produced by PBMCs from individuals with chronic schistosomiasis correlates with protection against hepatic fibrosis and portal hypertension.

Hepatic fibrosis in individuals infected with *S. mansoni* or *S. Japonicum* manifests as central fibrosis (CentF) around the central vein, and in *S. japonicum* infections, a network fibrosis pattern

(NetF) in the parenchyma is also present. In this study, CentF was graded as light (+/-), advanced (+) severe (++) and very severe (+++). Severe and very severe fibrosis were associated with portal blood hypertension and splenomegaly. We found that IL-22 concentrations in resting (p=0.005) and in egg-stimulated (p=0.04) cultures of PBMCs were inversely correlated with CentF (Fig.2b) in PBMC donors infected with *S. japonicum*. Moreover, multi-linear regression analysis showed that both severe and very severe CentF (p=0.01) and NetF (p=0.004) were independently associated with low IL-22 concentrations (Fig.2c). Furthermore, patient portal vein diameter (an indicator of portal hypertension) was negatively correlated (p=0.02) with IL-22 concentrations (Fig.2d).

Polymorphisms in *IL22RA2* are associated with severe hepatic fibrosis in Chinese fishermen exposed to *S.japonicum*. The aggravating genotypes are associated with an increase of IL-22RA2 transcripts.

We determined whether polymorphisms in *IL22RA2* affect the risk of severe HF to test further the hypothesis that high IL-22 production protects against schistosome-induced HF. *IL22RA2* is located on Chr.6q23, which controls HF (25). Mutations in *CTGF* account for part of the control exerted by this locus (26). *IL22RA2*, the gene encoding IL-22BP, is located in the same region and may also be involved in the control. To test this hypothesis, we genotyped SNPs representative of the six major SNP correlation bins (TagSNP , r^2=0.8) in *IL22RA2* (Supporting Fig.2a, supporting Table 1) in 327 Chinese fishermen (Table 1) with long exposure to *S. japonicum* infections. For this analysis, we have used a binary fibrosis phenotype that included as cases, subjects with severe CentF or severe NetF and subjects with advanced CentF if they also had advanced or severe NetF. Control groups included individuals who exhibited no NetF and light CentF or less (see methods). The genotypes AA, GG of SNP rs6570136 (p=0.003; OR=2), and CC, TT of SNP rs7774663 (p=0.004; OR=2.1), both in bin I, and the genotypes CC, TT of SNP rs2064501 (p=0.01; OR=2) in bin VI were significantly associated with HF (Table 2, Fig.3a-b). Multivariate analysis could not separate the effects of these SNPs due to the high linkage disequilibrium (LD) between these SNPs (Fig.3c).

Thus, rs6560136 and/or rs2064501, or other variants highly correlated with either of these SNPs, modulate susceptibility to HF in fishermen. We built a map of all SNPs correlated with either rs6560136 or rs2064501 in a region extending 5 Mb from the 3' and 5' end of *IL22RA2* to rule out the possibility that the casual variants may lie outside of *IL22RA2*. Supporting figures 2 b,c,d show the various common SNPs (MAF >5%) in this region and their correlation with the SNPs of interest (Y axis). None of these SNPs outside *IL22RA2* were strongly correlated with rs6560136 or rs2064501 and could account for the association. Then, the causal SNPs must lie in *IL22RA2*.

Next, we evaluated whether rs6560136 and rs2064501 modulate *IL22RA2* transcripts. We performed this analysis in healing skin tissue that highly expresses *IL22RA2*. We found a significant association of the genotypes GG, AA of rs6570136 (p=0.005) and a suggestive association of the genotype TT of rs2064501 (p=0.065) with the highest *IL22RA2* mRNA levels (Fig.3d-e). This evaluation shows that the genotypes of rs6560136 that are associated with aggravation of fibrosis (Fig.3a) are also associated with enhancement of IL22RA2 transcripts (Fig.3d); they also suggest that the genotype TT of rs2064501 that is also associated with HF (Fig.3b) also enhances IL-22RA2 transcripts (Fig.3e).

***Validation / extension of the associations observed in Chinese subjects to* Sudanese and Brazilian infected with *S. mansoni*.**

We sought to confirm the association between HF and SNPs in *IL22RA2* in Sudanese and Brazilian populations infected with *S. mansoni*. We genotyped 201 Sudanese subjects (described in Table 1) and found that SNPs rs6570136 GG (p=0.008, OR=3.2), rs11154915 TT (p=0.07; OR=4.9) and rs2064501 CC, TT (p=0.03; OR=2.2) were associated with severe HF (Table 3). The best multivariate model included SNP rs6570136 GG (p=0.0005; OR=8.1) and rs2064501 TT (p=0.008; OR=3.6).

We then genotyped 186 Brazilians (Brazil (1), described in Table 1) and found that SNPs rs6570136 GG (p=0.001; OR=4.8), rs7774663 TT (p=0.02; OR=2.5), rs779054 TT (p=0.02; OR=2.4) were associated with the aggravation of HF (Table 3, Fig.4a). No significant association was found between rs2064501 TT and HF in univariate analysis. Nevertheless, the best multivariate model included SNP rs6570136 GG (p=0.003; OR=26) and rs2064501 TT, TC (p=0.03; OR=11). Thus, both rs6570136 and rs2064501 independently contribute to the association with severe HF, with rs6570146 GG and rs2064501 TT corresponding to the aggravating genotypes. In conclusion, variants of *IL22RA2* that are associated with a high abundance of *IL22RA2* transcripts are also associated with severe hepatic disease in three genetically different populations.

The same genetic variants of *IL22RA2* are associated with susceptibility to fibrosis and cirrhosis in individuals infected with HCV.

We evaluated whether these same polymorphisms affected fibrosis and cirrhosis in HCV-infected patients (n=532, cohort Brazil (2); Table 1) with different grades of HF (from F0=no fibrosis to F3=advanced fibrosis and F4=cirrhosis). We found that the genotypes GG and AG of SNP rs6570136 (p=0.04; OR=1.6) and TT, CT of genotype rs2064501 (p=0.02, OR=2) and the genotype AA of the SNP rs202563 (p=0.07; OR=1.8) were associated with advanced fibrosis or cirrhosis (F3, F4, n=210), and were less prevalent in individuals with mild or no fibrosis (F0+F1+F2, n=322) (Table 4). Multivariate analysis demonstrated that rs6570136 GG, AG (p=0.007; OR=1.7) and rs2064501 TT, CT (p=0.004; OR=2.4) were independently associated with severe fibrosis (F3+F4). The data obtained in comparing of F3+F4 with F0+F1 are shown on Fig 4b. These associations in HCV-induced HF in Brazilians are similar to the associations we have observed in schistosome-induced HF in Brazilians (Fig.4a) The strong LD between the two associated SNPs (Fig.4c), explains why the strength of the association is high when both SNPs are analyzed simultaneously.

We confirmed these results in an independent cohort (Brazil (3) Table 1) of 149 (F0+F1, F3+F4) patients infected with HCV, which showed that SNP rs6570136 GG (0.04) and rs2064501 TT (0.05) were independently associated with severe fibrosis (F3+F4).

Discussion

We evaluated the production of IL-22 in a mouse model of schistosome infection to examine the role of IL-22 / IL-22 BP in HF. In a previous study, IL-22 was not detected in the liver and spleen of

schistosome-infected mice (22). Although we confirmed this result, we also showed that the abundance of *IL22* transcripts is high in Peyer patches when schistosome eggs reach the mouse intestine. IL-22 is thought to protect epithelia against damage and mediates tissue repair (5, 12, 13). IL-22 also stimulates the production of anti-microbial peptides that limit the invasion of microbes across epithelial lesions (2, 6, 7, 11). Schistosome eggs perforate the intestinal wall, therefore epithelial lesions caused by eggs, which allow the entry of bacteria and toxic bacterial products, should induce a strong IL-22 response. Our failure to detect IL-22 in mouse liver is probably due to a lack of sensitivity because cells producing IL-22 in the liver are outnumbered by hepatocytes that do not produce it. We also found that the abundance of *IL-22RA2* transcripts in the intestine was reduced after the arrival of eggs. Thus, the balance between IL-22 / IL-22BP is tipped in favor of IL-22 when eggs lodge in the liver and perforate the intestine. This indicates that the biological activity of IL-22 is high when hepatic and intestinal diseases begin to develop. A previous study found no evidence to suggest that IL-22 is involved in HF in schistosome-infected mice (22). However, there are many differences between schistosomiasis in mice and humans. Liver disease in mice is evaluated within a few weeks of infection whereas we examined hepatic disease in humans after more than 10 years of infection. Furthermore, the parasite load in mice is hundreds of times higher than in infected humans. For these reasons, we evaluated IL-22 in chronically infected individuals who have been living in an endemic region for their whole life. IL-22 was produced by PBMCs obtained from these individuals; IL-22 production was stimulated by schistosome eggs. Eggs had no effect on IL-22 production by PBMCs from local individuals who had no exposure to schistosomes. IL-17A production in these cultures was low and was not stimulated by eggs(9, 16, 17). Our findings suggest that synergy between IL-22 and IL-17 does not occur in humans with long chronic infections; although we cannot fully exclude the possibility that IL-17 may be produced locally in particular tissues and not in the blood. Our data indicate that IL-22 in PBMC cultures, was produced by at least two cell types: $CD3^+CD4^+$ and $CD3^-CD4^-$. This suggests that IL-22 is probably produced by Th22 cells (23, 24) and NK-like innate lymphoid cells (1). However, more work is required to characterize IL-22-producing cells in schistosome-infected patients. IL-22 was not produced by Th17 because little IL-17 was produced in cultures and IL-22 producing cells were IL-17 negative. There may be a high abundance of ILCs in the intestine because inflammation of the intestine strongly stimulates the multiplication of ILCs producing IL-22 as also indicated by our data in mice. We found that IL-22 production in cultures was positively correlated with protection against HF. Indeed, certain fishermen presented with no disease or only mild disease, although they have been exposed daily to infection for more than 20 years. This was due to the effective control of HF and not due to inherent protection against infection. The portal vein diameter of the studied fishermen was inversely correlated with IL-22 levels. This measure is used to detect portal hypertension. Thus, high IL-22 levels are associated with protection against the most severe stages of HF.

To investigate further the link between IL-22 / IL-22BP and HF, we performed a genetic analysis of *IL22RA2* to search for genetic variants associated with HF. We first studied *IL22RA2* because it is located in the 6q23 locus that exerts major control on HF in schistosome-infected populations (25). Mutations in the *CTGF* gene, which is present at this same locus, contribute to this major genetic

effect (26). Nevertheless, *CTGF* cannot account for the entire effect of the locus. We obtained convincing associations between HF and two polymorphisms in *IL22RA2*. The observation that heterozygous individuals are better protected from HF than homozygous individuals of either genotype has been found in other infectious diseases. We believe that the strong LD between rs657136 and rs2064501 explains this effect. Our study in Sudan and China shows that the aggravating homozygous genotypes are rs657136 GG and rs2064501 TT. The protective homozygous genotype of one SNP is almost always associated with the aggravating homozygous genotype of the other SNP due to the strong LD between these SNPs. This association between homozygous genotypes with opposite effects neutralizes the effects of the protective genotypes, and gives rise to a genotype that is associated with disease aggravation. The association of variants in *IL22RA2* with HF directly implicates IL-22BP in HF. Homozygous genotypes, which are associated with susceptibility to severe HF, are also associated with high levels of *IL22RA2* transcripts, strongly suggesting that IL-22BP aggravates HF. This finding is consistent with the association we observed between IL-22 and protection against both HF and portal hypertension.

The underlying mechanisms that lead to HF in schistosome and HCV infections are similar in many aspects. However, in HCV infections, liver fibrosis and viral hepatotoxicity are associated with a vigorous multiplication of hepatocytes, which is not observed in schistosome infections. IL-22 promotes liver cell regeneration by increasing cell proliferation and hepatocyte migration (27). However, such proliferation contributes to the regeneration nodules that greatly augment the loss of liver architecture and organization and the impairment of liver function in cirrhosis. Hepatocyte proliferation may be aggravated by IL-22, which stimulates tissue regeneration, inhibits apoptosis (10, 28); Furthermore, uncontrolled IL-22 activity may promote the development of hepatocarcinoma (14). For these reasons, we investigated these same polymorphisms in the context of HCV-induced cirrhosis. We showed that genetic variants of *IL22RA2* that are associated with susceptibility to severe HF in schistosomiasis are also associated with HCV-induced cirrhosis, indicating that IL-22 / IL-22BP exerts hepatoprotective effects in both HCV and schistosome infections.

Several biological effects may account for the protective action of IL-22 against schistosome-induced HF. IL-22 may stimulate the production of anti-inflammatory molecules (29, 30), promote liver repair by limiting apoptosis, or stimulating mitosis, cell migration (20, 29) and progenitor cell growth (31). IL-22 may also promote stellate cell senescence (32). The regulation of IL-22 by IL-22BP may also limit the entry of pro-fibrogenic bacterial products such as LPS by stimulating anti-bacterial innate immunity (4, 33),reducing intestinal inflammation (34, 35) and promoting the healing of the intestinal epithelium (12, 36), which is perforated by thousands of schistosome eggs. LPS is known to stimulate hepatic fibrogenesis through direct effects on hepatic stellate cells which express the TLR-4 receptor. Finally, IL-22 may stimulate the liver to produce LPS binding protein (37). Intestinal damage does not typically occur during HCV infections; therefore, the protective effects of IL-22 are likely to result from a direct protective action on the liver (10, 38), and probably involve tissue repair and regeneration (21) and stellate cell senescence (32) which was shown to be crucial for limiting HF. Hepatic tissue repair is probably more critical in HCV infections than in schistosome infections because HCV is very cytotoxic

for hepatocytes whereas schistosomes are not because eggs are trapped in the liver sinusoids and toxic substances are prevented from diffusing by sequestration in the granuloma.

In conclusion, we show IL-22 is associated with protection against liver fibrosis in human schistosomiasis and mutations that promote *IL-22BP* expression, the physiological inhibitor of IL-22, aggravate fibrosis and cirrhosis in both schistosome and HCV infections. These results strongly suggest that IL-22 protects against hepatic fibrosis and cirrhosis. This is also the first direct evidence that IL-22BP plays a significant regulatory role in human inflammatory diseases. IL-22 and IL-22BP do not act on hematopoietic cells; therefore, pharmacological intervention against these molecules should have fewer side effects than treatments that target classical cytokines like TNF. Thus, IL-22 and IL-22BP may be good therapeutic targets (3) in the prevention and treatment of fibrosis and cirrhosis.

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Figure Legends

Figure 1.

Schistosome eggs stimulate the production of *IL22* mRNA and impair that of *IL22RA2* in infected mice (a-d) (a). Abundance of *Il22*, *Il22ra2* and *Il22ra1* mRNA in the colon, intestine, Peyer's patches, liver and spleen of non-infected CBA/J mice **(b-d)** and mice infected with 30 *S. mansoni* cercariae. Transcript levels were evaluated at 4, 6, or 11 weeks post infection. One representative experiment out of two (5-6 mice / group) is shown. Data are the geometric mean **(a)** or the log (geometric mean) (**b-d**) of the fold change between infected and non-infected animals. Comparisons were performed by non-parametric analysis *$p < 0.05$.

Figure 2.

IL-22 response in humans infected with schistosomes. IL-22 concentrations correlate with protection against hepatic fibrosis and portal blood hypertension.

(a) Concentration of IL-22 produced in egg stimulated-cultures of PBMCs from 66 Chinese fishermen and 18 controls. A total of 10^6 PBMCs were cultured for 72 or 144 hrs with or without 500 schistosome eggs; **(b)** IL-22 concentrations in resting (p=0.005) and egg-stimulated (p=0.04) cultures at 144 hrs are inversely correlated with CentF severity. Light CentF CL^L is (+/-) (n=41), advanced CentF CL^M is (+) (n=18), severe CentF CL^H is (++) (n=7), and very severe CentF D+E+F is (+++) (n=3). **(c)** Multivariate analysis shows that CentF and NetF are independently correlated with IL-22 levels in egg-stimulated cultures (p=0.009 and p=0.004, respectively). CentF was divided into CL^L ,CL^M (n=51) and CL^H , D+E+F (n=7); NetF was divided into not detectable (n=35) and detectable (n=23). **(d)** Portal hypertension (portal vein diameter, PV) is inversely correlated with IL-22 produced in resting cultures at 144 hrs (p=0.02). IL-22 concentrations were divided into classes 11-100 ng/ml (n=26), 101-300 (n=16), 301-900 (n=16); >900 (n=8). Bars show SEM.

Figure *3.*

***IL22RA2* genotypes that are associated with severe fibrosis in Chinese Fishermen are associated with high *IL22RA2* mRNA levels in healing skin**.

(a-b) The genotypes AA and GG of SNP rs6570136 and the genotypes TT and CC of SNP rs2064501 are associated with susceptibility to severe HF. The sample of Chinese fisherman is described in

Supporting Table 1 and data are also shown in Table 1. The data are represented for men and women separately (p<0.001) and for fishermen who were exposed for more >20 years (longer exposure are not significantly associated with HF); **(c)** SNPs rs6570136 and rs2064501 are in strong linkage disequilibrium; **(d)** The genotypes AA and GG of SNP rs6570136 are associated (p=0.005) with the highest *IL22RA2* mRNA levels in healing skin of 34 individuals; **(e)** Suggestive association of the genotype TT of SNP rs2064501 (p=0.065) with the highest *IL22RA2* mRNA levels in the skin biopsies. The abundance of mRNA is expressed as arbitrary units (+/- SEM).

Figure 4.

The same alleles that are associated with susceptibility to severe hepatic fibrosis in Brazilians infected with schistosomes are also associated with a high risk of fibrosis and cirrhosis in Brazilians infected with HCV.

(a) The genotype GG of SNPs rs6570136 GG (*p*=0.003) and the genotype TT, TC of SNP rs2064501 (*p*=0.03) are independently associated with HF in Brazilians (Brazil (1)) infected with *S. mansoni*; **(b)** SNPs rs6570136 (*p*=0.007) and rs2064501 (*p*=0.004) are also independently associated with HF and cirrhosis in Brazilians (Brazil (2)) infected with HCV; The analysis compares F0+F1 with F3+F4 whereas data in Table 3 compares F0,F1,F2 with F3,F4. The SNP that shows the strongest association with HF is shown on the x axis. **(c)** The distribution of rs6570136 and rs2064501 genotypes among Brazilians reveals strong linkage disequilibrium between these two SNPs. The same genotype distribution was observed with schistosome- and with HCV-infected Brazilians inhabiting the same region of Brazil.

Supporting Figure 1.

Flow cytometry analysis of IL-22 producing cells in the blood of control and exposed individuals.

Data are from one representative experiment out of 20. Cell labeling was performed as indicated in Material and Methods.

Supporting Figure 2.

Description of the SNP bins in IL22RA2 (a); lack of high correlation (r^2<0.6) between SNP 6570136 or 2064501 and other SNPs in a 1Mb region surrounding *IL22RA2* (b-d).

SNPs shown in **(a)** have a minor allele frequency >10% in the Chinese population; the analysis extended 10 Mb from the 3' and 5' end of the gene. . Plot in **(b)** shows correlation values for all SNPs of the I0 Mb region surrounding IL22RA2, with SNPs 6570136 (closed symbols) and 2064501 (open symbols) whereas plots shown in **(c)** and **(d)** show data for each SNP respectively in the 1 MB region. Relevant genes located in the region are indicated (CCN2 encodes CTGF). There is no SNP outside *IL22RA2* that correlates significantly (r^2>0.6) with SNP rs6570136 and rs2064501 that could account for the observed association with HF. Data were collected from the Hapmap and 1000 Genomes databases. 185 mots

Table Legend

Table 1.

Description of the study samples.

Exposure to infection was evaluated with "years of fishing" (F. yrs) in Chinese fishermen and with age in Sudanese and Brazilians infected with *S. mansoni.* The duration of infection could not be accurately evaluated in HCV6-infected individuals. The analysis presented in Table 3 (Cohort Brazil (2)) compares F0, F1, F2 with F3 and F4. The analysis presented in Fig.4b and c (Cohort Brazil (2)) compares F0, F1 with F3 and F4.

Table 2.

***IL22RA2* genotypes associated with advanced fibrosis in Chinese Fishermen infected with *S. japonicum*.**

Cases and controls are described in Material and Methods and in Supporting Table 1. The association between genotypes and HF phenotypes was first tested separately (upper part of the table) and then simultaneously by logistic regression analysis (lower part). Significant covariates in the logistic regression analysis were sex ($p=10^{-3}$) and exposure ($p < 10^{-3}$). Bins represent correlation groups (r^2>0.8), and the aggravating genotype is shown. OR = odds ratio, CI = Confidence interval of OR.

Table 3.

***IL22RA2* genotypes associated with advanced fibrosis in Sudanese and Brazilian farmers infected with *S. mansoni*.**

The two principal schistosome strains that cause HF are *S. japonicum* in Asia, and *S. mansoni* in Africa and South America. We investigated whether allelic variants of *IL-22RA2* also affect susceptibility to severe HF in an *S. mansoni*-endemic region in Sudan and Brazil. *IL22RA2* polymorphisms that were associated with HF in Chinese fishermen were genotyped in both samples. The phenotypes of cases and controls and the cohort size are described in Material and Methods and in Supporting Table 1. We first tested for associations between the SNPs and HF phenotypes separately (upper part of the table) and then tested the SNPs simultaneously (lower part). The aggravating genotype is shown. OR = odds ratio, CI = confidence interval of OR. Age p=0.025 was a covariate in the Sudanese multivariate analysis.

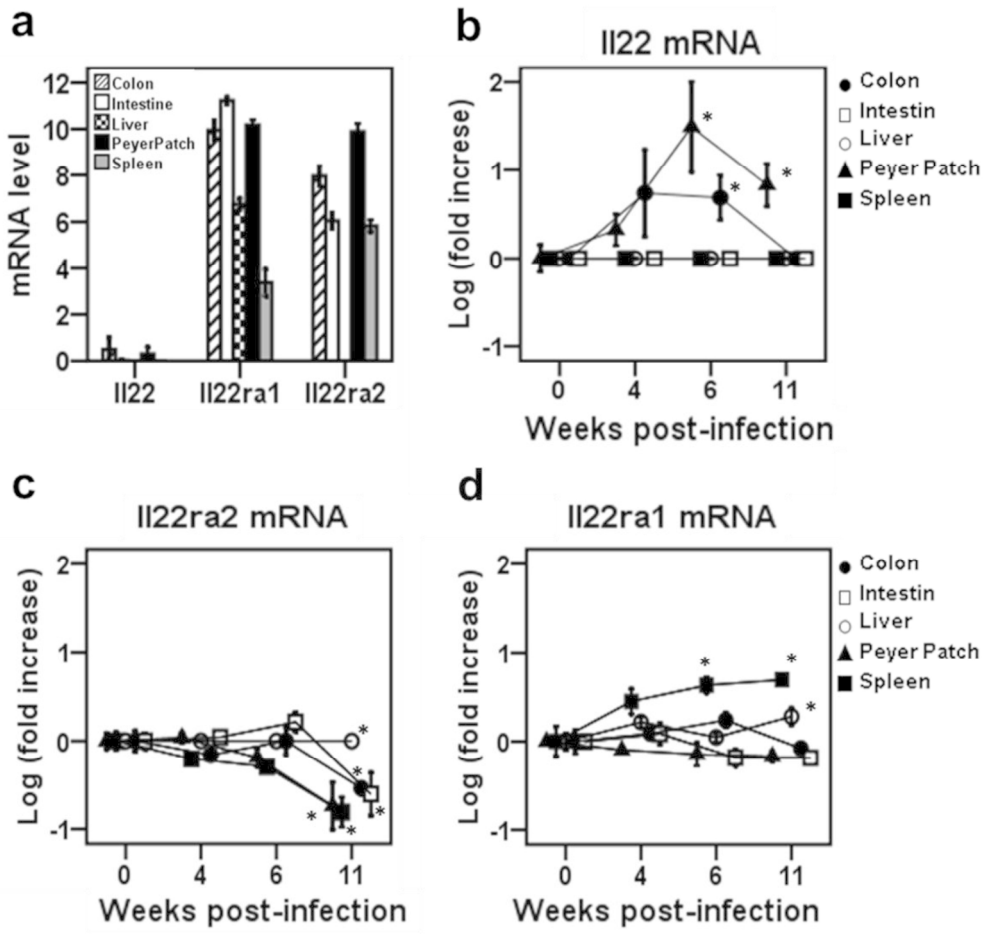
Table 4.

***IL22RA2* SNP rs6570136 and SNP rs2064501 are also associated with fibrosis and cirrhosis in humans infected with HCV**.

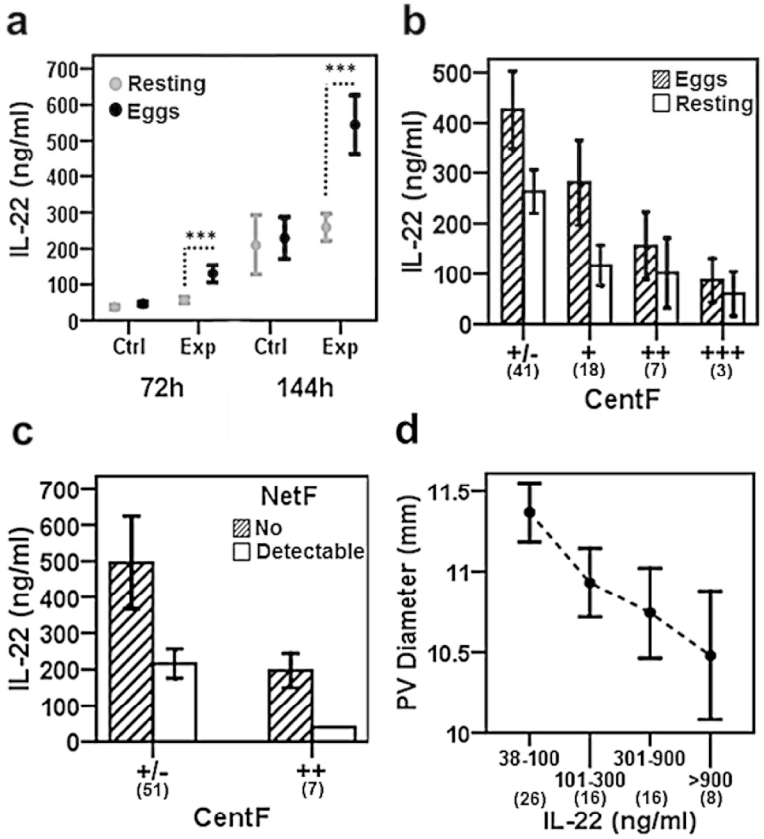
Severe and mild fibrosis phenotypes and the cohort size are described in Material and Methods and in Supporting Table 1. We first tested for associations between the SNPs and HF phenotypes separately (upper part of the table) and then tested the SNPs simultaneously by logistic regression analysis (lower part). Significant covariates (p<0.01) were age (45 yrs, <46 yrs) and alcohol intake. The aggravating genotypes are shown.

Supporting Table 1.

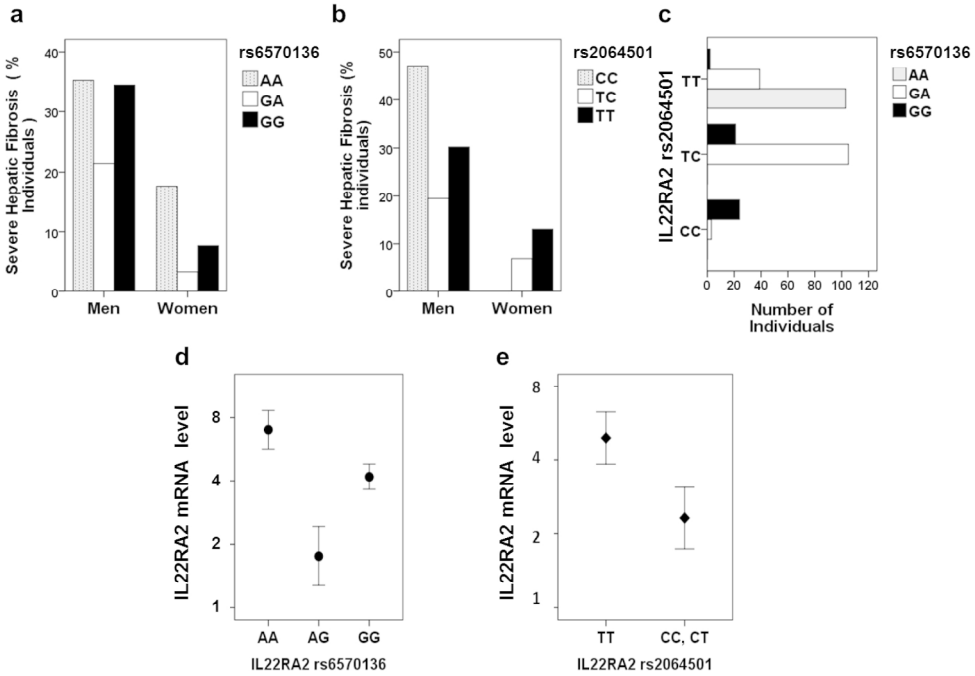
Primers used in the analysis of *IL22RA2*.



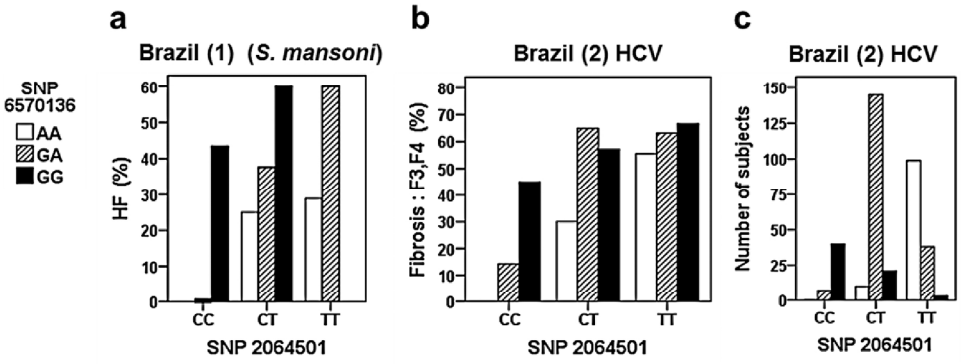
Schistosome eggs stimulate the production of IL22 mRNA and impair that of IL22RA2 in infected mice
190x187mm (300 x 300 DPI)



IL-22 response in humans infected with schistosomes. IL-22 concentrations correlate with protection against hepatic fibrosis and portal blood hypertension.
215x279mm (300 x 300 DPI)



IL22RA2 genotypes that are associated with severe fibrosis in Chinese Fishermen are associated with high IL22RA2 mRNA levels in healing skin.
190x129mm (300 x 300 DPI)



The same alleles that are associated with susceptibility to severe hepatic fibrosis in Brazilians infected with schistosomes are also associated with a high risk of fibrosis and cirrhosis in Brazilians infected with HCV.
192x79mm (300 x 300 DPI)

	Pathogen	N total (cases / controls)	Age (Mean) Case / controls Exposure (Mean) Case / Controls	Women: %case / %controls	Severe Fibrosis	Mild or No Fibrosis
Chinese	*S. Japonicum*	327 (122/205)	Age : 44.1 yrs / 52.2 yrs Fyrs : 25.5 yrs / 34.8 yrs	13.1 / 26.0	CL^H, D, E, F with GN^H CL^M with GN^M or GN^H	A, B, C, CL^L with GN0
Sudan	*S.mansoni*	217 (68/149)	Age = exposure 47.1 yrs / 50.7 yrs	17.2 / 28.7	CL, D, E, F with gall bladder wall thickening	A,B,C no gall bladder thickening
Brazil (1)	*S.mansoni*	186 (45/141)	Age : 46.6 yrs / 40 yrs	47.8 / 62.9	CL, D,E,F	A,B,C
Brazil (2)	HCV	532 (210/322) 364 (210/154)	F3F4 / F0F1 / F0: Age:57 yrs / 52.1 yrs / 53.7 yrs	57.0 / 53.8 / 52.9	F3+F4 (Cirrhosis)	F0+F1+F2 or F0+F1
Brazil (3)	HCV	149 (53/96)	F3F4 / F0F1 / F0: Age:57 yrs / 52.1 yrs / 53.7 yrs	39 / 46.2	F3+F4 (Cirrhosis)	F0+F1+F2

			Chinese Fishermen					
Analysis	**SNP**	**Bins**	**Genotype**	**Controls**	**Cases**	**p**	**OR**	**95% CI**
Univariate	**6570136**	I	**AA,GG**	45	62	0.003	2.07	1.5 - 5.4
	7774663	I	**CC,TT**	44	61	0.004	2.1	1.4 - 5.2
	7749054	II	**GG,TT**	46.2	54.8	0.12	1.45	0.9 - 2.3
	202563	III	**AA**	39.7	48.8	0.10	1.47	0.9 - 2.4
	276466	IV	**AA**	69.2	78.7	0.07	1.65	0.9 - 2.9
	11154915	V	**TT,TC**	25.6	34.6	0.10	1.5	0.9 - 2.6
	2064501	VI	**CC,TT**	53.6	66.4	0.01	2	1.2 - 3.3

			Sudanese Farmers						Brazilian Farmers					
Analysis	**SNP (rs)**	**Bins**	**Genotype**	**Controls**	**Cases**	**p**	**OR**	**95% CI**	**Genotype**	**Controls**	**Cases**	**p**	**OR**	**95% CI**
Univariate	**6570136**	I	**AA,GG**	59.7	75.4	0.03	2.1	1.1 - 3.9	**GG**	13	35.6	0.001	4.8	2.1 - 11
	7774663	I	**CC,TT**	47	67.7	0.006	2.4	1.3 - 4.4	**TT**	25.7	44.2	0.02	2.5	1.1 - 5.2
	7749054	II	**TT**	34.2	47.7	0.07	1.7	0.95 - 3.1	**TT**	47.2	67.4	0.02	2.4	1.2 - 4.9
	202563	III	**GG**	22.8	35.5	0.06	1.9	0.98 - 3.5	**GG,AG**	74.2	86	0.13	2.1	0.8 - 5.5
	276466	IV				>0.3			**AA**	26.7	41.9	0.07	2	0.9 - 4.2
	11154915	V	**TT**	0.7	6.3	0.04	9.4	1.1 - 85	**TT,TC**	75.4	87	0.1	2.1	0.8 - 5.6
	2064501	VI	**CC,TT**	65.1	80	0.03	2.1	1.1 - 4.3	**CC,CT**	56.7	66.7	0.05	2.1	1.0 - 4.6
Multivariate	**6570136**		**GG**			0.0007	8.2	2.4 - 28.0	**GG**			0.003	26	3.1 - 222
	2064501		**TT**			0.02	3.1	1.2 - 8.1	**TT,TC**			0.03	11	1.2 - 102

SUPPORTING MATERIALS AND METHODS

Study population and evaluation of hepatic fibrosis.

The human protocols were approved by the research ethics committees of the University of Gezira and the National Cancer Institute (Gezira, Sudan), the Hunan Institute of Parasitic Diseases (Yueyang, China), the University do Triangulo Mineiro (Uberaba, Brazil), Fiocruz (Salvador) and University Estadual de Pernambucco (Recife). The protocols were also approved by French INSERM ethics committees and by the CNIL.

All study samples are described in Table 1. Subjects infected with schistosomes were obtained from populations exposed to infection with *S. japonicum* (China) or *S. mansoni* (Brazil, Sudan) as described previously (26, 39). Individuals infected with HCV (genotypes 1, 2 or 3) were recruited among patients attending the outpatient clinic at the Instituto do Figado in Recife and at the Hospital das clinicas in Salvador (Brazil).

Evaluation of hepatic fibrosis.

Fibrosis in individuals infected with schistosomes was evaluated with Ultrasound (US) that was carried out with a portable ultrasound machine and a 3.5MHz convex probe (LOGIQ Book XP 2410786, Jiangsu, China), based on the standardized procedures of the World Health Organization (WHO) (40), Hepatic fibrosis in individuals infected with *S. mansoni* or *S. Japonicum* manifests as central fibrosis (CentF) around the central vein, and in *S. japonicum* infections, a network fibrosis pattern (NetF) in the parenchyma is also present. WHO guidelines graded CentF as A, B, C, CL, D, E, or F and NetF as GN if the diameter of the network mesh was less than 12mm (resembling fish scales) or GW if it is more than 12mm (resembling a tortoise shell). CentF A pattern is a normal liver struc A, diffuse echogenic foci or “starry sky” is graded CentF B, an uninterrupted thickness of the venous wall distinguished grade CL from grade C, a patchy pattern wa graded as D, E, and as F if around portal veins.

WHO grading was modified for *S. japonicum* infections as described (39) because 80% of Chinese fishermen had grade CL (CentF) and >40% of fishermen had grade GN (NetF). These large groups were inappropriate for further studies, so CL and GN were divided into three subgroups: light (CL^L/GN^L), advanced (CL^M/GN^M), and severe (CL^H/GN^H). CentF C^L was observed only in the left lobe of the liver, whereas CL^M and CL^H were observed in both liver lobes. Fibrosis in the right lobe of the liver extending to the second order branches was classified as CL^M, and thickness that extended further down the second order branches was classified as CL^H. For NetF, a network mesh < 2mm thick was classified as light GN (GN^L), a network mesh between 2 to 4 mm thick was classified as medium GN (GN^M), and a network mesh > 4mm thick was classified as heavy GN (GN^H). PV diameter (in mm) corresponded to the internal (inner to inner) diameter of the portal vein at the entry point of the portal vein into the liver. PV diameter was adjusted according to the average height of the healthy population from the same region. Only severe CentF CL^H and very severe CentF (D, E, F) and severe NetF (GN^H,

GW) were associated with portal hypertension. In Fig. 2.b, very light (B, C), light (CL^L) advanced (CL^M) and severe (CL^H) and very severe CentF (D,E,F) are coded CentF **+/-**, **+**, **++**, and **+++**, respectively. The genetic analysis uses binary phenotypes that included as cases, the Chinese fishermen with severe or very severe CentF or severe NetF and subjects with advanced CentF if they also had advanced or severe NetF. The inclusion of advanced CentF fishermen was necessary since the numbers of severe HF cases were too small (<100 individuals). Control groups included individuals who exhibited no NetF and light CentF or less. The HF cases in *S. mansoni* infections included D, E, and F grades. All other grades were considered as controls.

Fibrosis stages in HCV infections were determined on liver biopsies with the Metavir scale. All covariates (alcohol consumption, schistosome infections, mode of contamination, virus genotypes) were assessed either by interview or by genotyping. None of the HCV–infected individuals had active HBV or HIV infections.

Samples for immunological analysis (Fig.2) were obtained from the *S. japonicum* Chinese cohort, comprising 66 individuals with HF and 18 controls living in the same region. Controls had not been exposed to infection with *S. japonicum* and were negative when tested by ELISA using (IgG) schistosome Ag. None of the cases and controls had active HBV infections and all cases had been treated with Praziquantel fewer than 10 times over the last 15 years. The mean age of cases and controls did not differ significantly and all individuals were >30 and <66 years old.

Cell cultures and cytokine titration:

PBMCs were separated from heparin-treated venous blood by Ficoll-Hypaque gradient sedimentation (400g for 30min at 18°C). PBMCs were washed and placed in cultures as described (39) and stimulated with 500 eggs / 1ml well prepared as described (39) Culture plates were incubated at 37°C under 5% CO_2, in a humid incubator. Supernatants were collected at 72h and 144h and stored at -80°C. IL-22 and IL-17A concentrations were determined in the supernatant by ELISA (DuoSet kit, R&D, detection limits, 16 pg/ml).

Cellular staining and cytometry analysis.

PBMCs from Chinese patients were stained *ex vivo* for surface markers with FITC-conjugated anti-CD4 and PE-Cy7-conjugated anti-CD3 (BD biosciences) antibodies. The labeled cells were treated for 5 hrs with 100 ng/ml phorbol-12-myristate-13-acetate (PMA) and 1 µg/ml ionomycin in the presence of monensin (Golgistop, BD biosciences). Cells were then fixed and permeabilized with BD Cytofix/Cytoperm according to the manufacturer's instructions and incubated with Alexa647-conjugated anti-IL-17 (eBiosciences) and PE-conjugated anti-IL-22 (R&D) antibodies. Isotype control antibodies were obtained from the corresponding manufacturers. Compensation settings were determined with Comp Beads (BD biosciences). Data were collected on a FACS Calibur cytometer with Cellquest software (BD biosciences) and analyzed with DIVA software (BD biosciences). Cells in the lymphocyte region were gated based on FSC and SSC proprieties and $CD3^+CD4^+$ cells or $CD3^-CD4^-$ cells in the lymphocyte gate were analyzed for the expression of IL-17 and IL-22.

RNA extraction and quantitative RT-PCR.

Liver, spleen, Peyer patches and intestine biopsies were conserved in RNAlater (Ambion, Life Technologies) at -80°C. Tissue (20-30mg of tissue) homogenization was carried out with the Precellys-24 device (Bertin Technologies, Ozymee), with ceramic beads (1.4mm in diameter, CK14), in 400 µl of lysis buffer RLT plus (Qiagen) supplemented with 4µl β-mercapto-ethanol. RNA was recovered with the RNeasy plus mini kit for liver, spleen, Peyer patches and intestine biopsies or the RNeasy mini kit for skin biopsies following the manufacturer's instructions (Qiagen). RNA integrity was assessed with a 2100 Bioanalyzer (Agilent).

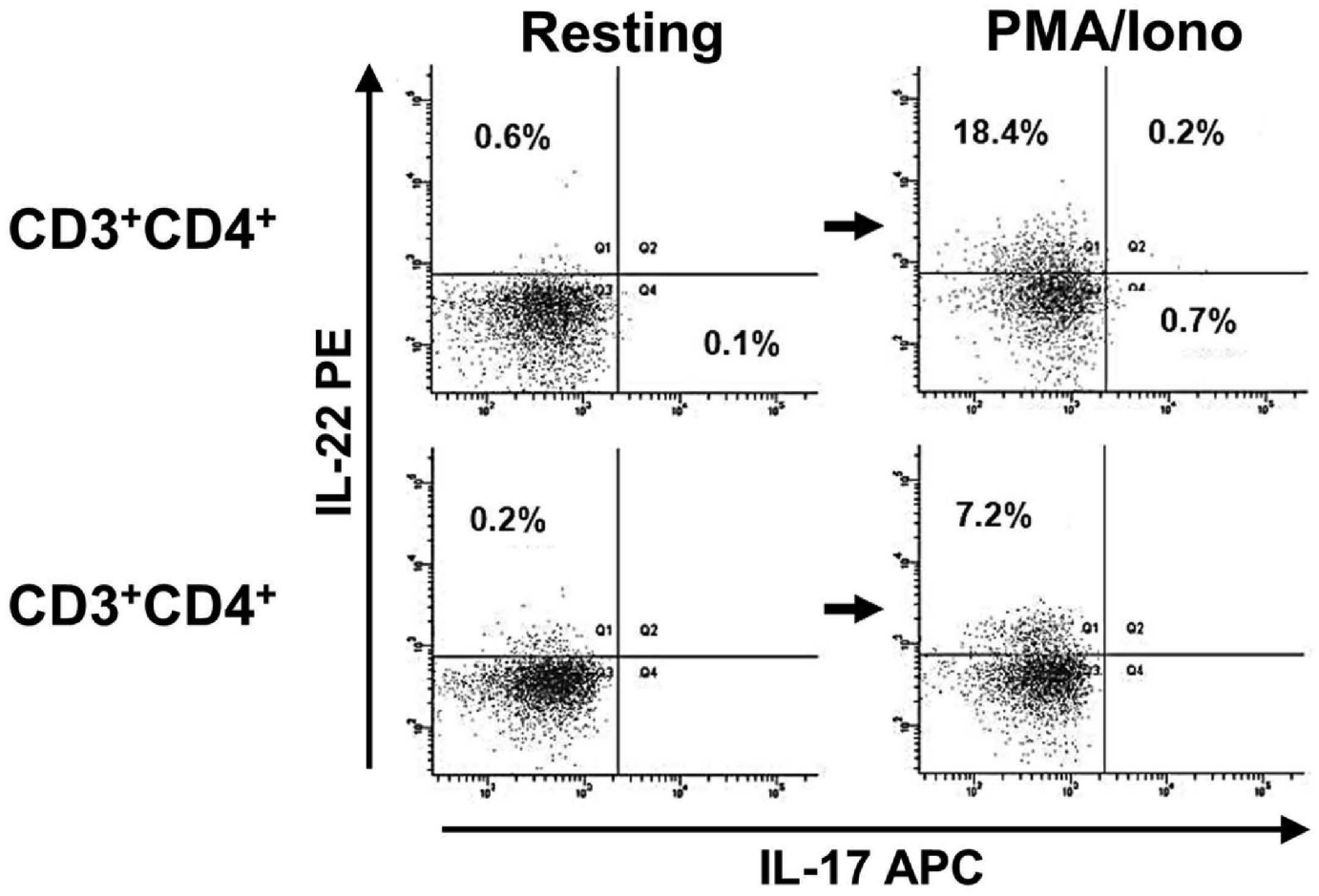
Total RNA (1µg), with a RIN > 7, was reverse-transcribed with the high Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time quantitative PCR, from 20ng of cDNA, was performed with the ABI 7900HT Fast Real Time PCR System and TaqMan Universal PCR Master Mix (Applied Biosystems, Life Technologies) following the manufacturer's protocols. The TaqMan gene expression assays: *Il22* (Mm00444241_m1), *Il22ra2* (Mm00617572_m1), *Il22ra1* (Mm00663697_m1), *Hprt1* (Mm00446968_m1) for mouse genes were from Applied Biosystems. Gene expression values were normalized to the value of the housekeeping gene *Hprt1* (hypoxanthine phosphoribosyltransferase).

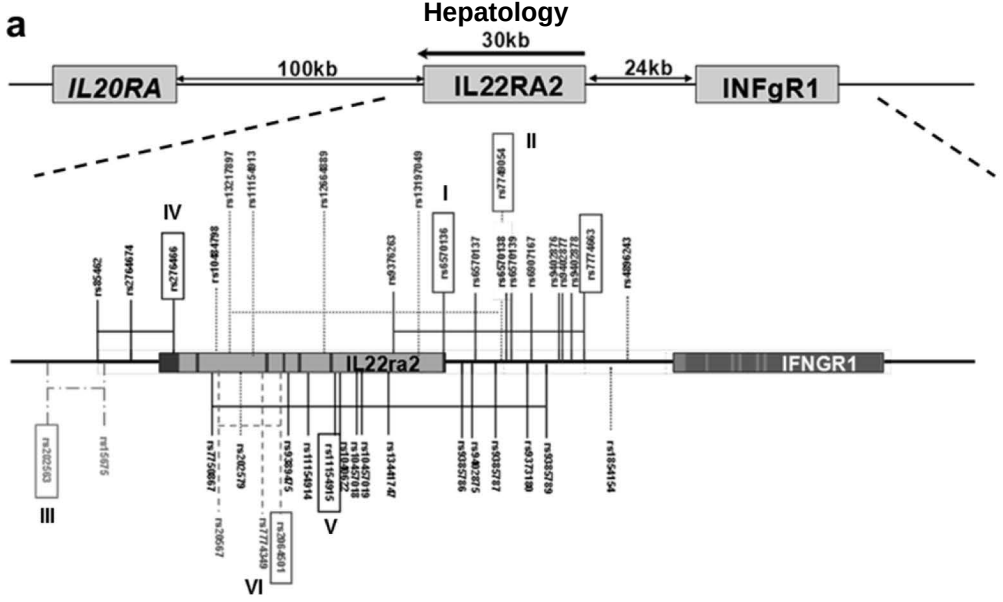
Genotyping

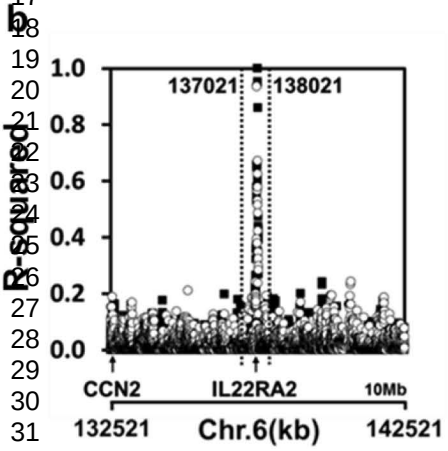
DNA extraction and genotyping were performed as in (26). Genotyped tag SNPs (Supporting Fig.2a) were selected from the 1000 Genomes Project and from the Hapmap database release #24, which contains three reference populations: Yoruba from Nigeria (YRI), Han Chinese from Beijing (CHB) and Utah residents of European ancestry (CEU). Genotyped SNPs had an R-square higher than 0.8 and minor allele frequency higher than 0.10. Primers are described in Supporting Table 2.

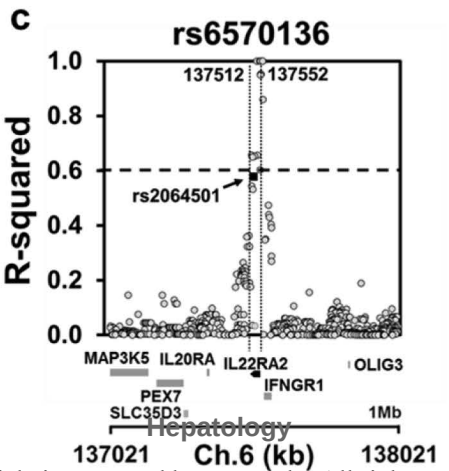
Statistical analysis.

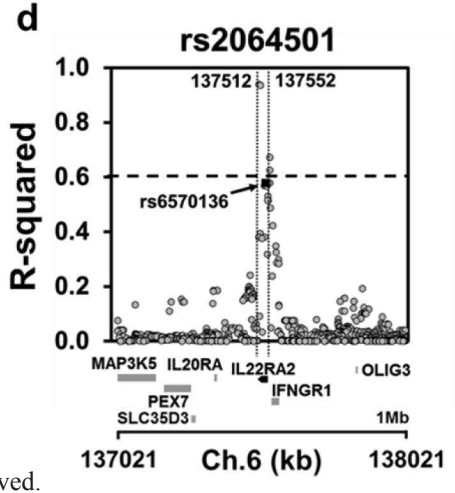
Group comparisons performed on data from Fig.1a-c were carried out with non-parametric Mann-Whitney tests with SPSS software. Linear regression analysis was performed to test for correlations between IL-22 concentrations, hepatic fibrosis and portal hypertension. CentF was divided into binary classes. NetF was divided in the same manner. Linear regression was performed with these binary variables (26) and with age, sex and exposure. Multivariate logistic regression (SPSS statistical software) was used to investigate possible associations of genotypes with advanced fibrosis or cirrhosis as described previously (26)











Bin	SNP	Position	Taqman Assay #	SNP sequence	MAF (HapMap) CEU	YRI	CHB
I	rs9376263	137489626	C__2523610_10	AGTAAA[C/T]AAATA	0.41 (T)	0.13 (C)	0.34 (C)
	rs6570136	137494622		TGGGAC[A/G]CCATGT	0.42 (A)	0.25 (G)	0.34 (G)
	rs6570137	137498645		CCCAGC[C/T]CTGCCT	0.38 (C)	0.13 (T)	0.37 (T)
	rs6570138	137501914		ACCCAC[G/T]CTACAT	0.41 (T)	0.25 (G)	0.34 (G)
	rs6570139	137502056		CTAAAA[A/G]AGTACA	0.41 (A)	0.12 (G)	0.31 (G)
	rs6907167	137503761		TTGTGA[G/T]TGATAG	0.41 (T)	0.25 (G)	0.33 (T)
	rs9402876	137509025		GAGTGA[C/T]TCATAA	0.41 (C)	0.09 (T)	0.32 (T)
	rs9402877	137509075		AACTAG[A/T]TCCTTG	0.41 (A)	0.13 (T)	0.34 (T)
	rs9402878	137509292		TGGAAA[G/T]AATTGA	0.37 (G)	0.29 (T)	0.33 (T)
	rs7774663	137510893	C__30217943_10	AAAAAA[C/T]CCTGGA	0.36 (C)	0.33 (T)	0.38 (T)
II	rs13217897	137471327		GGCAAT[A/G]CATGCA	0.18 (A)	0.26 (A)	0.44 (A)
	rs11154913	137474838		CAAGGC[A/G]TAATAT	0.17 (G)	0.27 (G)	0.44 (G)
	rs12664889	137481612		GCAGAG[A/C]CTGCCA	0.18 (A)	0.30 (G)	0.46 (G)
	rs13197049	137491211		TTTCTA[A/T]TCGGAA	0.18 (T)	0.26 (T)	0.44 (T)
	rs7749054	137500786	C__32241951_10	CCCTCT[G/T]CCTGGA	0.19 (G)	0.26 (G)	0.43 (G)
III	rs202563	137461492	C__3010272_10	TAAATT[A/G]TTCCAC	0.49 (G)	0.42 (A)	0.26 (G)
	rs156751	137463294		TCCACC[C/T]TTCTCC	0.49 (T)	0.19 (T)	0.26 (T)
IV	rs85462	137463154		AAACCT[C/G]GAAAGT	0.21 (G)	0.08 (G)	0.16 (G)
	rs276467	137464218		CTCCACT[A/G]ATAAGG	0.20 (A)	0.07 (A)	0.16 (A)
	rs276466	137466614	C__3010277_10	GAATGG[A/G]TAAACA	0.21 (G)	0.07 (G)	0.014 (G)
V	rs7750867	137470186		CCTTCC[C/T]GCATA	0.16 (T)	0.09 (T)	0.07 (T)
	rs9389475	137478484		TATCTC[C/T]AGCAAT	0.17 (T)	0.07 (T)	0.06 (T)
	rs11154914	137480411		GGTTCA[A/G]GGTTT	0.17 (G)	0.07 (G)	0.07 (G)
	rs11154915	137482982	C__9800072_30	CACTCC[C/T]GGGTTT	0.16 (T)	0.07 (T)	0.05 (T)
	rs1040622	137483258		AACTAG[C/T]GGGGCC	0.16 (C)	0.08 (C)	0.07 (C)
	rs10457018	137484893		CCAGAC[A/G]TAAGTG	0.16 (A)	0.08 (A)	0.07 (A)
	rs10457019	137484979		GGTCTC[A/G]GGAGAG	0.17 (A)	0.07 (A)	0.07 (A)
	rs13441747	137488608		AGGTGA[C/G]GTCTCG	0.17 (C)	0.09 (C)	0.07 (C)
	rs9385786	137497052		GTGTTT[C/T]GATTTT	0.16 (T)	0.07 (T)	0.06 (T)
	rs9402875	137498018		ATGAGC[A/C]GCCACC	0.18 (C)	0.08 (C)	0.06 (C)
	rs9385787	137500399		TGGAAG[C/G]CATTAA	0.17 (C)	0.08 (C)	0.07 (C)
	rs9373180	137503455		TCTATT[A/G]GTTCAG	0.17 (G)	0.04 (G)	0.07 (G)
	rs9385789	137505172		TGAGTG[A/T]TATAAG	0.17 (A)	0.08 (A)	0.07 (A)
VI	rs202567	137470844		GCTCCT[A/G]AATAAA	0.48 (G)	0.06 (A)	0.22 (A)
	rs7774349	137475858		ACAGAT[C/T]GCGAGA	0.48 (G)	0.06 (T)	0.21 (T)
	rs2064501	137477823	C__11693858_10	TTTATA[C/T]AATCTT	0.48 (T)	0.06 ©	0.23 (C)

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PLOS | NEGLECTED TROPICAL DISEASES

Extracellular Vesicles from *Leishmania*-Infected Macrophages Confer an Anti-infection Cytokine-Production Profile to Naïve Macrophages

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Abstract

Background: Extracellular vesicles (EVs) are structures with phospholipid bilayer membranes and 100–1000 nm diameters. These vesicles are released from cells upon activation of surface receptors and/or apoptosis. The production of EVs by dendritic cells, mast cells, macrophages, and B and T lymphocytes has been extensively reported in the literature. EVs may express MHC class II and other membrane surface molecules and carry antigens. The aim of this study was to investigate the role of EVs from *Leishmania*-infected macrophages as immune modulatory particles.

Methodology/Principal Findings: In this work it was shown that BALB/c mouse bone marrow-derived macrophages, either infected *in vitro* with *Leishmania amazonensis* or left uninfected, release comparable amounts of 50–300 nm-diameter extracellular vesicles (EVs). The EVs were characterized by flow cytometry and electron microscopy. The incubation of naïve macrophages with these EVs for 48 hours led to a statistically significant increase in the production of the cytokines IL-12, IL-1β, and TNF-α.

Conclusions/Significance: EVs derived from macrophages infected with *L. amazonensis* induce other macrophages, which *in vivo* could be bystander cells, to produce the proinflammatory cytokines IL-12, IL-1β and TNF-α. This could contribute both to modulate the immune system in favor of a Th1 immune response and to the elimination of the *Leishmania*, leading, therefore, to the control the infection.

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Introduction

Leishmaniases are a disease complex caused by about 21 trypanosomatid protozoa of the genus *Leishmania* [1–3]. Parasites of that genus have been co-evolving with their mammals and insect hosts for thousands of years. It is not surprising, therefore, that they are well adapted to these hosts and vectors, persisting and replicating in their tissues, subverting the immune response of the vertebrate host, and spreading to other hosts of the same or of another species. In the mammalian host, the *Leishmania* survives and replicates as amastigotes, mainly inside macrophages [4].

The immunity to *Leishmania* is associated with a parasite-specific Th1 immune response [5,6]. The release of IL-12 by macrophages and dendritic cells leads to production of IFN-γ by NK cells and differentiation of Th0 cells into Th1 cells, which also release IFN-γ. IFN-γ provides a key stimulus for the development of macrophage resistance to most intracellular pathogens, including the *Leishmania* [7,8].

Communication between cells occurs by different mechanisms. It can be, for instance, through growth factors, cytokines, nucleotides, lipids, nitric oxide, peptides, and adhesion molecules. An additional mechanism is by means of extracellular vesicles (EVs). The immune system comprises a group of often physically isolated cells that intensely communicate among themselves. The production of EVs by dendritic cells, mast cells, macrophages, and B and T lymphocytes has been extensively described in the literature [9]. These EVs carry the phenotypic characteristics of the cells they originate from. Thus, depending on their origin, they may express MHC class II molecules and may transport antigens [10,11].

Macrophages infected by microorganisms produce EVs, which can induce inflammatory responses *in vitro* and *in vivo* [12–15]. Because of their immune modulatory activities, EVs are being investigated as components of future vaccines [16–19]. Three main types of EVs have been described in the literature: exosomes, microparticles and apoptotic bodies [10].

Author Summary

Leishmaniases are a group of diseases—each one individually called leishmaniasis—that are caused by the protozoan *Leishmania*. They affect millions of people and thousands of dogs in tropical and mediterranean countries. Macrophages are the main cellular hosts of *Leishmania* in the mammalian host, where it is an obligatorily intracellular parasite. In this work, it is shown that mouse bone marrow-derived macrophages, when infected *in vitro* with *Leishmania*, release small (no larger than 300 nm) extracellular vesicles (EVs), in the same way as uninfected macrophages. The EVs from the infected macrophages, however, induce in other macrophages the production of some cell hormones, named cytokines, which are involved with protection of the macrophage against infection and with the development of a protective immune response against the parasite.

Exosomes are small vesicles of endosomal origin that are released by various cell types, including macrophages, and that are capable of modulating the immune response [20]. They range in size from 40 to 100 nm in diameter and have rounded and flattened morphology [21]. When derived from antigen-presenting cells, they can promote the adaptive immune response by presenting MHC class II molecule-peptide complexes to naïve $CD4^+$ T cells, as well as by inducing specific $CD8^+$ T-cell responses [22–25].

It has been demonstrated that exosomes may carry antigens from tumour cells [26] and from cells infected with intracellular microorganisms such as mycobacteria [27], cytomegalovirus [28], and *Leishmania* [29].

Microparticles are spherical bodies formed from the budding off of cell plasma membranes after cell activation and apoptosis, with diameters ranging from 100 to a 1000 nm, and which contain cytoplasmic components, such as proteins and nucleic acids [30]. They can be characterized by the presence of surface molecules from the cells from which they were derived [10,31]. Microparticles from macrophages infected with *Mycobacterium tuberculosis* carry mycobacterium antigens and promote inflammation by activating $CD4^+$ T cells [32]. Those from macrophages infected with *Listeria monocytogenes* also carry microorganism antigens and elicit a protective immune response [33]. Some studies suggest that macrophages are activated by microparticles via the toll-like receptor 4 (TLR4) [34].

Unlike other EVs, which are released either by cell activation or in the early stages of apoptosis, apoptotic bodies are formed in the final stages of the apoptotic process. They have larger diameters (1–5 μm) than the other EVs and display phosphatidylserine molecules on their membranes [35]. They have also been shown to induce immune responses [36].

In the present work, EVs obtained from *Leishmania amazonensis*-infected macrophages from BALB/c mice were compared with EVs from uninfected macrophages in terms of eliciting the production of cytokines by naïve macrophages. The BALB/c mouse-*L. amazonensis* combination was chosen because that *Leishmania* species causes a progressive cutaneous disease in that strain of mice, and we hypothesized that macrophage-derived EVs could have infection promoting properties.

It was found, on the contrary, that EVs from *L. amazonensis*-infected macrophages promote the release by other macrophages of cytokines that usually induce inflammatory responses.

Methods

Ethics Statement, Animals and Parasites

Four to 8 week-old BALB/c mice were used for collecting resident macrophages and bone marrow cells. The animals received balanced feed and water *ad libitum*. All procedures were performed in accordance with the ethics principles in animal research of the Brazilian law 11784/2008 and were approved by the Ethics Committee for Animal Research of the Gonçalo Moniz Research Centre, Oswaldo Cruz Foundation, Salvador, Brazil (protocol no. 003/2013). *L. amazonensis* (MHOM/BR88/BA/125) parasites were maintained by regular passages in Golden hamsters. The promastigotes derived from tissue amastigotes were grown under sterile conditions at 24°C in Schneider's medium (Schneider's drosophila medium, Sigma-Aldrich Chemical Co., St. Louis, MO, USA) containing 50 $\mu g.mL^{-1}$ gentamicin and 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA).

Infection of Macrophages and Preparation of Extracellular Vesicles

Bone marrow cells were differentiated into macrophages by 7–8 days incubation with RPMI (supplemented with 10% FBS, 50 μg/mL of gentamicin, 3.6 g/L of sodium bicarbonate, 25 mM HEPES, 2 mM glutamine and 30% supernatant of culture of GM-CSF–expressing cells), as described in the literature [37]. Two$\times 10^5$ macrophages were incubated with stationary-phase *L. amazonensis* promastigotes at parasite to macrophage ratio of 50:1 in 1 mL volumes of RPMI supplemented with 10% FBS, 50 μg/mL gentamicin, 3.6 g/L sodium bicarbonate, 25 mM HEPES, 2 mM glutamine (supplemented RPMI) during 6 hours, followed by a washing to remove non-internalized parasites. To isolate the EVs, the infected and control non-infected macrophages were cultured for 9 days in supplemented RPMI. After the 9-day incubation, the culture supernatants were centrifuged at 500 g for 10 minutes at 4°C, 1500 g for 10 minutes at 4°C and 8,000 g for 5 minutes at 4°C to remove residual cells and cellular debris. The EVs present in the supernatants were washed once with Hanks' balanced salt solution (HBSS) by centrifugation at 100,000 g for 45 minutes at 4°C and resuspended in cold HBSS. The protein concentrations in the extracellular vesicle preparations were determined with the BCA kit (Thermo Scientific, Rockford, USA) in accordance with the manufacturer's instructions.

The mean yields of EVmedium and EVLa obtained from 1-mL cultures of 2×10^5 macrophages, plus or less one standard deviation, were $5.2\times10^7\pm0.4\times10^7$ and $5.0\times10^7\times1.2\times10^7$ EVs, respectively. These numbers were determined by flow cytometry as described below.

Cells were also cultured over glass coverslips and stained with hematoxylin and eosin (H&E) for determining the infection rate.

The FBS used in the cultures was previously ultracentrifuged at 100.000 g for 4 hours in order to precipitate the EVs it probably had in suspension.

Characterization of EVs and Bone Marrow-Derived Macrophages, and Enumeration of EVs, by Flow Cytometry

For characterization of the EVs, the macrophages (in cultures inside wells of 24-wells culture plates) were washed twice with 0.15 M phosphate-buffered saline, pH 7.2 (PBS) and incubated with a 2 μM PKH26 (Sigma-Aldrich, St. Louis, MO, USA) solution or with a 20 μM carboxyfluorescein diacetate (CFDA; Sigma-Aldrich, St. Louis, MO, USA) solution in PBS for

2–5 minutes at room temperature with gently shaking. The staining reaction was stopped by adding an equal volume of FBS and incubating for 1 minute at room temperature. The macrophages were then washed once with PBS to remove the staining solution and cultured with supplemented RPMI. The culture supernatants were centrifuged for isolation of the EVs as described above, and the EVs were mixed together with 1 μm-diameter fluorescent beads (Invitrogen, Carlsbad, CA, USA) and analyzed in a flow cytometer.

Monoclonal antibody (mAb)-fluorochrome conjugates (clone M1/70 phycoerythrin—anti-CD11b mAb, clone BM8 phycoerythrin-cyanine—anti-F4/80 mAb, and clone NIMR-4 FITC—anti-mouse MHC II mAb) were from eBioscience (San Diego, CA, USA). Annexin V-FITC and propidium iodide were from Sigma (Apoptosis Detection Kit; Sigma-Aldrich, St. Louis, MO, USA). The flow cytometry technique was carried out as described in the literature [38]. Briefly, the cells or EVs were washed once with flow cytometry buffer (PBS containing 5% FBS), incubated with the conjugates on ice for 20 min and washed twice with flow cytometry buffer. The cell or extracellular vesicle suspensions were then washed once with PBS and analysed in a flow cytometer.

The enumeration of EVs was done by flow cytometry, based on the number of events occurring in a gate for the right-size particles in the time that 500 fluorescent beads (Invitrogen, Carlsbad, CA, USA) were counted. This was performed in four extracellular vesicle preparations from *L. amazonensis*-infected macrophages and four control preparations from non-infected macrophages.

Treatment of Macrophages with Membrane Vesicles

Peritoneal cells without stimulation with thioglycollate were obtained by washing the peritoneal cavity with 0.9% NaCl containing 20 IU.mL heparin. The cells were centrifuged at 300 g for 10 minutes at 4°C and resuspended in supplemented medium. One million large mononuclear peritoneal cells were distributed in each well of 24-well plates. After a 24-hour incubation period at 37°C, the non-adherent cells were discarded by replacement of the culture supernatants by new medium. More than 90% of the adherent cells were shown to be positive for CD11b and F4/80 by the flow cytometry technique described above (data not shown), a fact that characterized them as being constituted mostly by macrophages. These peritoneal macrophages were incubated for 48 hours with EVs from uninfected bone marrow-derived macrophages (EVmedium) and with EVs from *L. amazonensis*-infected bone marrow-derived macrophages (EVLa) with or without 1 ng/mL bacterial lipopolysacharide (LPS; Escherichia coli 0127:B8, Sigma-Aldridge, St. Louis, MO, USA), at 37°C and 5% CO_2. In cultures that received EVmedium and EVLa in the absence of LPS, 10 μg/mL of polymyxin B (Sigma-Aldridge, St. Louis, CA, USA) were added to rule out the possibility that a putative LPS contamination could compromise the interpretation of results.

The extracellular vesicle concentrations used in the treatment were the same concentrations in which the EVs were in the macrophage culture supernatants from which they were isolated. The mean numbers of EVmedium and EVLa added to the cultures, plus or less one standard deviation, were $5.2\times10^7\pm0.4\times10^7$ and $5.0\times10^7\pm1.2\times10^7$, respectively. Approximately 52 EV medium and 50 EVLa were, therefore, added per macrophage. The total amounts of protein contained in the added preparations were 11.4±1.1 μg for EV medium and 10.0±1.0 μg for EVLa.

Macrophage Viability Analysis

The supernatants of cultures in 24-well plates of bone marrow-derived macrophages were replaced by RPMI containing 10% Alamar blue (Invitrogen, Carlsbad, CA, USA) on the 1st, 3rd, 6th, 9th and 12th days after infection with *L. amazonensis*, or to cultures of control uninfected macrophages, in triplicates. After incubation for 4 hours at 37°C and 5% CO_2, 200 μL volumes of the supernatants were transferred to wells of 96-well microtiter plates and the absorbance for light with wavelengths of 570 and 600 nm was read. As assay controls, wells of 96-well microtiter plates received only culture medium or only medium with Alamar blue.

Cytokine Detection

The concentrations of cytokines (IL-6, TNF-α, IL-12p70, IL-1β and IL-10) in culture supernatants were determined with the Ready-Set-Go kit (eBioscienses, San Diego, CA, USA), in accordance with the manufacturer's instructions.

Electron Microscopy

EVs were fixed with sodium cacodylate buffer and 2% paraformaldehyde for 2 hours at room temperature. After fixation, the EVs were centrifuged at 100,000 g for 45 minutes, resuspended in PBS, and passed through filters with 0.22-μm diameter pores. The EVs were adsorbed to copper (Formvar) grids for 20 minutes. Two % phosphotungstenic acid was used for negative staining. The EVs were visualized in a transmission electron microscope (Zeiss EM 109; Munich, Germany).

Macrophages were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer. Post-fixation was performed with 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM calcium chloride in 0.1 M sodium cacodylate buffer. Dehydration was performed with acetone and the material mounted in resin (Polybed 812). The resin block was cut in an ultramicrotome, contrasted, and the material observed in the transmission electron microscope.

Statistical Analyses

To compare the levels of a given cytokine in cultures treated with either EVLa or EVmedium, the following assumptions were made:

1. the distribution of the data was non-gaussian [this assumption was made because the number of different EVLa and EVmedium preparations tested (n = 6) was too small to allow the determination of the type of distribution];
2. the EVLa would stimulate the production of either proinflammatory cytokines or regulatory cytokines, or of both, more than the EVmedium, and the EVs would stimulate the production of one or more of these cytokines so that their concentrations in the culture supernatants would be higher than in the supernatants of unstimulated macrophage cultures.

These two assumptions determined that a directional non-parametric method should be applied. The Wilcoxon's signed-rank test was therefore used to determine the statistical significance of differences in cytokine levels in six independent experiments, and the Mann-Whitney's U test was used to determine the statistical significance of differences in macrophage viability and extracellular vesicle numbers. The differences were considered significant when P was ≤ 0.05.

Results

Characterization of Macrophages of Myeloid Origin and of Their Infection by *L. amazonensis*

The cells differentiated from bone marrow cells were stained with surface antibodies specific for murine monocytes (anti-CD11b

and anti-F4/80) for the purpose of evaluating their phenotype by flow cytometry. A percentage of 88% of cells double–labelled for anti-F4/80 and anti-CD11b was observed (Fig. 1A), indicating that at least the vast majority of them were macrophages.

The infection of macrophages by *Leishmania* was evaluated by optical microscopy of H&E stained slides. The number of intracellular parasites increased with time. After nine days of culture, at the moment that the EVs were collected, about 100% of the cells were infected (Fig. 2A), with an average of 16 parasites per macrophage (not shown).

Characterization of EVs and Annexin V-Staining of Macrophages

The EVs had diameters smaller than 1 μm, as demonstrated by flow cytometry in the presence of 1 μm-diameter beads (Fig. 1B). Their presence was demonstrated, by flow cytometry, in the supernatant of macrophages that had been labelled with PKH26 (Fig. 1C). The EVs were also stained with CFDA (not shown).

The EVs derived from these macrophages, infected or not with *L. amazonensis* and incubated for a period of 9 days, stained with antibodies specific for murine monocytes (anti-CD11b and anti-F4/80), as well as for the antigen-presenting molecule MHC II (Fig. 1D, E, F). About 45% of the EVs stained with Annexin V (Fig. 1G). There were no statistically significant differences between EVLa and EVmedium in terms of binding to Annexin V.

About 23% of infected macrophages stained with Annexin V, whereas a smaller proportion (14%) of uninfected macrophages stained with that reagent (not shown). In accordance with this finding, there was a more prominent reduction of macrophage numbers in infected cultures than in uninfected cultures (Fig. 2B).

Transmission electron microscopy by negative contrast allowed the visualization of EVs and the observation of their heterogeneity in size. This visualization method has been described as appropriate for showing the presence of EVs [39]. The visualized EVs had spherical shapes and diameters ranging from 50 to 300 nm (Fig. 3A).

The macrophages infected with *L. amazonensis* were also evaluated by transmission electron microscopy, through which it was possible to visualize the formation of microparticles by blistering of the infected cell after nine days of culture (Fig. 3B).

The content of the vesicles had similar density to the cytosol and they were surrounded by cytoplasmic membrane (Fig. 3B).

Leishmania-Infected and Uninfected Macrophages Release Comparable Amounts of EVs

The number of EVs was measured in the supernatants of infected and uninfected macrophages by flow cytometry in four independent experiments. The values acquired in the supernatants of 9-day cultures of 2×10^5 infected macrophages (median = 5.0×10^7; range = 3.5×10^7 to 6.0×10^7) and of the same number of uninfected macrophages (median = 5.2×10^7; range = 4.7×10^7 to 5.8×10^7) did not differ significantly ($p>0.05$, Mann-Whitney test).

EVs Derived from *L. amazonensis*-Infected Macrophages Induce the Production of Proinflammatory Cytokines by Naïve Macrophages

The ability of EVs derived from macrophages, infected or not with *L. amazonensis* for 9 days, to stimulate cytokine production by resident peritoneal macrophages was evaluated. The peritoneal macrophages were incubated with the EVs in the presence or absence of LPS for 48 hours. Cultures to which LPS was not added also contained polymyxin B in order to rule out the effect of endotoxin that could be contaminating the preparations. Similar results were observed in experiments with and without polymyxin B (data not shown).

Increased levels of IL-12p70 (i.e., levels of IL-12p70 above zero in the graphs) could be observed in cultures of EVLa-treated, unstimulated or LPS-stimulated macrophages in all six experiments (Figs. 4A and 4B). These increases (in relation to cultures to which no EVs were added) were statistically significant ($p<0,025$, Wilcoxon's signed-rank sum test). There was no statistically significant differences ($p>0.05$, Wilcoxon's signed-rank sum test) between the results obtained in the groups of cultures that received EVmedium in the six experiments, with and without LPS, and the results of their respective control groups (that received no EVs), despite the fact that increases in IL-12p70 levels could be observed in 2 out of 6 experiments in cultures of EVmedium-treated macrophages to which no LPS was added (Fig. 4A) and in 3 out of 6 experiments in LPS-stimulated cultures of EVmedium-treated macrophages (Fig. 4B).

The addition of both EVmedium and of EVLa to cultures without LPS led to the increased production of IL-1β in all six experiments (Fig. 4C; $p<0.025$, Wilcoxon's signed-rank sum test). On the other hand, the addition of EVmedium to LPS-stimulated cultures increased the production of IL-1β in only three out of six experiments (Fig. 4D, $p>0.05$, Wilcoxon's signed-rank test), whereas the addition of EVLa increased the production of IL-1β in all six experiments (Fig. 4D; $p<0.025$, Wilcoxon's signed-rank sum test).

When the cultures of macrophages treated with EVLa were compared with those treated with EVmedium in terms of the levels of IL-12p70 and IL-1β that were present in their supernatants in all six experiments, the addition of EVLa to both unstimulated and LPS-stimulated cultures led to higher levels of IL-12p70 and IL-1β than the addition of EVmedium (Figs. 4A, 4B, 4C, and 4D; $p<0.025$, Wilcoxon's signed-rank sum test). Notwithstanding this statistically significant difference, the difference in IL-1β levels in cultures of macrophages not stimulated with LPS when they were treated with EVLa or treated with EVmedium was minimal in 3 out of 6 experiments (Fig. 4C).

TNF-α levels were increased in cultures of EVLa-treated macrophages, treated or non-treated with LPS, in all six experiments (Figs. 4E and 4F; $p<0.05$, Wilcoxon's signed-rank sum test). TNF-α levels were also higher in cultures of EVmedium-treated macrophages, treated or non-treated with LPS, than in cultures to which no EVs were added, in 4 out of 6 and in 3 out of 6 experiments, respectively (Figs. 4E and 4F). However, these increases were not statistically significant ($p>0.05$, Wilcoxon's signed-rank sum test).

Clearly higher levels of TNF-α in cultures of EVLa-treated macrophages than in cultures of EVmedium-treated macrophages were observed in only two experiments in the absence of LPS (Fig. 4E) and in 5 out 6 experiments in the presence of LPS (Fig. 4F; $p<0.05$, Wilcoxon's signed-rank test).

No statistically significant differences in the levels of IL-6 and IL-10 in cultures to which EVmedium or EVLa were added were seen (Figs. 4G, 4H, 4I and 4J). Despite that, it can be noted that in 3 out of 6 cultures containing LPS, the concentrations of IL-10, an immune regulatory cytokine [40,41], were lower with the addition of EVLa than when EVmedium was added (Fig. 4J). In the case of IL-6, a proinflammatory cytokine [42], the contrary was seen: in 3 out of 6 cultures containing LPS, the concentrations of that cytokine were markedly higher with the addition of EVLa than when EVmedium was added (Fig. 4H).

Discussion

EVs have different membrane compositions, depending on the cell from which they have originated (reviewed in [10]). The fact

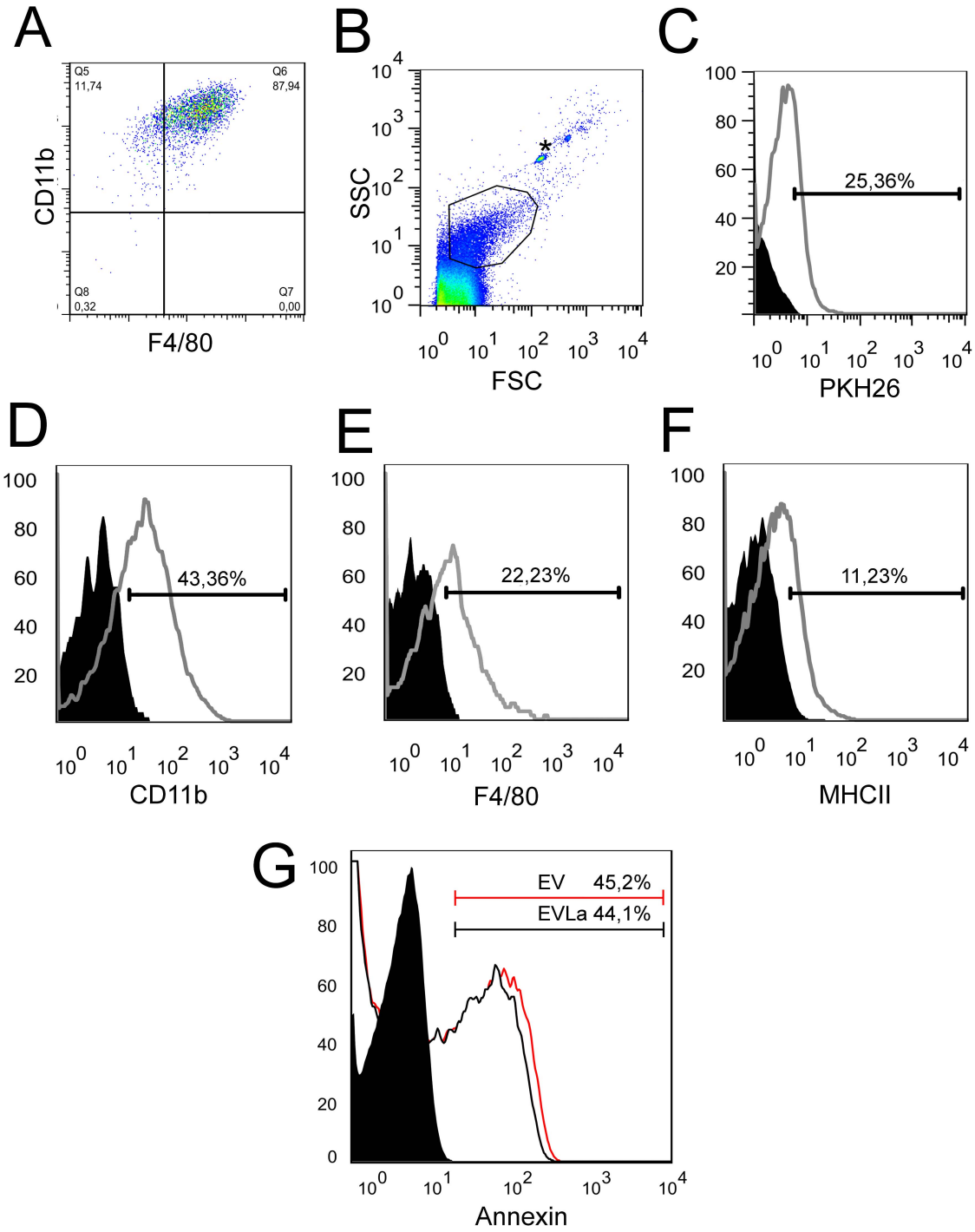

Figure 1. Flow cytometry analysis of bone marrow-derived macrophages and their extracellular vesicles (EVs). A, macrophages obtained by differentiation from bone marrow cells treated with GM-CSF were phenotyped with anti-F4/80 and anti-CD11b. 87.9% of the cells were double labeled. B, side scatter (SSC) and forward scatter (FSC) analysis showing the selection of the EV population, which is demarcated in the highlighted area. Beads with 1 μm in diameter (whose position is indicated by the asterisk) were used as reference. C, EVs derived from cell membranes labelled with PKH26. Curves with areas underneath in black were produced by unlabelled EVs and with areas underneath in white were produced by labelled EVs. D, E, and F, EVs were labelled with anti-CD11b, anti-F4/80 and anti-MHC type II, respectively. The curves with areas underneath in black were produced by unlabelled EVs and curves with areas underneath in white were produced by labelled EVs. G, annexin V-labelled EVs that had been collected on the 9th day of macrophage culture. The curves with areas underneath in black were produced by unlabelled EVs from uninfected macrophages and from infected macrophages, curves with areas in gray were produced by labelled EVs from uninfected macrophages, and curves with areas in red were produced by labelled EVs from infected macrophages.
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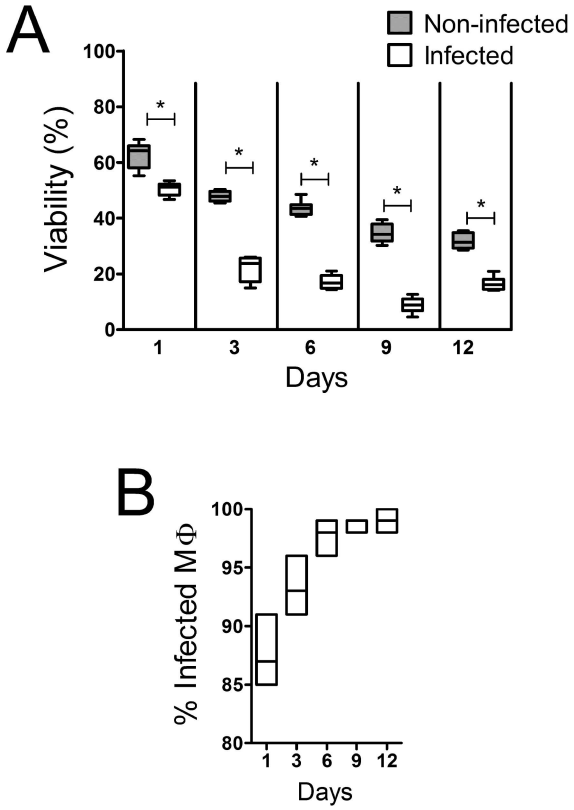

Figure 2. Macrophage viability and infection rate by *L. amazonensis*. A 50 parasites per macrophage ratio was used during the infection. A, percentage of chemical reduction of Alamar blue, relative to control, in macrophages infected with *L. amazonensis*. B, kinetics of infection. To determine the rate of infection, the macrophages were grown on coverslips, stained with HE, and the amastigotes visualized by optical microscopy (at least 200 macrophages were analysed per coverslip). The horizontal lines represent the median values of hexaplicates (A) or triplicates (B), the boxes the interquartile intervals (A) or the range (B), and the vertical bars the ranges (A). * $p < 0.05$, Mann-Whitney test.
doi:10.1371/journal.pntd.0003161.g002

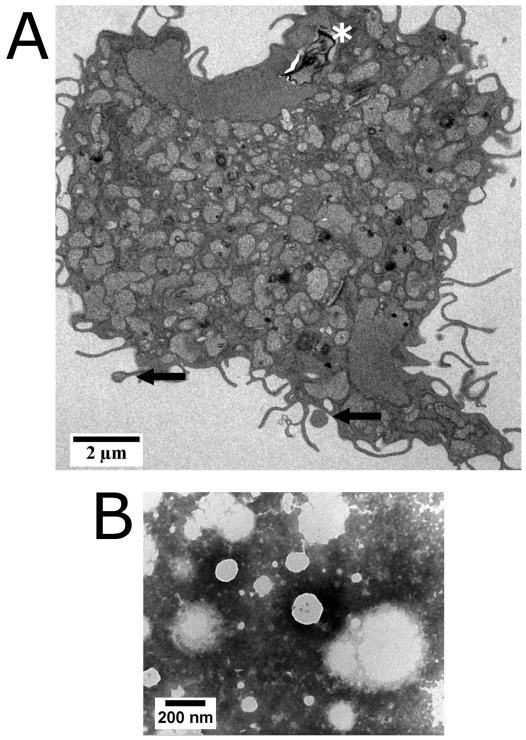

Figure 3. Transmission electron microscopy of extracellular vesicles (EVs) from supernatants of infected macrophage cultures and of *L. amazonensis*-infected macrophages presenting budding EVs. A, a macrophage was fixed with 2.5% glutaraldehyde and 2% paraformaldehyde and post-fixed with 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM calcium chloride. The arrows indicate EVs being formed; the dark asterisk at the top of the figure indicates the position of the remainings of a *Leishmania* amastigote. Scale bar = 2 µm. B, EVs were adsorbed to copper grids (Formvar) for 20 minutes and negatively stained with 2% phosphotungstenic acid. Scale bar = 200 nm.
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that the EVs present in the supernatants of macrophages in this work had the surface markers CD11b and F4/80, as well as MHC class II, indicates their macrophage origin.

At least part of the EVs seems to have originated from apoptotic cells, since about 45% of both EVLa and EVmedium stained with Annexin V, which binds to phosphatidylserine molecules that are exposed in cell membranes during the apoptotic process [43]. The observation that some of the EVs were stained by Annexin V is consonant with the finding that a proportion of the macrophages were also stained by that reagent.

Staining by PKH26 confirmed the presence of lipid membranes associated with the EVs. The integrity of the EVs was demonstrated by flow cytometry with CFDA, a substance that permeates intact cell membranes and enters the cytosol, where it is converted by esterases into a green fluorescent derivative [44].

The formation of microparticles by blistering of macrophages infected with *L. amazonensis* could also be visualized by transmission electron microscopy. A similar formation of EVs in dendritic cells treated with LPS after 6 hours of cultivation was described by Obregon and collaborators [45].

The presence of *bona fide* EVs in the 100,000 g pellet of macrophage supernatants used in the present work was therefore confirmed by several parameters. Judging by their size (50 to 300 nm as shown by negative-staining transmission electron microscopy), the EVs could consist exclusively of microparticles or of microparticles and exosomes. Apoptoptic bodies could be ruled out, despite the staining of a proportion of the EVs with Annexin V, because they have diameters larger than 1 µm. As was mentioned in the Introduction, microparticles are also released by apoptotic cells. As around 45% of the EVs studied in this work stained with Annexin V, one can conclude that at least that amount of EVs are microparticles.

An interesting finding in the present work was that EVLa stimulated the production of IL-12p70, IL-1β, and TNF-α by uninfected macrophages. These macrophages could have been previously treated or not with LPS. Similar results were observed in experiments with macrophages not treated with LPS in the presence or in the absence of polymyxin B, indicating that putatively contaminating endotoxin did not participate in the obtained results. The carrying out of some experiments in the presence of bacterial LPS, which activates macrophages by leading to signal transduction by TLR4, was done in order to better emulate the *in vivo* situation, in which neutrophil elastase may stimulate macrophages via TLR4 [46].

The EVLa used in the present work were obtained from macrophages that were infected with a 50:1 *Leishmania*:macrophage ratio and cultured for 9 days *in vitro*. The possibility that the

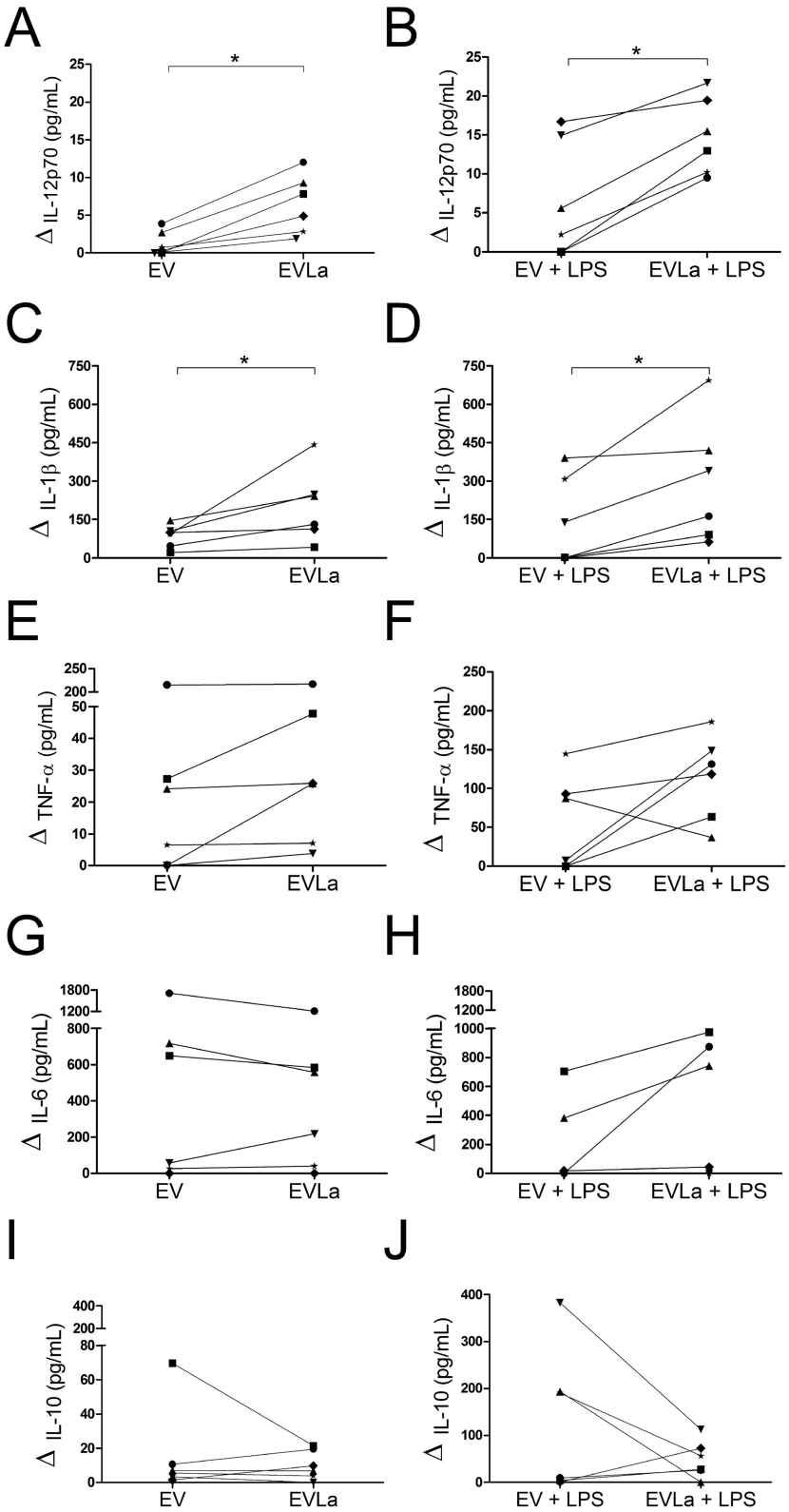

Figure 4. Effect of membrane vesicles from macrophages infected with *L. amazonensis* (EVLa) or not (EV) and stimulated with LPS or not on cytokine production by naïve macrophages. A and B, IL-12p70. C and D, IL-1β. E and F, TNF-α. G and H, IL-6. I and J, IL-10. A, C, E, G, and I, unstimulated macrophages; B, D, F, H, and J, macrophages stimulated with bacterium lipopolysacharide for 48 hours. The symbols and lines represent the averages of replicates of individual experiment. Data from six independent experiments are represented. Values obtained from cultures to which no EVs were added were subtracted from the data shown. *, $P<0.025$; Wilcoxon's signed-rank sum test.
doi:10.1371/journal.pntd.0003161.g004

EVs released by macrophages infected with different numbers of parasites, or collected at different times after infection, differ in terms of biological activity from the EVs studied in the present work, should justify the carrying out of additional investigation.

A certain degree of variation in the pattern of secreted cytokines was observed among the six independent experiments that were carried out, both with the preparations of EVmedium and EVLa. This fact, which could depend on the batch of the EVs, on the responsiveness of the naïve macrophages, or on both, could have hindered the obtaining of statistically significant results for some cytokines. A great variation in the response of individual macrophage preparations to LPS was observed (data not shown), indicating that the responsiveness of the naïve macrophage preparation was, at least in part, responsible by the observed variations in the response to EVs. This variation did not, however, affect the acquisition of consistent results for IL-12p70, IL-1β, and TNF-α when the EVLa were tested.

One of the mechanisms of resistance of *L. amazonensis* to the immune system is an inhibition of IL-12 production by infected macrophages [47]. The effect of EVLa seen in this work suggests that a mechanism, namely the release of EVs might, at least in part, counteract this effect by inducing the production of IL-12, IL-1β and TNF-α by bystander macrophages before they are infected.

Thus, the contact of EVs from infected macrophages with non-infected macrophages, perhaps entailing the incorporation of these EVs, together with the possible carrying over of *Leishmania* antigens by the EVs to the naïve macrophages, could promote the differentiation of Th0 to Th1 lymphocytes, which would release IFN-γ, resulting in the Th1 response that is associated with resistance to the *Leishmania* infection [5,48]. In addition, an increased production of IL-1β and TNF-α, which would also be induced in naïve macrophages by the EVs, is associated with host's resistance to *Leishmania* infection through the production of nitric oxide [49,50]. Consistent with this, it has been reported that the injection of anti-TNF-α into mice worsened the *Leishmania* infection [51]. The phenomena described above could perhaps underlie the protective response that normally keeps the *Leishmania* parasites in check in most infected human beings [52,53].

EVLa, therefore, are proinflammatory to macrophages. Macrophages infected with *Mycobacterium bovis* (BCG) release exosomes with capacity to generate proinflammatory immune responses *in vitro* and *in vivo* and that contain mycobacterial proteins [12,27]. It could well be, therefore, that the proinflammatory nature could be a general feature of EVs originated from infected cells.

As mentioned above, the extracellular vesicle populations studied in the present work had sizes compatible with their being formed by a mixed population of microparticles and exosomes. It was recently reported that purified exosomes from both naïve and *Leishmania mexicana*-infected macrophages induce the activation of the pro-inflammatory transcription factors NF-kB and AP-1 [54]. Those infected macrophage-derived exosomes, however, induced slightly less activation of NF-kB than the naïve macrophage-derived exosomes. This apparent discrepancy with the present results could result from differences in the time after infection that the EVs were obtained, or from the fact that in the present work the total population of EVs was assayed, rather than only purified exosomes.

One possibility that was not ruled out in the present work is that the biological activity of the EVLa would be mediated by *Leishmania*-derived EVs, which has been described in the literature [29,55]. However, contrary to the present observations, these *Leishmania* EVs had a suppressive effect on antigen-presenting cells [29,55].

Since *Leishmania* parasites readily sediment at 1.500 g (unpublished observation), the centrifugation of the EVLa-containing supernatants at 8.000 g during the process of EVLa preparation eliminated the contamination of the EVLa preparations with *Leishmania*.

The naïve macrophage arming process described herein could perhaps be more intense during a *Leishmania* infection, in which EVLa would be released, but it seemed also to occur, in a smaller extent, with EVs from uninfected macrophages in the absence of LPS, at least as far as IL-1β is concerned (Fig. 4C). Whether this apparently spontaneous release of EVs that stimulate the production of IL-1β is an *in vitro* artefact or a relevant phenomenon *in vivo* is open to speculation. EVs are certainly released in physiological contexts, as can be exemplified by the finding of circulating EVs in healthy human beings' plasma samples [56].

In the present work, in an attempt to perhaps emulate what could occur in a natural infection, the concentrations in which the effect of the EVs were assessed on naïve macrophages were the same ones that were present in the supernatants from the macrophages that released them (i.e. the EVs were not concentrated nor diluted). Similar amounts of EVLa and EVmedium were added to the cultures, showing that the higher induction of production of pro-inflammatory cytokines by EVLa than by EVmedium is due to qualitative differences between the two kinds of EVs.

One of the possible mechanisms that could account for the observed effect of the EVs would be their ligation to receptor of danger-associated molecular patterns (DAMPs) on the macrophage surface. EVs could present DAMPs from apoptotic or necrotic cells, such as lectin C, which is expressed in dead cells or in cell in death process [57]. As presented above, there is evidence that apoptosis might be involved in the generation of at least part of the EVLa. It has been reported that apoptotic bodies induce immune responses (reviewed in [36]). Other components that could be carried over by the EVS, such as uric acid and cholesterol crystals, are recognized by NLRP3 receptors [58], activating the NLRP3 inflammasome and leading to the production of proinflammatory cytokines. The activation of that inflammasome has been shown to lead to the production of IL-1β e IL-1α [59]. Another possibility that is open to investigation is that *Leishmania* molecules, possibly carried out by the EVs, could be triggering the pro-inflammatory cytokine production.

It has been reported in the literature that the *in vitro* infection by Leishmania increased the resistance of macrophages to the development of apoptosis [60,61]. These reports are in apparent contradiction with the present findings that a larger proportion of infected macrophages had exposed phosphatidylserine on their cell membranes (i.e., they stained with Annexin V), a fact that is an indication of apoptosis, than uninfected macrophages. However, the cited reports studied macrophages that have been infected for no more than 24 hours, whereas in the present work the macrophages were infected for 9 days, in an attempt to emulate what probably occurs *in vivo*, when heavily infected macrophages are found and the *Leishmania* lesions usually last for months. It is possible that, early in the infection, the *Leishmania* may induce anti-apoptotic mechanisms that are important to allow the host cell to survive long enough to allow the parasite to multiply and to spread to other cells and to the insect vector. However, late in the infection, when the host cells are heavily loaded with parasites, this anti-apoptotic activity would perhaps no longer prevail — anyway,

it would no longer be relevant for the parasite life cycle, and maybe would even hinder its continuity.

One possible exploitation of proinflammatory EVs is their use in vaccination and immunotherapeutic procedures, since they may carry MHC-antigenic peptide complexes, unbound antigens [10], cytokines that intensify the immune response and/or change its nature, costimulatory molecules [13,19,27], and, as shown in the present work, a still unidentified factor that induces the formation of proinflammatory cytokines, including IL-12, which may serve as an adjuvant for Th1 immune responses.

The ability of infected macrophage-derived proinflammatory EVs in conferring a resistant phenotype to *Leishmania* infection *in vitro* and *in vivo*, and the possible presence of *Leishmania* antigens in them, are currently under investigation.

Conclusions

As shown in the present work, EVs from macrophages infected with *L. amazonensis* induce the production of proinflammatory cytokines by naïve macrophages, and may, therefore, play a role in stimulating the immune response and in conferring a resistant phenotype to naïve, bystander macrophages.

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Author Contributions

Conceived and designed the experiments: ACA PRO PSTV LPdC. Performed the experiments: ACA LAF CFdA VJR MdCBS CPF. Analyzed the data: ACA LAF CPF LARdF LPdC. Contributed reagents/materials/analysis tools: LPdC. Wrote the paper: ACA LPdC. Wrote the first draft of the manuscript: ACA. Wrote the final version of the manuscript: LPdC.

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FOXP3+ regulatory T cells in hepatic fibrosis and splenomegaly caused by *Schistosoma japonicum*. The spleen may be a major source of Tregs.

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Running title: FOXP3$^+$ Tregs in *Schistosoma japonicum* infection.

Abstract

Schistosome eggs cause hepatic inflammation, fibrosis and splenomegaly in infected individuals. $FOXP3^{+}$Tregs regulate inflammation and homeostasis; therefore, these cells may influence liver and spleen disease occurring in human schistosomiasis. To evaluate the role of Tregs in liver and spleen disease, we investigated both the properties and fate of blood and spleen $FOXP3^{+}$Tregs in fishermen with lifelong exposure to *Schistosoma japonicum* infection. The proportion of blood effector Tregs ($CD45^{-}FOXP3^{hi}CD4^{+}$T cells, eTregs) among $CD4^{+}$Tcells were higher in individuals with severe hepatosplenic schistosomiasis than in those with mild schistosomiasis. Hepatic fibrosis and splenomegaly were independently associated with these high proportions of blood eTregs, which were in part reverted by splenectomy. Analysis of Tregs in the spleen of fishermen with splenomegaly showed a high proportion of eTregs expressing homing receptors (CXCR3, CCR5) for Th1-infiltrated tissues The transcripts of the ligands of these receptors were also highly abundant in egg-infected livers. These data suggest that the spleen produces eTregs which then could migrate to hepatic egg granulomas. Moreover, the proportions of $CXCR3^{+}$eTregs among blood $CD4^{+}$Tcells were less in severe than in mild hepatic fibrosis, suggesting that the altered migration of eTregs in the liver may aggravate disease. However, *IL2RA* variants that promote the induction of eTregs were associated with splenomegaly, suggesting that eTregs could contribute to spleen disease in the hepatosplenic patients. More studies are required to evaluate further these hypotheses.

Introduction

Regulatory T cells that express the transcription factor Forkhead box protein P3 (Foxp3) are crucial regulators of immunological self-tolerance and homeostasis (1, 2). They suppress the activation, proliferation and effector functions of many immune cells including $CD4^+$ and $CD8^+$ T cells, natural killer cells, NKT cells, B cells, and antigen presenting cells. The Treg phenotype results from two major regulatory events: the up-regulation of genes associated with Treg function including FOXP3, CTLA4, IL2RA, TNFRSF18 (encoding GITR), IKZF2 (encoding Helios) and IKZF4 (encoding Eos), the expression of which are regulated epigenetically (3, 4), and the FOXP3-mediated down-regulation of several genes, including IL2 and IFNG (5-8). $FOXP3^+$Tregs regulate inflammation and immunity to infectious pathogens (9) and therefore could play important roles in limiting hepatic inflammation and fibrosis caused by schistosomes. However, convincing evidence of such a role is lacking. Schistosome worms lay their eggs in the mesenteric and portal veins; the eggs are trapped in liver sinusoids where they lead to intense inflammation and fibrosis tissue in the portal spaces. This in turn causes an increase of portal blood pressure and the development of varicose veins. Here, we evaluated the properties and the fate of $FOXP3^+$Tregs in both the blood and spleen of *Schistosoma japonicum*-infected patients with schistosomiasis of varying severity. We analyzed naïve and effector $FOXP3^+$Tregs because human blood Tregs are divided into $CD45RA^+FOXP3^{low}$ $CD4^+$ naïve Tregs and CD45RA-$FOXP3^{hi}$ $CD4^+$ effector Tregs (eTregs), whereas blood $CD45RA^-FOXP3^{low}$ $CD4^+$ T cells are effector T cells without suppressive activity (10, 11). $FOXP3^+$Tregs are induced either in the thymus (tTregs), mostly by self-antigens, or in the periphery (pTregs) after stimulation by conventional antigens (12-14). However, these events have been difficult to document in humans due to the absence of specific markers of pTregs and tTregs. The induction of pTregs should occur during inflammation induced by eggs and could also take place in the hyperstimulated spleen of schistosome-infected individuals.

Naïve Tregs express homing receptors for lymphoid organs (CCR7), whereas eTregs expressing high levels of CCR5, CXCR3, CCR6, and CCR8 (15) are attracted to non-lymphoid, inflamed tissues. Under these conditions, $FOXP3^+$Tregs become phenotypically and functionally specialized and develop into Th2, Th1 or Th17 cells (16). It is unclear how mediators produced in the environment created by schistosome eggs influence Tregs. Here, we evaluated homing receptors on Tregs and determined whether alterations in Treg migration are associated with disease aggravation.

Our results shed light on the potential role of Tregs during human schistosomiasis, particularly in patients with severe hepatic disease and massive splenomegaly.

Material and Methods

Study subjects

The study was approved by the ethical committee of the Hunan Institute of Parasitic Diseases, Hunan Province, China and by the WHO. Only compliant participants were recruited into the study and they were free to drop out at any point. Written informed consent was obtained from each subject.

Evaluation of hepatic fibrosis

Hepatic fibrosis was evaluated by ultrasound and the WHO grading scale (17), which was modified as described . The WHO scale grades peripheral (NetF) and central fibrosis (CentF) separately. CentF is graded A, B, C, CL, D, E or F. The C linear thickening pattern (CL) of CentF represents uninterrupted fibrosis thickness of the linear wall of the portal vein extending from the portal vein to its branches. The uninterrupted nature of fibrosis distinguishes CL from grade C (discontinuous thickness), and the linear pattern differentiates it from the patches observed in grades D, E, and F. More than 60% of the fishermen had grade CL; therefore, we subdivided CL into CL^{L} (CL light), CL^{M} (CL medium) and CL^{H} (CL heavy): CL^{L} was observed in the left lobe of the liver only and CL^{M} and CL^{H} were observed in both lobes. Subjects with right lobe fibrosis extending to second order branches were classified as CL^{M} and CL^{H} if CentF extended a long way down the second order branches. Only CL^{H} was associated with evidence of portal hypertension, and was therefore grouped with grades D, E and F to define a severe CentF phenotype. The WHO grades peripheral fibrosis (network fibrosis, NetF) as narrow mesh (GN) if the net is <12mm and wide mesh (GW) if >12mm. We also refined GN grading into three categories: GN^{L} (or GW^{L}) if the mesh streak (or band) was <2mm thick, GN^{M} (or GW^{M}) if 2-4mm, and GN^{H} (or GW^{H}) if >4mm. Patients were divided into three hepatic fibrosis (HF) groups according to CentF: HF+/- (B, C), HF^{++} (CL^{L}) and HF^{+++} (CL^{M}, CL^{H}, D, E). Multivariate regression analysis showed that NetF did not influence any of the dependent variables studied; nevertheless, we indicate the NetF grade in our analysis, which was either absent (G0) or light (GN^{L}) in the $HF^{+/-}$ group, and intermediate (GN^{M}) or high (GN^{H}, GW) in the HF^{++} and HF^{+++} groups. Study subjects were also assigned to normal spleen (Spl, spleen size <110 mm) and splenomegaly (SplM, >110 mm) groups. Patients who had undergone splenectomy were included in the Spl- group. Finally, individuals with moderate or severe hepatic fibrosis and splenomegaly were historically described as hepatosplenic (HSP) patients.

Study Groups

All study subjects were fishermen working on the Dong Ting Lake who were recruited from the same region, and were highly exposed to infection with S. japonicum. All individuals were recruited and interviewed by the same people. There is no overlap between the groups (i.e. no patient belonged to more than one group).

Group 1: Chinese fishermen for the FACS analysis of blood Tregs

Chinese fishermen (n=76) were described previously **(18).** Controls (*n*=20) were from the same region but reported no contact with lake water, they were negative by ELISA with schistosome antigens and showed no signs of spleen or liver disease. All study subjects were aged between 30 and 65 years. Eleven of the 16 HF^{+++} patients, seven of the 23 HF^{++} patients and six of the 29 $HF^{+/-}$ patients had splenomegaly and therefore also belonged to the SplM group. The splenectomy group (Spl-) included subjects with liver disease HF^{+++}.

Group 2. Spleen and liver biopsies for the study of transcripts of chemokine receptors and chemokine genes in infected livers:

RNA abundance (Figure 3) was determined in biopsies obtained from eight subjects who underwent a splenectomy at the Yueyang Hospital. These individuals (four men and women) were 25-59 years old (48.2+/-3.8). None had been infected with neither HCV nor HBV and all exhibited schistosome eggs in liver biopsies. All but three had advanced or severe CentF or

NetF. Three patients exhibited milder but nevertheless significant CentF, which was associated with advanced NetF in two patients. These patients showed severe splenomegaly. Control healthy tissues were obtained from a tissue bank in France.

Group 3. Spleen and blood from subjects with hepatosplenic disease for the study of FOXP3$^+$ Tregs in the spleen.

FACS analyses shown in Figure 4 were performed on the spleen and blood cells from five patients (including four men) who underwent a splenectomy. All subjects had HF++ or HF+++ and showed signs of infection (eggs in the biopsy, seropositivity). They were infected with neither HCV nor HBV.

Group 4. Cohort of patients used for the case control study that tested the association of *IL2RA* SNPs with splenomegaly

Genetic analysis was performed on a sample of 274 Chinese fishermen and farmers who were described previously (19). Of this cohort, 16.9% were women, 177 had mild or no liver fibrosis (control group) and 97 (cases) had advanced (CLM, 53.6%) or severe (CLH, D, E, F, 46.4%) CentF. None of the controls and 76.3% of the cases had **been** splenectomized.

Cell cultures

PBMC were purified from venous blood as described previously ((18)). Spleen biopsy specimens were teased with forceps in FBS/RPMI 1640 medium supplemented with 10% FBS, 200 U/ml penicillin, 10000 U/ml streptomycin and 10 mM HEPES. The suspension was then passed through a filter with 70 µm pores, and washed in phosphate-buffered saline (PBS). Red blood cells were lysed with red cell lysis buffer (eBiosciences) and washed twice in PBS. PBMCs and splenocytes were maintained in FBS/RPMI 1640 medium until use for flow cytometry.

Induction of eTregs in vitro: We used this assay to test the effects of IL2RA variants on the induction of eTregs. We obtained PBMCs from healthy donors carrying one of the three possible genotypes at the IL2RA locus that was tested and we cultured the cells using eTregs induction culture conditions: we cultured PBMCs for 72 hrs with beads coupled to antiCD3 and antiCD28 antibodies (1:20 or 1:200 bead/cell ratio; Miltenyi Biotec) in the presence of 10 U/ml IL-2 (BD Biosciences) and 2 ng/ml TGF-b1 (PeproTech). Both IL-2 and TGFβ are required to induce eTregs in vitro.

Antibodies and flow cytometry

All cell labeling was performed on cells right after purification from the blood without any stimulation excepted for the experiments described Fig.5 where cells were cultures 72 hrs as described. PBMCs or spleen cells were dispensed (4 x 10^5 cells/tube) into 5 ml polystyrene tubes (Falcon®) for surface and intracellular staining with the Human FOXP3 Buffer Set (BD Pharmingen). Quadruple staining was carried out with FITC-conjugated anti-CD45RA (BD Pharmingen), PE-Cy7-conjugated anti-CD4 (eBiosciences), PE-conjugated anti-FOXP3 (BD Pharmingen) antibodies in association with one of the following APC-conjugated antibodies: anti-CD25 (BD Pharmingen) antibodies; anti-CCR7 (eBiosciences), anti-CXCR3 (BD Pharmingen), anti-CCR5 (BD Pharmingen) or anti-CTLA-4 (BD Pharmingen; intracellular staining) antibody. For the analysis of IFN-γ production, cells were first double-stained with FITC-conjugated anti-CD3 (BD Pharmingen) and PE-Cy7–conjugated anti-CD4 antibodies and then stained with APC-conjugated anti-IFNγ intracellular markers (eBiosciences). They were incubated with 100 ng/ml PMA, 1 µg/ml ionomycin and monensin (BD GolgiStop) for 6 hours at 37°C before intracellular cytokine labeling. Isotype controls were obtained from the manufacturers. All antibodies were used according to the manufacturers' recommendations. Flow cytometry was carried out with a FACScalibur® flow cytometer (BD Biosciences) and CELLQUEST™ software. DIVA™ was used for analysis.

RNA extraction and quantitative RT-PCR

Liver and spleen biopsy specimens were stored in RNA Later (Life Technologies, Courtaboeuf, France) at -20°C. Tissue homogenization was carried out with a Precellys-24 device (Bertin Technologies, Ozyme, Saint-Quentin-en-Yvelines, France), with ceramic beads (1.4 mm diameter, CK14), in 350 µl RLT lysis buffer (Qiagen SAS, Courtaboeuf, France) supplemented with 3.5µl β-mercaptoethanol. A total of 400 µl of Tri-reagent (Life Technologies) and 150 µl of chloroform were added. The aqueous phase was mixed with 500 µl of 50% ethanol (liver) or 70% ethanol (spleen), and RNA was purified on an RNeasy spin column (Qiagen SAS, Courtaboeuf, France). RNA integrity was assessed with a 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). Liver and spleen "controls" were from the Biological Resource Center, Curie Institute, Paris, and from Stratagene (Agilent), Clonetech (Ozyme), Panomics (Ozyme), and INSERM U1040, Montpellier. Biopsies were collected either from deceased individuals with no known history of infection (i.e. from untransplanted organs) or from liver biopsies carried out for diagnostic purposes. We verified that the donors were healthy by examining the expression of inflammatory cytokines in these tissues. If a "healthy tissue" showed an abnormal pattern of inflammatory cytokine expression (compared with the other biopsies), we discarded it. Total RNA (1 µg), RIN > 7, was reverse-transcribed with the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Courtaboeuf, France). Real-time quantitative PCR, with 20 ng of cDNA, was performed with the ABI 7900HT Fast Real-Time PCR System and *Taq*Man Universal PCR Master Mix (Applied Biosystems, Life Technologies). The *Taq*Man gene expression assays: *CCL3* (Hs00234142_m1), *CCL5* (hs00174575_m1), *CCL19* (Hs00171149_m1), *CCL20* (Hs00171125_m1), *CCL21* (Hs99999110_m1), *CXCL9* (Hs00171065_m1), *CXCL10* (Hs00171042_m1), *CXCL11* (Hs00171138_m1), *IFNG* (Hs99999041_m1), *IL12B* (Hs00233688_m1), *IL12RB2* (Hs00155486_m1), *RPLP0* (Hs99999902_m1), *TBX21* (Hs00203436_m1), were from Applied Biosystems. Gene expression values were normalized to those of the housekeeping gene RPLP0 (ribosomal phosphoprotein large P0). Transcripts of this housekeeping gene were stable in all study groups. An abnormal deviation of the abundance of RPLP0 transcripts from the mean abundance of RPLP0 transcripts in other samples was taken as an indication of problems in RNA extraction.

Genotyping

Genomic DNA was extracted as described previously ((19)). The SNP rs3118470 (TGGGGTCTCTCTCCCTGGAATCTCA[C/T] TGATGGAAATTTACTGGTCCCTTGG) was genotyped with *Taq*Man probe assays (Applied Biosystems) as in ((19)).

Statistical analysis

Group comparisons were performed with nonparametric analysis in SPSS software. We assessed how hepatic disease (fibrosis), spleen disease (splenomegaly) and splenectomy affected subpopulations of Tregs, by carrying out linear regression analysis on these dependent variables. Hepatic fibrosis was divided into three binary classes as described previously(20). The variables introduced into the regression model were made binary to make no assumption on the linear relationship between the dependent variable and the independent ones. Thus, a three category variable (a, b, or c) yields two binary variables a+b versus c and a versus b+c . It makes no sense to introduce a+c versus b for HF but this makes sense if we were dealing with genotypes such as AA, AC, CC, which yield three binary variables: AA/ AC+CC; AA+AC /CC and AA+CC/ AC. Spleen disease was also divided into three binary classes. All binary variables were included in the linear regression model. Age and sex were not significant covariates in most models. Results are given as the non-standardized slope (A),

its 95% confidence interval (CI) and the *p* value of the association. The statistical significance of the effect of splenectomy was always assessed by comparison with the splenomegaly group or the HF^{+++} group, because splenectomized subjects belonged to this group before surgery.

Results

Effector and naïve Tregs in the blood of individuals infected with *S.japonicum*

We evaluated the proportion of Tregs among blood FOXP3$^+$ T cells in fishermen altogether. (group 1). These had been exposed for >10 years to infection with *S. japonicum*. Tregs (CD45RA$^+$FOXP3^{low} and CD45RA$^-$FOXP3^{hi}) made up 4.3± 0.26% (SEM) of all CD4$^+$ T cells and 41.2 ± 0.16% of all FOXP3$^+$CD4$^+$Tcells (Fig. 1A and B). The remaining FOXP3$^+$CD4$^+$cells, CD45RA$^-$FOXP3^{low} T cells, which are FOXP3$^+$ non-regulatory T cells, made up 6.1 ± 0.4% of all CD4$^+$ T cells and 58.7 ± 0.15% of the FOXP3$^+$CD4$^+$ T cells.

We phenotyped patient CD45-FOXP3^{hi} and CD45+FOXP3^{low} Tregs for CD25, CCR7, CXCR3, CCR5 and CTLA-4 (splenectomized patients were excluded) (Fig.1C). CCR7 directs Tregs to lymphoid organs and CXCR3 and CCR5 direct them to Th1-infiltrated tissues (21). Strong CD25 expression is a marker of Treg activation, whereas CTLA-4 expression strongly correlates with suppressive activity. Most CD45RA$^+$FOXP3^{low} Tregs expressed CCR7 (80.5 ± 1.2 %), but few expressed CD25 (18.3 ± 2.3%), CXCR3 (11.6 ± 2%), CCR5 (3.2 ± 0.8%) and CTLA-4 (8.3 ± 0.7%). By contrast, the proportion of cells expressing CCR7 (34.1 ± 2.2%) was lower in CD45RA-Foxp3^{hi} than in CD45RA+ Foxp3^{low} Treg ($p<0.01$), by contrast the proportions of cells expressing CD25^{hi} (51.2 ± 2.2%), CXCR3 (48.1 ± 2%), CCR5 (45.8 ± 2.1%) or CTLA-4 (71.7 ± 1.5%) were higher for CD45RA-Foxp3^{hi} than for CD45RA+ Foxp3^{low} Treg (p <0.01 for all comparisons). These patterns were used to characterize CD45RA$^+$FOXP3^{low} naive Tregs and CD45RA$^-$FOXP3^{hi} eTregs respectively in normal blood (10). Thus, blood Tregs in schistosome-infected patients can be divided into eTregs and naïve Tregs.

Both severe fibrosis and splenomegaly are independently associated with an increase in eTreg blood levels.

Splenomegaly (SplM) and hepatic fibrosis (HF) often develop in the same patients. Tregs may be produced and/or attracted to both the liver and spleen because these organs are sites of intense inflammation and cell proliferation. We evaluated Tregs blood counts in individuals with spleen and/or liver disease of varying severity to investigate a possible link between SplM, HF and Tregs. Study subjects were assigned to three groups according to the severity of HF and to two groups according to spleen size as described in methods.

Figure 2A shows Tregs blood levels according to HF and spleen disease. FOXP3$^+$ Tregs blood levels were positively correlated with both the severity of HF (+1.3 +/- 0.4; p=0.004) and the presence of SplM (+1.03 ±0.44%, p=0.03). The simultaneous testing of HF and SplM in the regression model showed that HF (+0.85 +/- 0.3%, p=0.01) and SplM (+0.97 +/-0.49%, p=0.05) were independently associated with high Treg blood levels. Interestingly, eTregs and naïve Tregs were not equally affected: multivariate regression showed that eTreg blood levels (Fig. 2B) were higher both in patients with HF (p=0.05) or SplM (p=0.08) than in healthy controls, but naïve Treg blood levels were similar (Fig. 2C). The largest differences were between eTreg levels in HF^{++} and HF^{+++} patients (+0.9 ± 0.4 %, p=0.04) and between SplM and patients with a normal spleen (+0.9 ± 0.36 %, p=0.04).

The proportions of blood CXCR3$^+$ eTregs are lower in patients with severe hepatic fibrosis. The proportions of CCR5$^+$ eTregs are increased in patients with splenomegaly

We analyzed the expression of molecules that are crucial for the activation and homing of Tregs to evaluate the migratory capacities of these cells. We also evaluated CTLA4, a marker of suppressor activity. The proportions of eTregs expressing CD25^{hi}, CCR7, and CTLA-4 were similar between the Spl and SplM group and among HF groups. However, the proportion of CXCR3$^+$ eTregs was lower in the HF^{+++} group (37.4 +/- 5.9%) than in patients with milder HF (51.7 +/- 2 %; p=0.009) and was not affected by SplM (Fig. 2D). The

proportion of $CCR5^+$ eTregs (Fig. 2E) was higher in the SplM group than in the Spl group (p=0.04) and we observed a similar trend in the HF^{++} group (p=0.09 in the multivariate analysis, or p=0.07 taking into account SplM). We observed no statistically significant differences between clinical groups with markers on naïve Tregs.

The genes encoding IFN-γ-dependent chemokines, ligands for CXCR3 and CCR5, are highly transcribed in the liver of HSP subjects.

CXCR3 and CCR5 direct $FOXP3^+$ Tregs to T_H1 cell-infiltrated sites; therefore, our results suggest that CXCR3 and CCR5 regulate the traffic of eTregs toward Th1-infiltrated egg granulomas in the liver. To test this hypothesis, we evaluated the production of CCR5 and CXCR3 ligands in liver biopsies from HSP patients who were splenectomized (study group 2). We also evaluated the production of CCR7 and CCR6 ligands because these receptors are also expressed by $FOXP3^+$ Tregs (This study and (15)). Transcript levels of *CCL5* (CCR5 ligand), *CXCL9, 10, 11* (CXCR3), *CCL19*, *CCL21* (CCR7) and *CCL20* (CCR6) were four to 33 times higher (p<0.01) in infected than in controls livers (Fig. 3A). *CCL20* showed the largest difference in expression (33-fold, p<0.001). By contrast, in the spleen, only *CXCL11* (CXCR3) and *CXCL10* (CXCR3) transcripts were more abundant in HSP patients than in control individuals (p<0.01) (Fig. 3B).

We also evaluated transcripts of T_H1-related genes in infected livers: *IL12RB2* mRNA levels (p=0.004) were between four and five times higher in infected than in control livers *IFNγ, IL12B* and *TBX21* mRNA levels showed a similar trend (p<0.15) (data not shown). Moreover, transcripts of *CXCR3*, *CXCL9* (r=0.93, p=0.02) and *CXCL11* (r=0.88, p=0.05) were correlated with *IFNG* transcript levels in the liver but not in the spleen of infected subjects (*CXCL10* also showed a trend of correlation; r=0.79 p=0.1) (data not shown). No such correlation was found for the other chemokines tested: *CCL3* (ligand for *CCR1, 5*), *CCL5* (*CCR1, 3, 5*), *CCL19* (*CCR7*), *CCL20* (*CCR6*), *CCL21* (*CCR7*) and *CXCL9* (*CXCR3*). Similarly, $FOXP3^+$ Tregs blood levels were negatively correlated (r=-0.73, p=0.002) with T_H1 blood levels (Fig. 3 C), consistent with the negative regulation exerted by $FOXP3^+$ Tregs on T_H1 cells.

The spleen of HSP patients contains high proportions of effector Tregs.

We found that blood levels of $FOXP3^+$Tregs (-2.5 ± 0.56, p<0.001) and eTregs (-1.3±0.6, p=0.03) were low in HSP patients who had undergone a splenectomy (Fig. 2 A, B) as compared to HF+++ subjects with splenomegaly. This observation, in addition to high blood counts of Tregs in SplM, suggests that the spleen may be involved in eTregs production (induction or proliferation). We thus analyzed Tregs in the spleen removed from HSP patients (group 3, Fig. 4). The proportion of naïve Tregs among $CD4^+$ T cells was lower in the spleen (0.95 ± 0.2) than in the blood (2.68 ± 0.7) (p=0.02) (Fig. 4A). In addition, the proportion of naïve Tregs expressing CCR7 was lower in the spleen than in the liver (p=0.006) (Fig. 4B). However, the proportion of naïve Tregs expressing CCR5 tended to be higher in the spleen than in the blood (p=0.07) (Fig. 4B). There was no statistically significant difference in the proportion of eTregs in the spleen (2.8 ± 0.64) and blood (5.2 ± 1.6) (Fig. 4A). However, the proportion of eTregs expressing CD25 or CCR7 was lower in the spleen than in the liver (p=0.05 and p=0.04, respectively) whereas the proportion of eTregs expressing CCR5 tended to be higher in the spleen than in the blood (p=0.08) (Fig. 4C). Thus the composition of naïve Tregs and eTregs populations are different in the spleen and blood. Spleen eTregs may be less activated than blood eTregs; nevertheless, spleen eTregs exhibit a profile of higher CCR5 and CTLA-4 expression, which is typical of cells that are committed to migrate to inflamed tissues. The observation that spleen naïve Tregs express less CCR7 and more CCR5 than blood naïve Tregs suggests their activation, which may ultimately transform them in eTregs.

The high production of eTregs in HSP patients is associated with genetic variants in *IL2RA*

The induction of $FOXP3^{+}$Tregs is stimulated in PBMCs cultures by IL-2 and TGF-β cytokines and anti-CD3 and anti-CD28 antibodies. Using this culture assay, we found that PBMCs from healthy individuals carrying the genotype TT of the rs3118470 polymorphism in *IL2RA* produced more Tregs than PBMCs from healthy individuals carrying the TC or CC genotypes of this SNP (p=0.001) (Fig. 5A). We found that IL2RA 3118470 TT was more frequent (p=0.005) in patients with severe SplM (who exhibit high eTregs blood levels) than in subjects with milder schistosomiasis (who exhibit low eTregs blood levels) (sample 4, Fig. 5B). We evaluated whether variants outside *IL2RA* but in strong linkage disequilibrium with *IL2RA* variants could account for the association, and found no such variants in a 1MB region surrounding *IL2RA* (data not shown). Thus, the causal variant that modulates Tregs production *in vitro* and is associated with splenomegaly is in the *IL2RA* gene itself.

Discussion

Few studies have analyzed FOXP3$^+$ Tregs in patients infected with schistosomes and no work has been carried out on FOXP3$^+$Tregs without the interference of FOXP3$^+$ non-Tregs. Here, we analyzed FOXP3$^+$ Tregs with markers that discriminate these cells from FOXP3$^+$ non-Tregs. We report that effector Tregs blood levels in patients with severe hepatic fibrosis and/or splenomegaly. Both fibrosis and spleen disease were independently associated with high FOXP3$^+$ eTreg blood levels. Others have shown that CD4$^+$CD25$^+$FOXP3$^+$CD127^{low} T cell levels in the blood of *S. haematobium*-infected children and those of FOXP3$^+$ T cells are high in the blood of *S. mansoni*-infected individuals after anti-helminthic treatment (22, 23). These results, in addition to our work, indicate that schistosome infection stimulates the production of FOXP3$^+$ Tregs, although the effects observed in these studies may be due in part to non-Tregs. The positive correlation between eTreg blood counts and the severity of both hepatic and spleen disease suggests that the spleen and the liver either produce or regulate the induction of Tregs. We found that the spleen of HSP patients contains a high proportion of Tregs and thus may be an important source of Tregs, especially given its large size in HSP patients. Alternatively, spleen Tregs may be produced in the blood and captured by the spleen. However, this seems unlikely because splenomegaly was associated with the highest eTreg blood levels and splenectomy resulted in a drop in eTreg blood levels. Moreover, the properties (high expression of CXCR3, CCR5 and CTLA4) of spleen eTregs indicate that these cells do not stay in the spleen but are instead targeted to inflamed tissues such as the liver, where CXCR3 and CXCR5 ligands are highly abundant. In addition, *IL2RA* variants that promote the induction of eTregs, are frequently carried by patients with splenomegaly, suggesting a link between splenomegaly and the induction of Tregs. Thus, our data suggest that at least some of the eTregs detected in the blood of schistosome-infected patients are produced in the spleen (pTregs), either by induction from naïve Tregs or by proliferation of eTregs. The induction of peripheral FOXP3$^+$Tregs has been demonstrated in mice (12-14); it is mostly triggered by conventional antigens and results in the selection of high affinity TcRs. These cells have been less studied in human diseases due to the lack of markers to distinguish human pTregs from tTregs, until the recent report of neuropilin as a tTreg marker (24, 25). In future studies, the use of neuropilin and proliferation markers such as Ki67 will help to distinguish between tTregs and pTregs and to demonstrate definitively the active production of eTregs in the spleen, involving their induction, proliferation or both.

Our findings also provide an explanation for high eTreg blood levels in both HF and SplM: first, low CXCR3$^+$ eTreg counts may limit the recruitment of eTregs to the liver; second, the spleen of HSP patients is probably a considerable source of Tregs; third, the production of eTregs may be stimulated by *IL2RA* allelic variants in HSP patients; and finally, the liver could also contribute to the production of Tregs, as suggested in patients infected with HCV (26). It is important to determine the role of Tregs in human schistosomiasis because splenectomy, which is performed in HSP patients, could remove an important source of Tregs. Removal of CD25$^+$ T cells (which comprise >50% of non-Tregs) promotes collagen deposition in the liver of *S.mansoni*-infected mice (27). However, the same treatment reduces worm and egg load in *S.japonicum*-infected animals, suggesting that Tregs may reduce the clinical manifestations of schistosomiasis but inhibit sterile immunity (28, 29). Nonetheless, these results await confirmation and no study has tested the effects of highly enriched preparations of FOXP3$^+$ Tregs in schistosome-infected mice.

Our finding that CXCR3 expression is low in patients with severe HF suggests that eTregs protect against liver disease by reducing liver inflammation. This view is consistent with several other studies on the role of Foxp3+Tregs in chronic liver inflammation. These studies have shown that CXCR3 is crucial for the localization of Tregs in inflamed liver (30, 31). Helbig and coll. (32) reported that the CXCR3 chemokines are the most significantly

expressed chemokines in chronic hepatitis C liver. Others have more directly implicated Foxp3+ Tregs in protection against chronic hepatitis: in a model of autoimmune inflammation of the liver, Lapierre and coll (33) observed that adoptive transfer of ex vivo expanded CXCR3+ Tregs to the mice with auto-immune hepatitis deficiency targeted the inflamed liver and restored peripheral tolerance, inducing remission of auto-immune disease. Furthermore Hasegawa et al(34)showed that acute GVHD could be ameliorated in intestine, liver and lungs by accumulation of CXCR3-expressing CD4+CD25+ regulatory T cells (but not CXCR3-Tregs) in target organs. CXCR3+Treg cells accumulated into Th1-associated chemokine-expressing target organs, resulting in stronger suppression of alloreactive donor T cells. Interestingly, Oo et al (35) comparing blood-derived Tregs and liver-derived Tregs fund that the latter express high levels of the chemokine receptors CXCR3. In flow-based adhesion assays using human hepatic sinusoidal endothelium, Tregs used CXCR3 to bind and transmigrate. The authors proposed that CXCR3 mediates the recruitment of Tregs via hepatic sinusoidal endothelium. Finally, Erhardt et al(36) reported that CXCR3+ Foxp3+ Tregs that are generated in ConA –induced hepatitis in mice, disseminate into the organism and specifically migrate into the liver, where they limit immune-mediated liver damage. Thus, the low proportion of $CXCR3^+$ eTregs in individuals with severe schistosomiasis may impair the influx of eTregs into the liver, thus contributing to HF.

The role of $FOXP3^+$ Tregs in the spleen is less clear. The *IL2RA* allele that promotes eTreg induction *in vitro* is associated with the aggravation of spleen disease. This raises the intriguing possibility that eTregs generated in the spleen of HSP patients may be pathogenic. Tregs are normally stable due to both TSDR demethylation and the FOXP3-mediated suppression of IL2. However, IL-2 activation may cause these cells to lose FOXP3 expression and their suppressive capacities (8), but in normal physiological conditions, they conserve their TSDR demethylation pattern that prevents them from becoming pathogenic. However, it is not known whether Tregs produced in the massive hyperplasic spleen of HSP patients acquire the epigenetic demethylation pattern of normal Tregs. Tregs in lymphopenic mice develop pathogenic properties (37). Thus, Tregs generated in a huge, hyperactive spleen could be unstable and develop into pathogenic T cells (e.g. Th17 cells) that aggravate spleen disease. We are currently evaluating this possibility.

Here, we show that splenomegaly is associated with high counts of Tregs and we have discussed the hypothesis that these Tregs may be unstable and develop pathogenic properties. These findings may have implications for diseases such as malaria and visceral leishmaniasis, which are also associated with marked splenomegaly. In both infections, the liver and the spleen play important role in the control of parasite multiplication. If our observations can be extended to the spleen of malaria and leishmaniasis patients, Tregs produced in the spleen may interfere with parasite control. Data obtained in the murine model of schistosomiasis but also of Leishmaniasis (38) suggest that Tregs protect against the inflammation but interfere with the control of the parasite. Thus, it is essential to confirm that the spleen of individuals with splenomegaly overproduce eTregs and to verify whether these eTregs present the epigenetic signatures of stable suppressive Tregs.

In conclusion, we propose the following model: in individuals chronically infected with *S. japonicum*, the spleen produces eTregs, which then migrate to the inflamed liver. In patients with severe hepatic disease, CXCR3 is poorly expressed on blood eTregs, which limits their access to the liver where they may otherwise play a protective role, in particular by inhibiting Th1 cells. However, the abnormally large spleen contributes to an excess of eTregs. These cells may have altered properties that may be detrimental for the organ. Further investigation will be necessary to confirm these hypotheses.

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Footnotes:

1. A. Romano and X. Hou contributed equally to this work

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4. Abbreviations: pTregs: peripherically-induced Tregs, tTregs: thymus-induced Tregs, eTregs: effector Tregs, HF: hepatic fibrosis, CentF: central fibrosis, NetF: network fibrosis, HF+/-: mild HF, HF++: moderate HF, HF+++: severe HF, Spl: subjects with normal spleen size, SplM: subjects with splenomegaly, Spl-: splenectomized subjects, HSP: hepatosplenic subjects.

Figure legends

Figure 1: A) Gating strategy for the accurate determination of the proportion of regulatory T cells in PBMCs 1. Lymphocytes; 2. $CD4^+$ T cells; 3. $CD4^+CD45RA^+FOXP3^{low}$ T cells; 4. $CD4^+CD45RA^-FOXP3^{low}$ T cells; 5.$CD4^+CD45RA^-FOXP3^{high}$ T cells); **B**) Percentage of blood $CD45RA^-FOXP3^{low}$ non-Tregs (gray bars), $CD45RA^-FOXP3^{high}$ T cells (black bars) and $CD45RA^+FOXP3^{low}$ T cells (white bars) among blood $CD4^+$ T cells of infected patients; **C**) Proportion of $CD45RA^-FOXP3^{high}$ T cells and $CD45RA^+FOXP3^{low}$ T cells expressing CD25, CCR7, CXCR3, CCR5, or CTLA-4 among blood $CD4^+$ T cells in 45 patients (excluding splenectomized patients). * $p<0.01$.

Figure 2: (A) Proportions of total $FOXP3^+$Tregs; **B**) eTregs; **C**) and naïve Tregs in the blood of study subjects divided according to clinical status (hepatic fibrosis grade and splenomegaly); **D**) Proportion of $CXCR3^+$ cells among eTregs by clinical status, **E**) Proportion of $CXCR5^+$ cells among eTregs by clinical status.
Gray bars show subjects with a normal spleen and black bars show subjects with splenomegaly. Subjects with HF+++ who have undergone a splenectomy are shown by dashed bars. Controls are shown by a white bar. Fig 2.A: $FOXP3^+$ Treg blood levels were positively correlated with both the severity of HF (p=0.004) and SplM (p=0.03).In the multivariate regression, HF (p=0.01) and SplM (p=0.05) were associated with high Treg blood levels. Fig.2B: eTreg blood levels (Fig. 2B) were higher both in patients with HF (p=0.05) or SplM (p=0.08) than in healthy controls.

Figure 3: A-B) Expression of the ligands for *CXCR3*, *CCR5* and *CCR7* in the liver and spleen of eight hepatosplenic patients (sample 2). Messenger RNA levels are expressed relative to the arithmetic mean values obtained for 11 healthy controls. **C**) The proportion of $IFN\gamma^+$ cells among blood $CD4^+$ T cells is negatively correlated (r=-0.73, p=0.002) with blood counts of eTregs in the study population. The proportion of $IFN\gamma^+CD4^+$Tcells was determined after 6h of stimulation with PMA, Ionomycin and monensin. * $p<0.01$, ** $p<0.001$.

Figure 4: Proportion of eTregs and naïve Tregs in the blood and spleen of hepatosplenic patients. Grey bars represent eTreg and squared bars represents naïve Tregs (A); proportion of eTregs (B) and Naïve Tregs (C) expressing various markers in the blood and spleen are shown. Data from five HSP patients is shown (Group 3).

Figure 5: *IL2RA* genetic variants that stimulate the induction of eTregs are associated with severe splenomegaly. (A) The effect of *IL2RA* rs3118470 on Tregs induction were evaluated in cultures of blood from healthy donors carrying the indicated genotypes. The percentage of eTregs detected in cultures with TGF-β ± IL-2, antiCD3 and antiCD28 Ab (Materials and Methods) is shown. (B) The association was assessed in 456 individuals (179 with hepatosplenic schistosomiasis and 277 with milder schistosomiasis), as previously described (19). Data are expressed according to rs3118470 C/T genotype.

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Figures

Figure 1

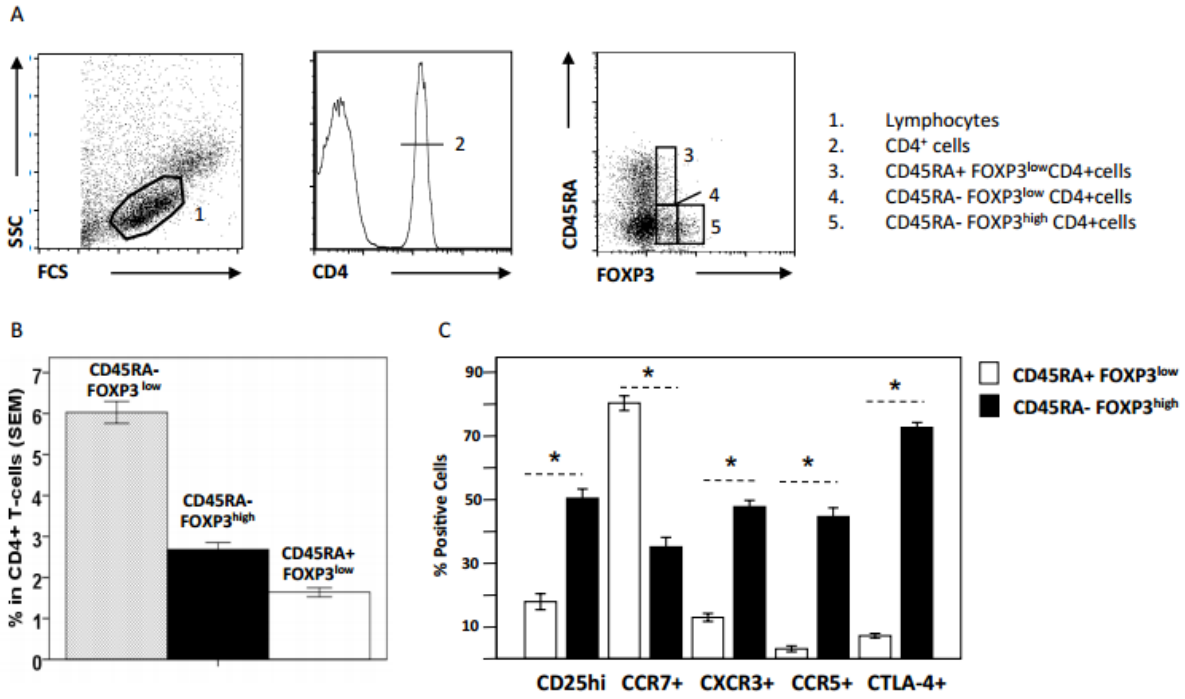
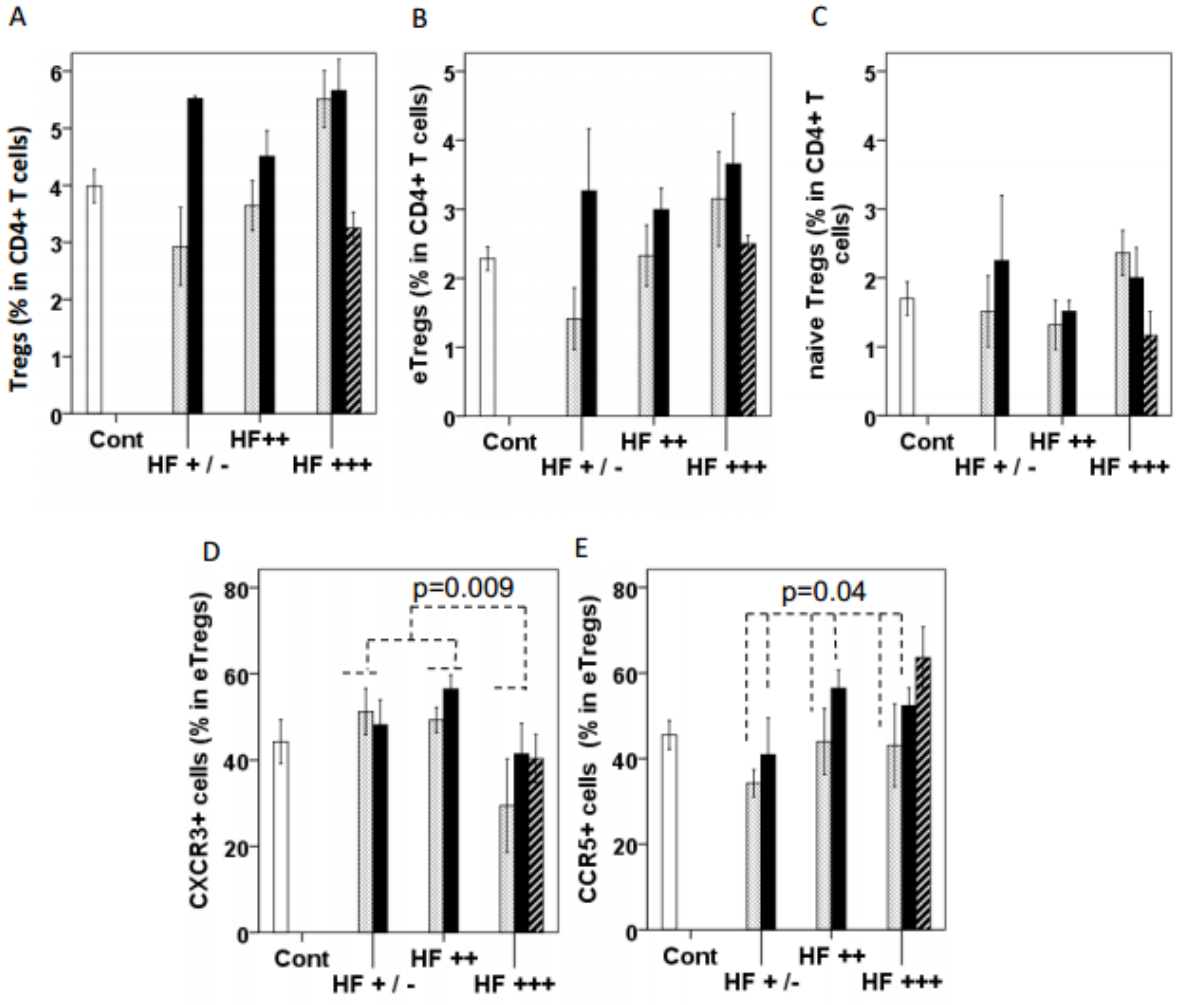


Figure 2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Cont
HF + / -
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HF +++
B
eTregs (% in CD4+ T cells)
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HF + / -
HF ++
HF +++
C
naive Tregs (% in CD4+ T cells)
Cont
HF + / -
HF ++
HF +++
D
CXCR3+ cells (% in eTregs)
p=0.009
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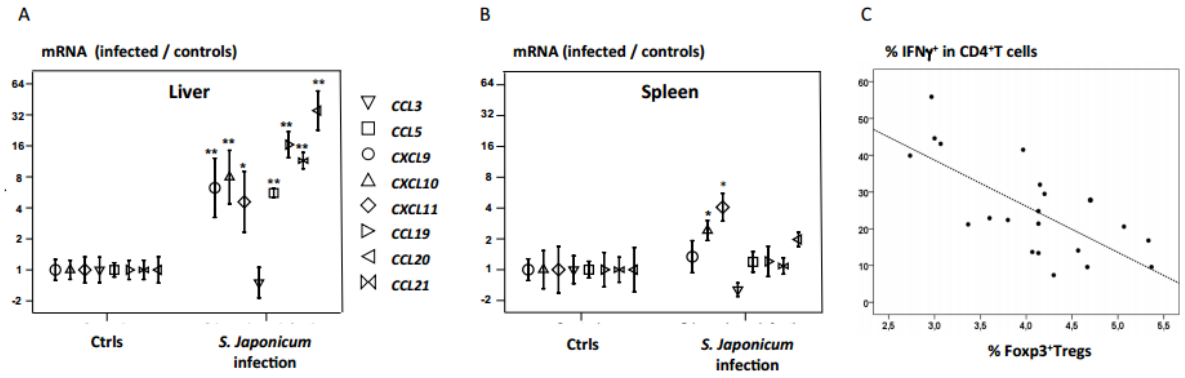
Figure 3


A
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CCL3
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CXCL9
CXCL10
CXCL11
CCL19
CCL20
CCL21
B
mRNA (infected / controls)
Spleen
64
32
16
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4
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1
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Ctrls
S. Japonicum infection
C
% IFNγ+ in CD4+T cells
60
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% Foxp3+Tregs

Figure 4

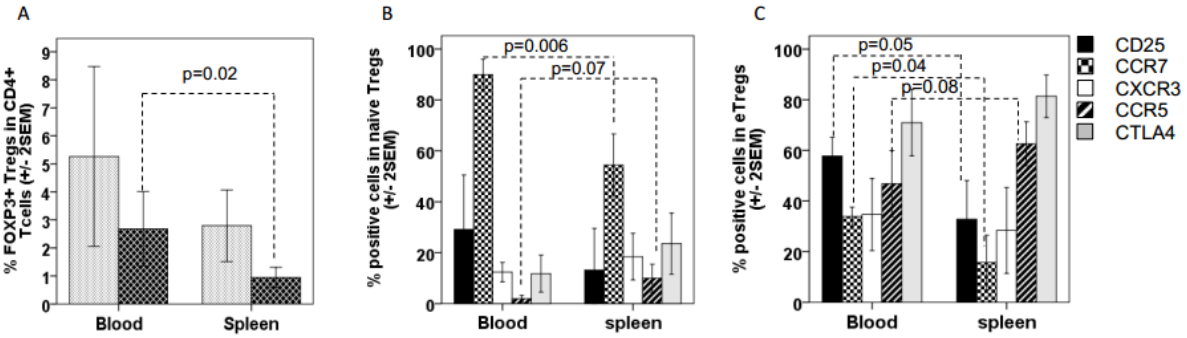
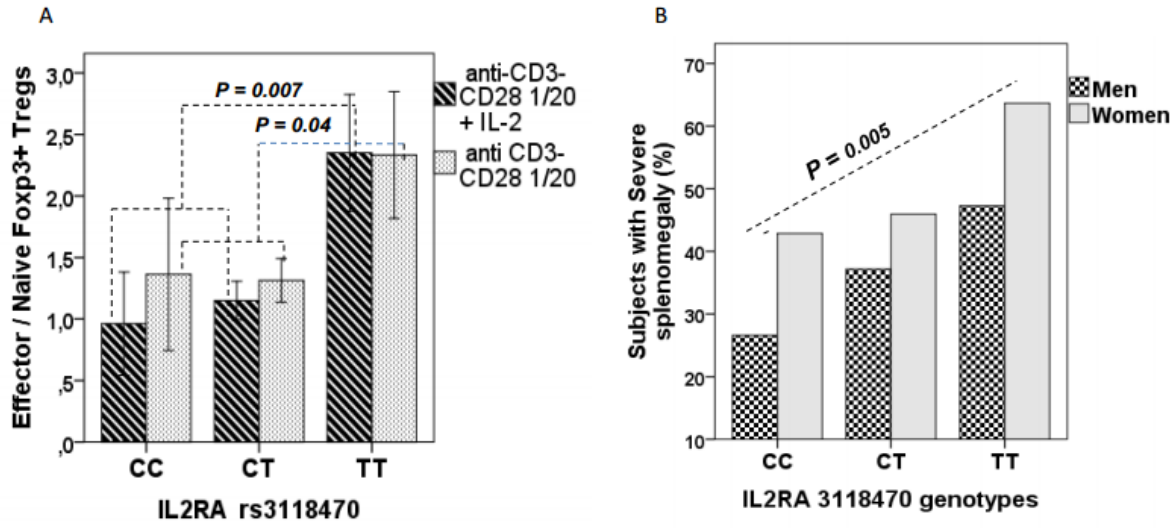

A
% FOXP3+ Tregs in CD4+ Tcells (+/- 2SEM)
p=0.02
Blood
Spleen
B
% positive cells in naive Tregs (+/- 2SEM)
p=0.006
p=0.07
Blood
spleen
C
% positive cells in eTregs (+/- 2SEM)
p=0.05
p=0.04
p=0.08
Blood
spleen
CD25
CCR7
CXCR3
CCR5
CTLA4

Figure 5


A
Effector / Naive Foxp3+ Tregs
3,0
2,5
2,0
1,5
1,0
,5
,0
P = 0.007
P = 0.04
anti-CD3-CD28 1/20 + IL-2
anti CD3-CD28 1/20
CC
CT
TT
IL2RA rs3118470
B
Subjects with Severe splenomegaly (%)
70
60
50
40
30
20
10
P = 0.005
Men
Women
CC
CT
TT
IL2RA 3118470 genotypes

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DETECTION OF PARASITE ANTIGENS IN *LEISHMANIA INFANTUM*–INFECTED SPLEEN TISSUE BY MONOCLONAL ANTIBODY-, PIEZOELECTRIC-BASED IMMUNOSENSORS

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ABSTRACT: Diseases such as leishmaniases are important causes of morbidity and mortality in Brazil, and their diagnoses need to be improved. The use of monoclonal antibodies has ensured high specificity to immunodiagnosis. The development of an immunosensor, coupling a monoclonal antibody to a bioelectronic device capable of quickly detecting *Leishmania* sp. antigens both qualitatively and quantitatively, is a promising alternative for the diagnosis of leishmaniasis due to its high specificity, low cost, and portability, compared with conventional methods. The present work was aimed at developing an immunosensor-based assay for detecting *Leishmania infantum* antigens in tissues of infected hosts. Four hybridomas producing monoclonal antibodies against *L. infantum* had their specificity confirmed by enzyme-linked immunosorbent assay. These antibodies were immobilized on a gold surface, covered with a thin film of 2-aminoethanethiol (cysteamine) and glutaraldehyde, blocked with glycine, and placed into contact with extracts of *L. infantum*–infected and noninfected control hamster spleens. The assay was able to detect 1.8×10^4 amastigotes/g of infected tissue. These results demonstrated that this assay may be useful for quantifying *L. infantum* amastigotes in organs of experimental animals for studies on pathogenesis and immunity and that it is a promising tool for the development of a diagnostic method, based on antigen detection, of human and dog visceral leishmaniasis.

Visceral leishmaniasis (VL) is endemic in 62 countries, where 200 million people are at risk of acquiring the disease. About 90% of the cases are concentrated in 5 countries, namely Bangladesh, India, Nepal, Sudan, and Brazil (Desjeux, 2004). VL is mainly caused by 2 species of *Leishmania*: *Leishmania donovani* and *Leishmania infantum*. *Leishmania donovani* causes anthroponotic VL in the Old World, and *L. infantum* causes zoonotic VL in both the Old and New World (Desjeux, 2004). VL in the New World uses canines as its main reservoir and is also called American visceral leishmaniasis (AVL) (Grimaldi and Tesh, 1993; Romero and Boelaert, 2010). AVL has not been dealt with successfully by the public health services in many of the affected countries, and it still causes high morbidity and death when not treated appropriately and in a timely manner (Grimaldi and Tesh, 1993).

The serodiagnosis of AVL is usually performed by immunofluorescence, enzyme-linked immunosorbent assay (ELISA), or both, but neither of these methods has yet shown good specificity (Reithinger and Dujardin, 2007); the diagnosis is only conclusive with the identification of amastigote forms of *L. infantum* in bone marrow smears (usually in humans) and spleen aspirates (usually in dogs). However, due to economic difficulties, the diagnosis of AVL may be based exclusively on clinical criteria or serology. Indeed, in the absence of parasitological diagnosis, the therapeutic response to toxic drugs is sometimes the only way to diagnose AVL (Marsden, 1984; Bryceson et al., 1985; Chappuis et al., 2007).

Advances in the serodiagnosis of leishmaniasis have been related to the use of recombinant proteins as antigens (Braz et al., 2002; Sreenivas et al., 2002; Maalej et al., 2003). However, the detection of antibodies, despite having large relevance for epidemiological studies, is less useful for clinical diagnosis not only due to cross-reactions with other pathogens but also because high antibody levels may be related to previous or asymptomatic infections (Maia and Campino, 2008). Based on these assumptions, the detection of parasite antigens has a great advantage over detection of antibodies because, in sensu lato, it is a parasitological test, revealing the presence of parasite molecules and therefore the presence of the pathogen (Cruz et al., 2006; Gomes et al., 2008). A great advance in the diagnosis of leishmaniasis may potentially arise from the detection of parasite DNA by the polymerase chain reaction, but this technique requires a specialized technician, above-average laboratory facilities, and is relatively time-consuming (Cruz et al., 2006; Maide et al., 2008).

An immunosensor is a device made up of an antigen or antibody species coupled to a signal transducer that detects the binding of the complementary species. Biosensors based on piezoelectric transducers can replace commonly used tests, decreasing the time of performance and cost, by using serum and tissue samples. Quartz crystal microbalance (QCM) piezosensors are considered excellent for detection of immunoreactions, in that changes in the electrode–electrolyte interface are of a sufficient magnitude to reveal the presence of the desired adsorbed components by changes on the electrical frequency (Chu et al., 2006). The principle of operation of the QCM biosensor depends on the piezoelectric effect that is governed by relationships between mass and frequency variations (Luo et al., 2007). The selectivity of the immunosensor is achieved by the proper choice of a chemically modified surface and immobilized biocomponents on the electrode surface (Marx, 2003; Dutra and Kubota, 2007). Self-assembled monolayers (SAMs) using alkanethiol films have been presented as adequate structuring for the antibody immobilization (Dutra et al., 2007). These QCM sensors can be easily packaged for routine use as portable units. The present work was aimed at developing a piezoelectric immunosensor from sensing elements (anti–*L. infantum* monoclonal antibodies) to be used as a tool to detect parasite antigens in tissues of experimentally infected animals and, as a medium-term goal, to contribute to the parasitological (sensu lato) diagnosis of human and canine VL.

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MATERIALS AND METHODS

Animals

Pathogen-free, 6- to 12-wk-old BALB/c mice and golden hamsters were maintained at the animal facilities of the Gonçalo Moniz Research Center, Oswaldo Cruz Foundation, Bahia (FIOCRUZ-BA), Brazil, and provided with rodent diet and water ad libitum. All procedures were approved and conducted according to the institutional Ethical Committee for the Utilization of Experimental Animals (protocol 036/2009) of FIOCRUZ, Salvador, Brazil.

Anti–*L. infantum* monoclonal antibodies (αLimAbs)

αLimAbs (5A9H8, 2B7B8, 4B6F7, and 5AB3A10B4)-producing hybridomas, previously obtained by fusing myeloma cells with *L. infantum*–infected mouse splenocytes in accordance with the methodology described by Fróes et al. (2004), were cultured and expanded in Iscove's modified Dulbecco's medium (Invitrogen, São Paulo, SP, Brazil), containing 10% fetal calf serum (FCS), 100 μg/ml glutamine, and 50 μg/ml gentamicin. The isotypes of the monoclonal antibodies (mAbs) present in supernatants of hybridoma cultures were determined by capture ELISA, using the Mono-Ab-ID kit (Invitrogen), according to manufacturer's recommendations. Large amounts of these mAbs were produced by inoculation of the hybrid cells into the peritoneal cavity of BALB/c mice, previously injected intraperitoneally with pristane (2,6,10,14-tetramethylpentadecane; Sigma-Aldrich, St. Louis, Missouri). The antibodies were semipurified from the ascitic fluid by precipitation with ammonium sulfate (pH 7.0), followed by dialysis against 0.15 M phosphate-buffered saline (PBS) at pH 7.4. The semipurified mAbs were assayed for protein content by Lowry's method (Lowry et al., 1951), aliquoted, and stored at –70 C until use.

Parasites and parasite extracts

Leishmania infantum (MHOM/BR2000/Merivaldo strain) promastigotes were obtained from in vitro cultures of homogenates from infected hamster spleens, in Schneider's medium supplemented with 10% fetal FCS (Invitrogen) and 50 μg/ml gentamicin at 25 C. Part of the promastigotes was washed by 3 centrifugations (1,620 *g*; 10 min) in cold PBS, counted in a Neubauer's chamber, and stored at –70 C for extract preparation. The remaining promastigotes were transferred to Schneider's medium containing 50 μg/ml gentamicin and 20% FCS (pH 7.2) and cultivated at 35 C for 10–13 days, in accordance with Teixeira et al. (2002), to obtain axenic amastigotes. After transformation, the axenic amastigotes were washed in cold PBS and stored at –70 C until use.

Trypanosoma cruzi amastigotes and trypomastigotes were obtained from in vitro culture of infected monkey epithelial cells (MK2) in the presence of RPMI-1640 medium (Invitrogen) containing 10% FCS and 50 μg/ml gentamicin (Piazza et al., 1994). The parasites were collected from the cell supernatants after 5 days of infection, washed, and kept frozen until use. All frozen parasites were lysed by ultrasound (Branson's Cell Disruptor, Branson Sonic Power Company, Danbury, Connecticut) in the presence of PBS and centrifuged (14,000 *g*; 10 min). The supernatants were filtered through a membrane of 0.45-μm-diameter pore size and stored at –70 C until use. The protein content was measured by Lowry's method (Lowry et al., 1951).

Extracts of *L. infantum*–infected hamster spleen

One hundred million *L. infantum* metacyclic promastigotes, obtained from cultivation in Schneider's medium, were inoculated into the peritoneal cavity of a 3-mo-old golden hamster. After 60–90 days, the animal was killed and its spleen was weighed. The spleen parasite load, estimated by a limiting dilution assay (Titus et al., 1985), performed in a culture that had been set up with 200 mg of a homogenized spleen fragment in Schneider's medium containing 20% FCS and 50 μg/ml gentamicin, was 4.9×10^9 amastigotes/g. Four hundred milligrams of the remaining infected organ fragment was homogenized in 56 ml (an 1:140 [w:v] dilution) of PBS containing 1% NP40 (Sigma-Aldrich) and a protease inhibitor mix (Amersham, São Paulo, SP, Brazil). The tissue was then sonicated, incubated at 4 C for 30 min, centrifuged (14,000 *g*; 10 min), filtered through a membrane with 0.45-μm-diameter pores, aliquoted, and stored at –70 C until use. Each milliliter of this spleen extract therefore contained the soluble antigens from 3.5×10^7 amastigotes. The spleen of a noninfected hamster was subjected to the same procedure and used as a negative control.

Determination of the reactivity and specificity of αLimAbs

The reactivity of the αLimAbs was determined by a sandwich ELISA using an extract of *L. infantum*–infected hamster spleen. In brief, wells of a microtiter plate were coated with 30 μg/ml of each semipurified monoclonal antibody, diluted in 0.15 M carbonate/bicarbonate buffer (pH 9.3). After blocking of possibly available binding sites with PBS containing 5% powdered skimmed milk (PBS-SM), 100 μl of the *L. infantum*–infected hamster spleen extract or control noninfected spleen extract was added to the wells, diluted at 1:125 in PBS-SM. The reaction was detected using *L. infantum*–infected dog serum, diluted at 1:200 in PBS-SM, followed by an anti-dog immunoglobulin antibody-peroxidase conjugate, diluted at 1:1,000; followed by a solution of 3,3′,5,5′-tetramethylbenzidine with hydrogen peroxide (Sigma-Aldrich) as substrate. Between all the steps, the plate was incubated for 1 hr at room temperature (except for the substrate that was incubated for 20 min) and washed 4 times with PBS-SM containing 0.05% Tween 20 (PBS-SM-T20). The reactions were read in a spectrophotometer at 450 nm.

An indirect ELISA was conducted to investigate the cross-reactivity of the αLimAbs against heterologous antigens (axenic amastigote and promastigote forms of *L. infantum*, and amastigote and trypomastigote forms of *T. cruzi*). In brief, parasite extracts, obtained as described above, were used at a concentration of 100 μg protein/ml to coat the wells of microtiter plates. Nonspecific reactions were blocked with PBS-SM. Undiluted αLimAbs-containing hybridoma supernatants were added to the wells, and the assays were developed using an anti-mouse immunoglobulin-peroxidase conjugate diluted at 1:2,000. The remainder of the assay was performed as described above.

Immunosensor assembly and detection the of *L. infantum* amastigote antigens in infected hamster spleen

We applied 4 μg/ml αLimAbs (5A9H8, 4B6F7, 2B7B8, and 5AB3A10B4) to quartz crystal electrode (QMC of 9 MHz; Maxtec Inc., Salt Lake City, Utah) that consists basically of a disk made of piezoelectric quartz crystal coated with a thin film of gold, with a phase lock oscillator circuit coupled to a microcomputer and a potentiostat-galvanostat (Metrohm Pensalab Instrumentação Analítica Ltda, São Paulo, SP, Brazil), that was, in turn, controlled by the GPES software (Eco Chemie B.V., AD Utrecht, the Netherlands). The αLimAbs were immobilized on the gold surface using a SAM, with 2-aminoethanethiol (cysteamine), followed by covalent binding with glutaraldehyde, according to Ramos-Jesus et al. (2011). Remaining unbound aldehyde groups were blocked with glycine. The immunosensor assembly was carried out at room temperature with the following incubation steps: cysteamine (2 hr), glutaraldehyde (45 min), and antibodies and blocking (2 hr each). Then, the *L. infantum*–containing spleen and the negative control spleen extracts, diluted 1:1,000, 1:2,000 and 1:4,000, were injected in the immunosensor surface and incubated for 15 min. Between all steps, 3 washings were performed with PBS, followed by 2 readings of the frequencies. The mean frequency of the 2 readings, expressed in hertz, is depicted in the figures. The sample was considered positive when its application on the surface of the crystal produced a reduction in the oscillation movement that, in turn, resulted in decreased resonance frequencies. The measurements of the frequency in real time permitted the monitoring of the interfacial phenomena that occur by the antigen binding to specific antibodies immobilized on the electrode surface.

RESULTS

The αLimAbs were isotyped as IgG1 (5AB3A10B4), IgG2a (5A9H8 and 4B6F7), and IgG3 (2B7B8) (data not shown). To assess their specificity to *L. infantum* amastigotes compared with hamster spleen antigens, the mAbs were assayed for reactivity against extracts from amastigote-containing and control hamster spleens in a sandwich ELISA. All reacted with the *L. infantum* antigen-containing extract more intensely than with the control extract (Fig. 1). The specificity of the mAbs, as far as *T. cruzi*, *L. infantum* epimastigote, and *L. infantum* axenic amastigote antigens is concerned, was assessed in an indirect ELISA, which is simpler than the sandwich ELISA mentioned above. None of

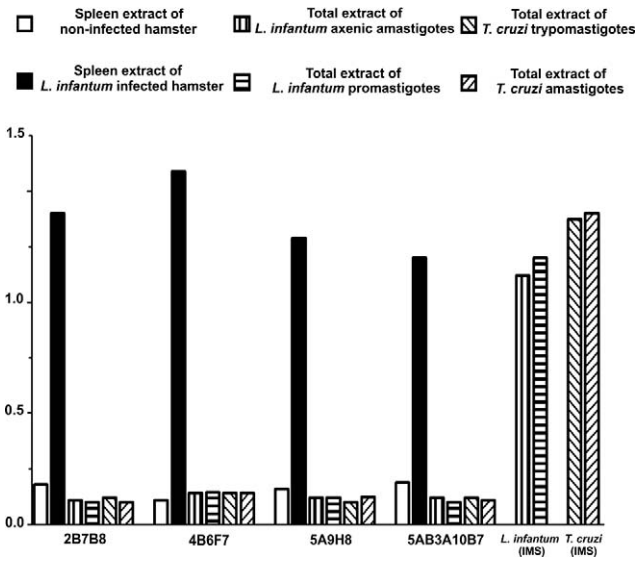

FIGURE 1. Lack of reactivity of anti–*Leishmania infantum* amastigote mAbs to heterologous antigens as determined by ELISA. To analyze the specific reaction against *L. infantum* tissue amastigotes, the wells were coated with the 4B6F7, 5A9H8, 2B7B8, or 5AB3A10B4 anti–*Leishmania* mAbs. The solid columns represent the results obtained when a hamster spleen extract containing *L. infantum* amastigotes (prepared from the spleen of an infected hamster) was added to the wells, and the open columns represent the results obtained with a control extract prepared from a noninfected hamster spleen. The presence of bound *Leishmania* antigens was shown by successive incubations with *L. infantum*–infected dog antibodies, an anti-dog immunoglobulin-peroxidase conjugate, and a chromogen–substrate mixture, as explained in Materials and Methods. To analyze the reactions against heterologous antigens, wells were coated with extracts from *L. infantum* axenic amastigotes or promastigotes and *T. cruzi* amastigotes or trypomastigotes. The reaction was developed using 2B7B8, 4B6F7, 5A9H8, or 5AB3A10B4 anti–*Leishmania* monoclonal antibodies. *Leishmania infantum*- or *T. cruzi*–infected mouse sera (IMS) were used as positive controls.

the mAbs reacted with any of these antigens (Fig. 1). Consecutive injections of 200 µl of *L. infantum*–infected hamster spleen extract, diluted 1:1,000, led to a progressive decrease in the resonance frequencies of the immunosensors coated with any of the 4 mAbs (Fig. 2). In contrast, injections of the noninfected hamster spleen extract had no effect on the frequencies; they remained stable (Fig. 2). The same data are shown in a single graph in Figure 3 to allow a better comparison among the assays performed with the different αLimAbs. The assay with the best signal was the assay that used the 2B7B8 αLimAb (Fig. 3), producing a positive result with a 1:2,000 dilution of the *Leishmania* antigen-containing containing extract (Fig. 4).

DISCUSSION

The development of diagnostic methods with high sensitivity, specificity, low cost, and easy portability, based on the detection of parasite products, may constitute an important achievement to improve the clinical management of suspected AVL cases, allowing the early initiation of specific treatment and avoiding the wrong administration of toxic drugs. In this work, the development of an immunosensor-based assay using αLimAbs and its application for the detection of *Leishmania* spp. antigens in the spleen of an infected hamster has been described. The spleen is the main organ that is infected by *L. infantum* in different mammal species (Rousseau et al., 2001).

First, it was determined that the mAbs reacted with a *L. infantum*–infected hamster spleen extract in a sandwich ELISA, producing optical densities (ODs) ranging from 1.2 to 1.6, values that were about 1 order of magnitude larger than those produced when the mAbs were incubated with a control, noninfected hamster spleen extract (range, from 0.12 to 0.19). This readily demonstrable reactivity to *Leishmania* antigens within a spleen extract indicated that the mAbs did not cross-react with a major hamster splenic antigen and could be used to detect *Leishmania* antigens in the extracts. After the characterization and semi-purification of the αLimAbs, they were successfully used to construct piezoelectric immunosensors using QCM with self-assembled, organized monolayers. These immunosensors produced clear signals when placed into contact with an extract of a spleen containing *L. infantum* amastigotes. In recent decades, QCM biosensors have found various applications in diagnosis, for example, in studies on the pathogenicity of microorganisms and in the investigation of molecular interactions, because of their attractive characteristics, such as high specificity, low cost, simplicity, and rapid generation of results (Prusak-Sochazewski et al., 1990; Plomer et al., 1992).

The mAbs used in the work reported here reacted with antigens of a *L. infantum*–infected mouse spleen extract in a sandwich ELISA, whereas they did not react with promastigote and axenic amastigote extracts of this parasite, nor with *T. cruzi* amastigote and trypomastigote extracts in an indirect ELISA. This indicates that, although similarly to cell-derived amastigotes in protein makeup (Teixeira et al., 2002), axenic amastigotes do not express the antigens that are recognized by the used mAbs. Indeed, differences in protein profile between cell-derived and axenic *Leishmania* sp. amastigotes, and between *Leishmania* sp. cell-derived amastigotes and promastigotes, have been reported previously (Teixeira et al., 2002).

As it is, the *L. infantum* antigen-reactive immunosensor described in this report could be of great clinical importance if it proved to be sensitive and specific enough to detect *Leishmania* sp. antigens in AVL patients' bone marrow aspirates. It would be also interesting to determine whether the αLimAbs recognize other *Leishmania* species, particularly *L. donovani* amastigotes, because this would extend their use to other important endemic areas of VL, such as those in the Indian subcontinent and in Sudan. The use of the developed immunosensor is a promising procedure to replace the visualization of amastigotes in bone marrow or spleen aspirates by microscopy, the method that is usually conducted to confirm the diagnosis of VL and requires a highly skilled technician (Chappuis et al., 2007).

The assay using the 2B7B8 αLimAb-coated sensor produced a positive result with a 1:2,000 dilution of a *Leishmania* sp.–infected hamster extract. This dilution corresponded to a 1:280,000 dilution of the soluble antigens that were present in the spleen tissue (4.9×10^9 amastigotes/g of tissue that was diluted 1:140 [w:v] in the neat extract). The assay should therefore be able to produce a positive result in an extract in which the tissue of a spleen containing 7×10^6 parasites/g was diluted 1:400 (w:v). The developed assay is already potentially useful for quantifying amastigotes in studies on pathogen-free experimental animals, such as those dealing with pathogenicity, and on assessing vaccination procedures. Nonetheless, an improvement in the

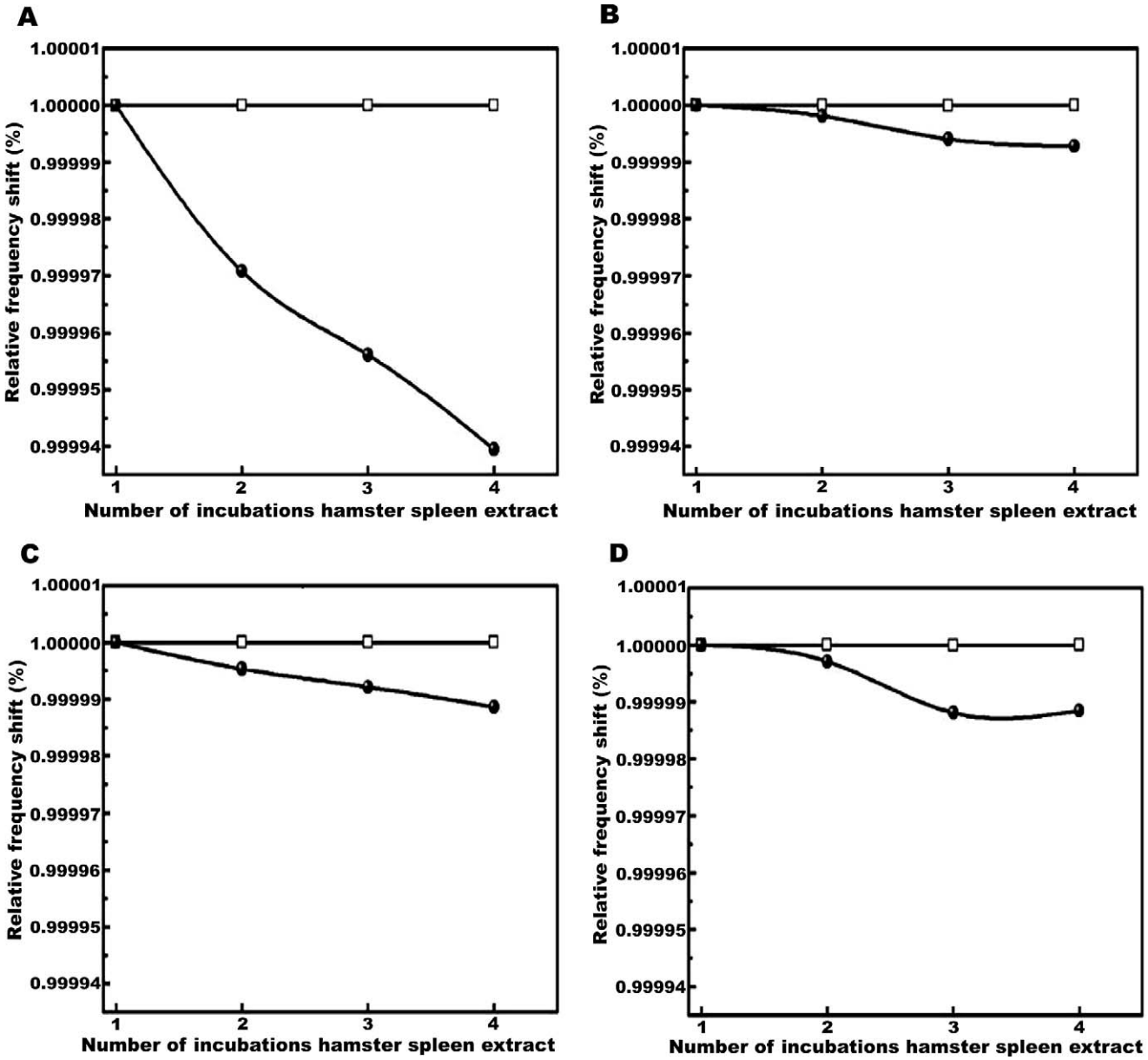

FIGURE 2. Reaction of αLimAbs against extracts of spleens from *Leishmania infantum*–infected (●) or noninfected (□) hamsters on a piezoelectric effect-based sensor. Four injections of 1/1,000 dilutions of spleen extracts were applied onto the immunosensor surface that had been precoated with the 2B7B8 (**A**), 4B6F7 (**B**), 5A9H8492 (**C**), and 5AB3A10B4 (**D**) αLimAbs. Each point of the curves represents the mean frequency of 2 reaction readings in the same immunosensor.

sensitivity of the immunosensor is highly desirable. One way to achieve this goal is to increase the QMC binding area for the biological component. This will allow an increase in the amount of antigen–mAb complex due to an increase in the amount of the solid phase mAb in the immunosensor, increasing the assay's sensitivity. An additional possibility is to test whether coating the QMC with more than 1 mAb would also increase the assay sensitivity. Still another possibility is to replace glutaraldehyde with a molecule directed to the carboxyl residue of the Fc region; glutaraldehyde does not bind specifically to the NH_2 residues in the Fc region of the antibody and therefore may interfere with the antibody antigen-binding region. This would increase the amount of operationally active mAb in the immunosensor. Future investigation aimed at decreasing the assay costs would also be desirable. Finally, the assay may also be useful in the following cases: (1) for the detection of amastigote antigens in bone marrow aspirates of suspected human cases of AVL, and in spleen aspirates of dogs suspected of being infected with *L. infantum*, replacing the parasitological examination, as discussed above; (2) detection of amastigote antigens in the blood of *L. infantum* and human immunodeficiency virus (HIV) co-infected patients; and (3) detection of *L. donovani* amastigote antigens in the blood of anthroponotic VL. Although *L. infantum* amastigotes are not easily found in the blood of patients with AVL, their presence in

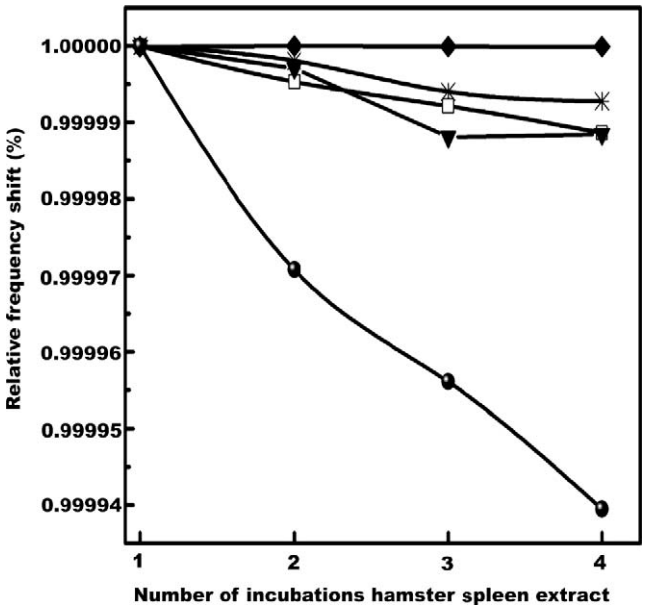

FIGURE 3. Signal strengths obtained by using different αLimAbs in a piezoelectric effect-based sensor. The curves represent data obtained with immunosensors prepared with the 2B7B8 (●), 4B6F7 (▼), 5A9H8 (*), and 5AB3A10B4 (□) αLimAbs, injected 4 times with 1:1,000 dilutions of extracts from *L. infantum*–infected (ISE) or noninfected (NiSE) hamster spleens. The curves obtained with the NiSE in the assays with the 4 different αLimAbs were identical and are shown as a single curve (αLimAbs + NiSE). Each point of the curves represents the mean frequency of 2 reaction readings in the same immunosensor.

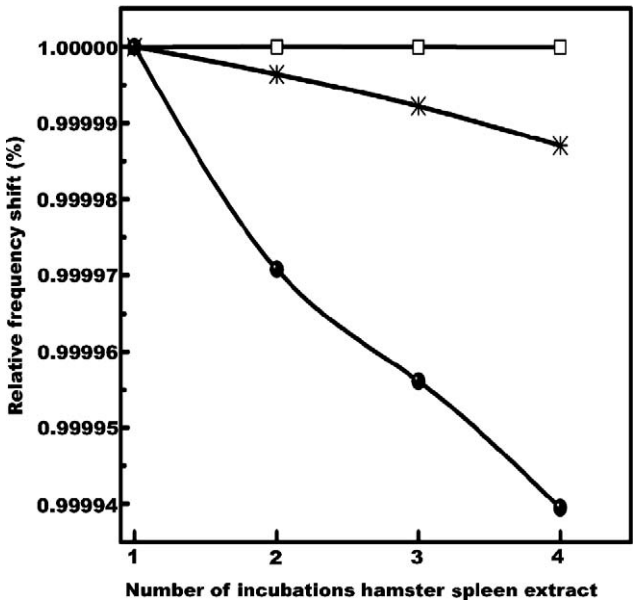

FIGURE 4. Dilution curve of the *Leishmania infantum*–infected hamster spleen extract assayed in an immunosensor prepared with the 2B7B8 αLimAb. *Leishmania infantum*–infected spleen extract was diluted 1:1,000 (●), 1:2,000 (*), and 1:4,000 (□) and applied 4 times onto the surface of an immunosensor prepared with the 2B7B8 αLimAb. Each point of the curves represents the mean frequency of 2 reaction readings in the same immunosensor.

the blood of *L. infantum* and HIV co-infected patients has been reported previously (Orsini et al., 2002; Catorze, 2005). In the case of anthroponotic visceral leishmaniasis, *L. donovani*–reactive mAbs would have to be used in the immunosensor. Due to the strong homology between *L. donovani* and *L. infantum* (Mauricio et al., 2001; Jamjoom et al., 2004), it is possible that the immunosensor whose development is described in the present paper could be used for the diagnosis of anthroponotic visceral leishmaniasis. Although the clinical features of anthroponotic VL are similar to those of AVL, *L. donovani* amastigotes are more frequently found in the blood than *L. infantum*, facilitating their transmission among humans (Chappuis et al., 2007). In addition, their secreted-excreted antigens circulate in the bloodstream and can be detected by antigen-capture methodology (Desjeux, 2004; Singh et al., 2006; Chappuis et al., 2007; Gorski et al., 2010). Thus, the use of immunosensors as described in the present work should be investigated as a diagnostic tool, both in the case of co-infection by *L. infantum* and HIV and of infection by *L. donovani*.

The immunosensor-based assay using αLimAbs that has been described in the present work is a promising tool for the detection of *Leishmania* antigens in infected experimental animals, and it may be useful for the development of a diagnostic method based on antigen detection, of AVL in humans and dogs and of anthroponotic VL.

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Antibody markers of infection susceptibility in *Leishmania* extract-injected mice with experimental cutaneous leishmaniasis

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Abstract

Background: *Leishmania braziliensis* and *Leishmania amazonensis* induce cutaneous leishmaniasis in BALB/c mice. However, whereas BALB/c mice die from a progressively increasing cutaneous disease when infected by *L. amazonensis*, the infection by *L. braziliensis* is spontaneously cured. We have found that intravenous injections of *L. amazonensis* amastigote extract (LaE) promote *L. braziliensis* infection in BALB/c mice, and the infection-promoting activity could be inhibited by adding protease inhibitors to the extract.

Results: In order to detect markers of disease evolution, in the present work we analyzed the specificity of the anti-*L. amazonensis* antibody response of BALB/c mice injected intravenously with saline or LaE, supplemented or not with proteases inhibitors, by the Western blot technique. IgG1 antibodies specifically recognizing an antigen with apparent MW of 116 kDa were specifically detected in BALB/c mice that had been turned susceptible to *L. braziliensis* infection by injections of LaE.

Conclusion: A Th2 immune response against this antigen, therefore, could be associated with a reduced resistance to *Leishmania* infection.

Keywords: *Leishmania*; Antibody response; Disease progression

Background

Leishmania parasites proliferate either as extracellular promastigotes, in the sand-fly vector, or as intracellular amastigotes, inside the phagolysosome of mammalian macrophages. Depending on the *Leishmania* species or isolate, and on the nature of the host immune mechanisms, the infection can cause distinct forms of disease, ranging from self-limiting cutaneous lesions to lethal visceral illness [1-3]. The leishmaniasis are difficult to treat, and parasite resistance against the currently available drugs is increasing [4].

Although *Leishmania braziliensis* causes a serious health problem in South America [5], leading in some cases to mutilating nasal and/or oral lesions [reviewed in 6], few experimental studies on the characterization of its antigens, and on the immune response against them, have been performed [7-9]. Contrasting to what can be observed in *L. braziliensis*-infected individuals, some *L. amazonenis*-infected individuals develop a progressive form of leishmaniasis, with multiple, heavily parasitized cutaneous nodules (diffuse cutaneous leishmaniasis), which is clearly associated with a failure of the patients' immune system to mount a parasite-specific Th1 immune response [10,11].

It has been demonstrated that IgG1 and IgG2a antibody isotypes are associated with either a Th2 or Th1 immune response, respectively [12]. This Th2 immune response has been associated with disease susceptibility, and the Th1 immune response to disease resistance, in murine leishmaniasis models [13]. As with human beings, different species of *Leishmania* cause different diseases in mice, depending also on the genetic background of the mice [14-17].

A study on amastigote antigens, using the Western blot technique, demonstrated that antibodies from infected, healthy individuals and from leishmaniasis patients reacted with different parasites antigens [18]. Along the same line, a *Leishmania chagasi* recombinant antigen, the k39, has been shown to discriminate antibodies from infected, asymptomatic individuals from those of patients with overt visceral leishmaniasis [19,20]. Antibodies, therefore, may serve as markers of active disease in leishmaniasis. Prospective studies would need to be carried out in order to ascertain whether some antibodies produced by asymptomatic, *Leishmania*-infected individuals could be markers of resistance or susceptibility to the development of disease. The early *L. infantum* infection in dogs was characterized by recognition of polypeptide fractions of low molecular weight, by IgG1 and IgG2, symptomatology was associated with recognition by both IgG subclasses of a 24 kDa fraction and other antigens belonging to the AG24 family [21].

In our laboratory, it was shown that BALB/c mice, which are normally resistant to *L. braziliensis* [22], become susceptible if they are treated with intravenous injections of a soluble extract of amastigotes of *Leishmania amazonensis* (and not of *L. braziliensis*), and that the supplementation of the extract with serine-protease inhibitors reduces this effect [23].

In order to identify possible antigenic markers of susceptibility to disease associated with the biologically active *L. amazonensis* extract, in the present work the specificity of the anti-*Leishmania* antibody response was assessed by Western blot in *L. braziliensis*-infected BALB/c mice that had been injected intravenously with the *L. amazonensis* extract, supplemented or not with protease inhibitors. Antibodies against an antigen with an apparent molecular weight of 116 kDa, that was only recognized when the

biologically active extract (and not the protease inhibitors-treated, biologically inactive extract) was injected, were identified.

Methods

Mice

Specific pathogen-free, 8-12 week-old, male BALB/c mice were maintained at the animal facilities of the Gonçalo Moniz Institute, Oswaldo Cruz Foundation, Salvador, Bahia, and provided with rodent diet and water *ad libitum*. All procedures performed on the animals were approved and conducted in accordance with norms of the institutional Committee for Animal Care and Utilization.

Parasites and amastigote extract

The MHOM/Br87/Ba125 *L. amazonensis* and MHOM/Br/3456 *L. braziliensis* strains were used. Their infectivities were maintained by regular inoculations of promastigotes in susceptible BALB/c mice and golden hamsters, respectively. Promastigotes, derived from tissue amastigotes, were cultured at 23^{o} C in Schneider´s medium (Sigma Chemical Co., Saint Louis, MO, USA), pH 7.2, supplemented with 50 μg/mL of gentamycin and 10% of heat-inactivated fetal bovine serum (FBS; HIFCS, Gibco Laboratories, Grand Island, NY, USA) for *L. amazonensis*, or 20% FBS for *L. braziliensis*. *L. amazonensis* axenic amastigotes were obtained by the differentiation of promastigotes in axenic cultures, as described elsewhere [24]. The amastigotes were washed three times in ice-cold sterile saline, resuspended in ice-cold saline and lysed by exposition to ultrasound (10 times for 1 min on ice). The lysates were centrifuged at 16,000 g for 10 minutes at 4^{o} C, the supernatants filtered on membranes with 0.22 μm-diameter pores (Millipore, São Paulo, Brazil) and immediately stored at -70^{o} C in small aliquots. These filtered saline supernatants are called in this report *L. amazonensis* extract (LaE). It was shown to be free of bacterial endotoxin by the *Limulus* amebocyte enzyme assay (Biowittaker, MD, EUA), and their protein content was determined by Lowry's method [25]. Part of the prepared LaE was supplemented with proteases inhibitors (10 mM phenylmethanesulfonyl fluoride, 5.7 mM N-p-tosyl-L-phenylalanine chloromethyl kNap-tosyl-L-lysyl chloromethyl ketone etone, 5.4 mM N-p-tosyl-L-lysine chloromethyl ketone hydrochloride, and 5.9 mM 4-nitrophenyl hepta-O-acetyl-1-thio-beta-lactoside; Sigma Chemical Co., St Louis, MO, USA).

Treatment of mice with *Leishmania* extract

Each animal from groups of 4 to 5 BALB/c mice received four 0.2-mL i.v. injections of either LaE or LaE with proteases inhibitors. The injections were separated by an interval of two weeks and each contained 200 μg of protein. Control groups consisted of mice that received four 0.2-mL injections of saline or saline treated with proteases inhibitors at identical times.

Murine model of cutaneous leishmaniasis

Ten million *L. braziliensis* promastigotes, obtained from stationary-phase culture, were subcutaneously inoculated into one of the hind footpads of BALB/c mice, seven days after the first injection of the LaE. Parasite loads in the footpads were estimated by limiting dilution [26]. Briefly, the infected footpads were macerated in Schneider´s medium and centrifuged at 50 g for 10 min, at 4^{o} C. The supernatants were re-centrifuged at 1540 g for 10 min at 4^{o} C and the pellets were resuspended in Schneider´s medium, supplemented with 50 μg.mL^{-1} of gentamycin, and 20% FBS. The

suspension was serially diluted in 10-fold dilutions and distributed in triplicates in 96-well culture plates. The number of viable parasites in each footpad was determined from the reciprocal of the highest dilution at which promastigotes could be detected after a 7-day culture at 23^{o}C, and was expressed as parasites per milligram of tissue.

Determination of antibody specificity by Western blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of LaE was performed on 12% polyacrylamide gels using a Mini Protean II apparatus (Bio-Rad, California, USA). Wells were loaded with LaE containing 20 µg/mL of protein, resuspended in SDS-sample buffer and boiled for 4 min and the electrophoresis carried out as described elsewhere [27]. Proteins were electrophoretically transferred from the gel to nitrocellulose membrane. The nitrocellulose membrane was cut into vertical strips and blocked for 12 h with 0.15 M phosphate-buffered saline, pH 7.2 (PBS) containing 10% FBS, at 4° C. Incubation with mice sera (diluted 1:1000 in PBS containing 0.05% of Tween 20 and 10% of FBS) was carried out during 1 hour at room temperature with mechanical agitation. After five 1-minute washes in PBS, the membranes were incubated during 1 hour with appropriately diluted horseradish peroxidase-conjugated anti-mouse IgG, IgG1 or IgG2a (Sigma Chemical Co., Saint Louis, MO, USA) in PBS containing 0.05% Tween 20 and 5% FBS, at room temperature. The nitrocellulose strips were finally incubated with a mixture of 3'3-diaminobenzidine (Sigma Chemical Co., Saint Louis, MO, USA) and H_2O_2 in PBS. Normal mouse sera were used as negative controls.

Statistical analysis

When the distribution of the data was found to be Gaussian by the D'Agostino and Pearson's method, comparisons among more than two experimental groups were performed by ANOVA followed by the Newman-Keuls's method. When the distribution was found to be non-Gaussian, comparisons among more than two experimental groups were performed by the Kruskal-Wallis method followed by the Dunn's post test. Results were considered significant when $P \leq 0.05$.

Results

Parasite burden in experimentally infected mice

In a previously published work we have shown that injection of LaE together with protease inhibitors abolished the enhancing effect of the LaE on the *Leishmania* infection in BALB/c mice [23]. We, therefore, in addition to antibodies from control, vehicle (saline)-injected mice, also studied the antibodies from a group of mice that had been injected with LaE together with protease inhibitors.

The number of parasites in the foot pad lesion of BALB/c mice, infected with *L. braziliensis*, that received four biweekly LaE i.v. injections, was significantly higher ($P \leq 0.05$) than in those of animals injected with saline supplemented with proteases inhibitors (Figure 1).

The infected footpads of the mice injected with protease inhibitors-treated LaE had on average 100-fold fewer parasites than the footpads of the mice injected with untreated LaE, as measured by limiting dilution. The addition of protease inhibitors also significantly reduced the ability of the LaE to increase the size of *L. braziliensis*-induced lesion in BALB/c mice (not shown). Mice injected with protease inhibitor-supplemented saline had the same sizes of lesions of mice injected with non-supplemented saline (not shown).

Specificity of the antibody response against *L. amazonensis* amastigote antigens

One protein of apparent MW of 28 kDa, and three proteins with apparent MW from 45 to 54 kDa were only detected by the IgG1 antibodies from mice injected with LaE, regardless of its supplementation with proteases inhibitors (Figure 2). Additional antigens were recognized only by the sera of the mice treated with the unsupplemented extract. These sera clearly recognized from 6 to 17 different antigens (Figure 2, columns 11 to 15), whereas the sera of the mice injected with protease inhibitor-supplemented extract recognized 4 to 5 different antigens (Figure 2, columns 6 to 10). An antigen of apparent MW of 116 kDa was recognized by all sera of the mice injected with unsupplemented LaE (Figure 2, columns 11 to 15), and by none of the sera from the protease inhibitor-supplemented extract (Figure 2, columns 6 to 10). The recognition pattern of the serum from a particular mouse injected with unsupplemented extract (Figure 2, column 14) only differed from the recognition patterns of the mice treated with the protease inhibitor-supplemented extract (Figure 2, columns 6 to 10) by the recognition of that particular antigen.

Similar antigens were recognized by IgG2a antibodies of the *L. braziliensis*-infected BALB/c mice, injected or not with supplemented or unsupplemented LaE (Figure 3). Three antigens of apparent MW from 34 to 38 kDa reacted with IgG2a antibodies only from the sera of mice treated with LaE, supplemented or not with protease inhibitors (Figure 3). No antigen was recognized specifically by IgG2a antibodies from animals that received unsupplemented LaE in relation to animals that received LaE supplemented with proteases inhibitors (Figure 3, columns 6 to 15).

Discussion

The transformation from the promastigote to amastigote forms provides the necessary adaptations for the *Leishmania* to survive in the mammalian environment, including mechanisms to evade or subvert the host immune response, such as the escape to safe, protease-free intracellular vacuole and the production of immunomodulatory molecules [28-30]. In this study, the presence of a *L. braziliensis* infection-potentiating activity in soluble extracts of *L. amazonensis* amastigotes, as observed previously in our laboratory [23,31], was confirmed: larger numbers of parasites were found in the foot pad of *L. braziliensis*-infected BALB/c mice that received LaE than in those from animals injected only with saline (Figure 1).

As also shown herein, the nature of the humoral immune response against *L. amazonensis* antigens differed in *L. braziliensis*-infected mice rendered susceptible by the intravenous injection of LaE from those of relatively resistant mice (injected with LaE supplemented with protease inhibitors or with saline). Quite interestingly, these differences were most evident for IgG1 antibodies (Figure 2), and not for IgG2a antibodies (Figure 3), a fact which is consistent with the LaE acting through the promotion of a Th2-dependent immune response. This again confirm previous results showing increased production of IL-4 by lymph node cells of LaE-treated mice and the absence of infection-enhancing effect of this extract in IL-4-knockout mice [23].

The observed differences in the production of IgG1 antibodies were both quantitative (antibodies of infected BALB/c mice injected only with saline, or even with protease inhibitor-supplemented LaE, reacted with fewer peptides than antibodies from mice injected with unsupplemented LaE), and qualitative: a 116 kDa protein band of *L. amazonensis* amastigotes was exclusively recognized by sera of unsupplemented LaE-treated BALB/c mice. It is possible that the presence of IgG1 antibodies against one or more of these antigens could be a marker of an infection-promoting effect of *L. amazonensis*, maybe through the generation and maintenance of a predominant Th2 immune response that would hinder an otherwise effective Th1 response, allowing the progression of the disease. It would be interesting to find out if sera from patients with diffuse cutaneous leishmaniasis differ from patients from localized cutaneous leishmaniasis caused by *L. amazonensis* by the presence of antibodies against these antigens.

The expression of a serine protease with an apparent molecular weight of 115 kDa by *L. amazonensis* has been described by Silva-Lopez and collaborators [32]. In an electrophoretic-zymographic study performed by our research group, a protein with the same apparent molecular weight and with protease activity was found in an *L. amazonensis* extract and not in an *L. braziliensis* extract (Oliveira & Navarro, unpublished data). It is possible that this serine protease is the protein recognized only by antibodies from mice with severe leishmaniasis, which had been injected with LaE without inhibitors of serine protease in the present work.

Another interesting fact observed in the present work was that the untreated *L. braziliensis*-infected animals produced antibodies, mainly of the IgG2a isotype, recognizing cross-reactive *L. amazonensis* antigens (Figure 3, columns 1-5). This is in accordance to the fact that these animals preferentially mounted a Th1-dependent immune response [23,33]. Moreover, these cross-reactive antibodies could be useful for

identifying, in *Leishmania* DNA libraries, genes encoding recombinant proteins able to elicit potentially protective Th1 immune responses.

Conclusions

In summary, we investigated the pattern of antigen recognition by antibodies from BALB/c mice that were rendered susceptible to *L. braziliensis* infection by intravenous injections of a *L. amazonensis* extract. We found that a protein of approximately 116 kDa was specifically recognised by antibodies from those mice. The immune response against this protein, therefore, might be detrimental to the development of a protective immune response.

Abbreviations

kDa: Kilodalton; LaE: *L. amazonensis* amastigote extract; MW: Molecular weight; PI: Protease inhibitors.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LPC, VMGS and PRSO designed the experiments; VMGS, PRSO, CFA and ICN conducted the experiments; LPC, VMGS and PRSO analyzed the data; LPC and VMGS wrote the paper. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Parasite burden, six weeks after infection, as assessed by limiting dilution, in mouse foot pads. The mice were treated with saline (Saline), saline supplemented with protease inhibitors (Saline + PI), or with *L. amazonensis* extract in the presence (LaE + PI) or absence (LaE) of protease inhibitors, as detailed in the Materials and Methods. The boxes represent the 10-90 percentile intervals and the median values of the results, and the vertical bars the value ranges (n per group = 5). The data are representative of five independent experiments. *P≤0.05 (statistical significance of differences between the indicated sets of data; Mann-Whitney test and Dunn's post test).

Figure 2. Specificity of the anti-*L. amazonensis* IgG1 antibodies present in the sera of *L. amazonensis* extract-treated, *L.braziliensis*-infected BALB/c mice, as assessed by Western blot. The sera were from blood samples collected five weeks after infection. The infected mice were treated with saline (Saline; columns 1 to 5), *L. amazonensis* extract supplemented with protease inhibitors (LaE + PI, columns 6 to 10) or unsupplemented *L. amazonensis* extract (LaE, columns 11 to 15), as detailed in the Materials and Methods. The positions of molecular weight markers are shown on the left of the figure.

Figure 3. Specificity of the anti-*L. amazonensis* IgG2a antibodies present in the sera of *L. amazonensis* extract-treated, *L.braziliensis*-infected BALB/c mice, as assessed by Western blot. Sera were from blood samples collected five weeks after infection. The infected mice were treated with saline (Saline; columns 1 to 5), *L. amazonensis* extract supplemented with protease inhibitor (LaE + PI, columns 6 to 10) or unsupplemented *L. amazonensis* extract (LaE, columns 11 to 15), as detailed in the Materials and Methods. The positions of molecular weight markers are shown on the left of the figure.

Figures

Figure 1

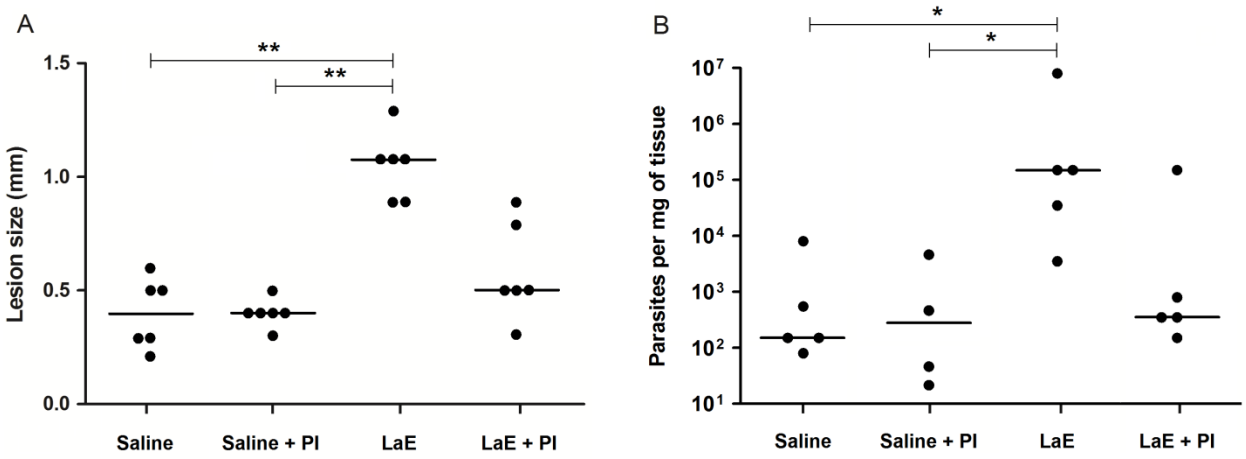

Figure 2

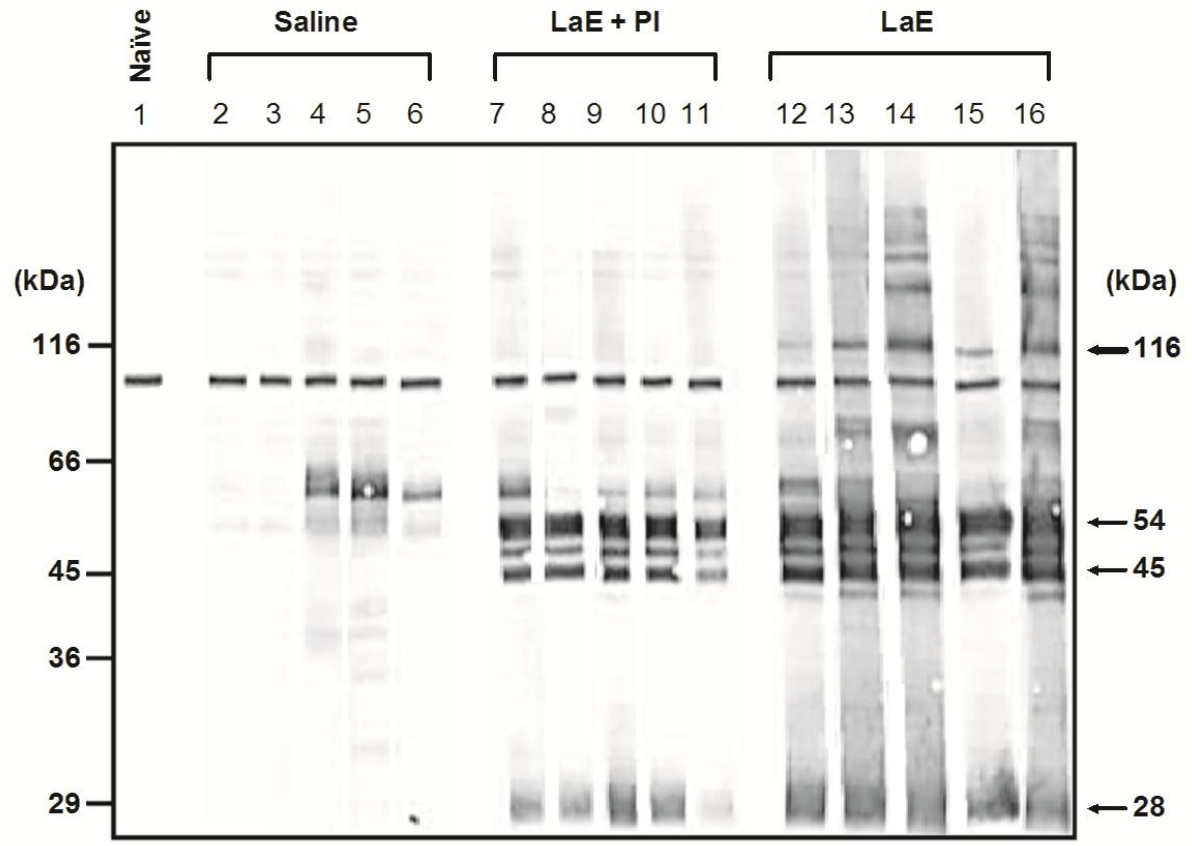

Figure 3