UNIVERSIDADE FEDERAL DE PERNAMBUCO
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MARINA DE ASSIS SOUZA

Quantificação de mediadores dos perfis Th1, Th2, Th17 e Treg na resposta imune contra Leishmaniose Tegumentar Americana ativa e após cura clínica

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Orientador(a): Prof[a]. Dr[a]. Valéria Rêgo Alves Pereira

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Dedico este trabalho à minha família e
aos portadores de leishmaniose tegumentar americana.

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“O destino do ser humano é moldado
segundo a imagem idealizada na mente.
Isto porque a imaginação é uma força concretizadora.

Utilize a força de sua imaginação!
Não dê atenção às críticas alheias!
Avance decididamente no caminho traçado por sua mente e não pare diante dos obstáculos que surgem durante o seu trajeto.

Aquilo que você realmente deseja já existe concretamente em seu subconsciente;
basta materializar-se no plano concreto.
Para tanto você deve lutar com coragem,
não desistindo no meio do caminho...
vá em frente e coragem!”

(Masaharu Taniguchi)

RESUMO

SOUZA, M. A. **Quantificação de mediadores dos perfis Th1, Th2, Th17 e Treg na resposta imune contra Leishmaniose Tegumentar Americana ativa e após cura clínica.** Tese (Doutorado). Universidade Federal de Pernambuco, Recife, Pernambuco, Brasil, 2014.

O modelo clássico de susceptibilidade e resistência à leishmaniose sugere que a expansão de células Th1 ou Th2 direcione o resultado da infecção. Recentemente, o perfil Th17 foi relacionado à doença e parece ser antagônico às células T regulatórias (Treg). Assim, este estudo teve como objetivo quantificar, por ELISA de captura ou qPCR, alguns mediadores dos perfis Th1, Th2, Th17 e Treg (IFN-γ, TNF-α, IL-10, IL-4, TGF-β, IL-6, IL-17, IL-22, RORC, Foxp3 e iNOS) em pacientes com leishmaniose tegumentar americana (LTA) ativa (AD) e após a cura clínica, tratados (AT) ou não (cura espontânea; SH). Os pacientes foram capazes de apresentar resposta imunológica específica frente aos antígenos solúvel (AgSol) e insolúvel (AgIns) de *L. (V.) braziliensis* em relação ao grupo controle. O primeiro artigo demonstrou que, em resposta ao AgSol, houve a predominância de IFN-γ ($P = 0,0061$) e TNF-α ($P = 0,008$) durante a doença ativa, indicando a presença de uma resposta inflamatória; IL-17 também é evidenciada nesse estado clínico ($P = 0,04$). Um aumento na secreção de NO foi observado em SH ($P = 0,023$), enquanto que IL-17 foi observada em baixos níveis nesses pacientes, sugerindo que esta parece ser regulada pelo NO. A presença de IL-10 e IL-22 foi observada em todos os grupos ($P > 0,05$). No segundo artigo, o AgIns estimulou produção significativa de IFN- γ em todos os grupos ($P < 0,05$). AD ($P = 0,007$) e AT ($P = 0,003$) produziram TNF-α. Em contrapartida, o grupo SH apresentou baixos níveis da citocina. De forma interessante, a secreção de NO foi significativa nesses indivíduos ($P = 0,04$), enquanto que IL-17 foi observada em baixos níveis. Produção significativa de IL-17 foi observada no grupo AT ($P = 0,04$). Embora IL-22 tenha sido detectada em AD ($P = 0,02$), seu papel é questionável. A presença de IL-10 em todos os grupos de pacientes sugere que a citocina desempenhe diferentes papéis na doença ($P > 0,05$). No terceiro artigo, PBMC de pacientes com lesões ativas expressaram mRNA para IFN-γ (AgSol: $P = 0,017$; AgIns: $P = 0,0004$) e TNF-α (AgSol: 0,0306; AgIns: $P = 0,027$), reforçando a possível presença de uma resposta inflamatória. A ausência de mRNA para a iNOS nesses pacientes pode ser devido à presença de NO secretado, representando um mecanismo de compensação. Este evento pode ajudar a evitar a exacerbação da resposta imune. Além disso, a presença significativa de mRNA para IL-10 (AgSol: $P = 0,0119$; AgIns: $P = 0,0114$) sugere que mecanismos imunomodulatórios favoreçam uma resposta imune efetiva contra a *Leishmania*. A expressão de Foxp3 frente ao AgIns ($P = 0,0208$) indica que células T regulatórias estejam presentes na resposta de pacientes com lesões ativas. Embora IL-17 e IL-22 pareçam ser importantes na resposta imune efetiva na LTA, Os níveis de mRNA para estas citocinas não foram significativos ($P > 0,05$).Entretanto, os níveis de IL-6 expressos pelos pacientes (AgSol: $P = 0,0189$) com doença ativa parecem ter sido insuficientes para induzir um perfil Th17 juntamente com TGF-β ($P > 0,05$), dada a expressão não significativa de RORC ($P > 0,05$). A expressão de TGF- β pode ter contribuído para a regulação da resposta imune pelas células Treg, o que reforça o suposto antagonismo entre este e o perfil Th17. Assim, a secreção/expressão desses mediadores reforça a complexidade da resposta imune celular no combate à *Leishmania*, e traz informações para o desenvolvimento de vacinas e esquemas imunoterapêuticos.

Palavras-chave: Leishmaniose tegumentar americana; resposta imune celular; ELISA; RT-qPCR.

ABSTRACT

SOUZA, M. A. **Quantitation of mediators from Th1, Th2, Th17 and Treg profiles in the immune response against active American Tegumentary Leishmaniasis and after clinical cure.** Thesis (Doctorate). Federal University of Pernambuco, Recife, Pernambuco, Brazil, 2014.

The classical model of susceptibility and resistance to leishmaniasis suggests that Th1 or Th2 expansion directs the outcome of the infection. Recently, the Th17 profile was related to the disease and seems to be antagonistic to the regulatory T cells (Treg). Thus, this study had as objective to quantify, through capture ELISA or qPCR, some mediators of Th1, Th2, Th17 and Treg profiles (IFN-γ, TNF-α, IL-10, IL-4, TGF-β, IL-6, IL-17, IL-22, RORC, Foxp3 and iNOS) in patients with active (AD) american tegumentary leishmaniasis (ATL) and after clinical cure, treated (AT) or not (spontaneous healing; SH). The patients were capable of presenting specific immune response to the soluble (AgSol) and insoluble (AgIns) of *L. (V.) braziliensis* in relation to the control group. The first article demonstrated that, in response to AgSol, there was the predominance of IFN-γ (P = 0,0061) and TNF-α (P = 0,008) during the active disease, indicating the presence of an inflammatory response; IL-17 is also evidenced in this clinical state (P = 0,04). An increase in NO secretion was observed in SH (P = 0,023), whereas IL-17 was observed in low levels in these patients, suggesting that it may be regulated by NO. The presence of IL-10 and IL-22 was observed in all groups (P > 0,05). In the second article, AgIns stimulated significant IFN- γ production in all groups (P < 0,05). AD (P = 0,007) and AT (P = 0,003) produced TNF-α. On the other hand, the SH group presented low levels of the cytokine. Interestingly, NO secretion was significant in these individuals (P = 0,04), whereas IL-17 was observed in low levels. Significant IL-17 production was observed in AT group (P = 0,04). Although IL-22 was detected in AD (P = 0,02), its role is questionable. The presence of IL-10 in all groups of patients suggests that the cytokine exerts different roles different roles in the disease (P > 0,05). In the third article, PBMC from patients with active lesions expressed mRNA for IFN-γ (AgSol: P = 0,017; AgIns: P = 0,0004) and TNF-α (AgSol: 0,0306; AgIns: P = 0,027), reinforcing the possible presence of an inflammatory response. The absence of mRNA for iNOS in these patients may be due the presence of secreted NO, representing a feedback mechanism. This event may help to impair the exacerbation of the immune response. Furthermore, the significant presence of mRNA for IL-10 (AgSol: P = 0,0119; AgIns: P = 0,0114) suggests that immunomodulatory mechanisms favor an effective immune response against *Leishmania*. Foxp3 expression to AgIns (P = 0,0208) indicates that regulatory T cells are present in the response of patients with active lesions. Although IL-17 and IL-22 seem to be important in the effective immune response in ATL, the mRNA levels for these cytokines were not significant (P > 0,05). However, IL-6 levels expressed by the patients (AgSol: P = 0,0189) with active disease seem to have been insufficient to induce a Th17 profile along with TGF-β (P > 0,05), given the non-significant expression of RORC (P > 0,05). TGF-β expression may have contributed to the regulation of the immune response by Treg cells, what reinforces the supposed antagonism between this and the Th17 profile. Thus, the secretion/expression of these mediators reinforces the complexity of the cellular immune response in the fight against *Leishmania*, and provides informations for the development of vaccines and immunotherapeutic schemes.

Keywords: American tegumentary leishmaniasis; cellular immune response; ELISA; RT-qPCR.

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LISTA DE ABREVIATURAS E SIGLAS

AD	Active disease (doença ativa)
ADP	Adenosina difosfato
AgIns	Antígeno insolúvel
AgSol	Antígeno solúvel
AhR	*Aryl hydrocarbon receptor*
AT	After treatment (após tratamento)
CD	*Cluster differentiation* (Grupamento de diferenciação)
cDNA	DNA complementar
CO_2	Dióxido de Carbono
DNA	Ácido desoxirribonucléico
DPDx - CDC	Laboratory Identification of Parasitic Diseases of Public Health Concern - Centers for Disease Control and Prevention
EDTA	ácido etilenodiaminotetracético
ELISA	*Enzyme-linked immunosorbent assay* (ensaio imunoenzimático)
Foxp3	*Forkhead box p3*
H_3PO_4	Ácido Fosfórico
IDRM	Intradermorreação de Montenegro
IFI	Imunofluorescência indireta
IFN-γ	Interferon-gama
IL	Interleucina
iNOS	Óxido nítrico sintase induzível
Irf-4	*Interferon regulatory factor 4*
LC	Leishmaniose cutânea
LCD	Leishmaniose cutânea difusa
LPG	Lipofosfoglicano
mRNA	RNA mensageiro
NETs	*Neutrophil Extracellular Traps*
NK	Células *natural killer*
NNN	McNeal, Novy & Nicole
NO_2^-	Nitrito
ODC	Ornitina descarboxilase

OMS	Organização Mundial de Saúde
PAGE	Eletroforese em gel de poliacrilamida
PBMC	Células mononucleares do sangue periférico
PCR	Reação em cadeia da polimerase
PHA	fitohemaglutinina
PMSF	metil-fenil-fluoreto
RNA	Ácido ribonucléico
RNAse	Ribonuclease
RORC	*Retinoid-related orphan receptor C*
RORα	*Retinoid-related orphan receptor alpha*
RORγt	*Retinoid-related orphan receptor gamma t*
RPMI	*Roswell Park Memorial Institute*
Sb5+	Antimonial pentavalente
Sb^{III}	Antimonial trivalente
SDS	Dodecil-sulfato de sódio
SFB	Soro fetal bovino
SH	*Spontaneous healing* (Cura espontânea)
STAT3	*Signal transducer and activator of transcription 3*
TCLE	Termo de Consentimento Livre e Esclarecido
TGF-β	*Transforming growth factor* (Fator de crescimento e transformação)-beta
Th	Célula T *helper* (auxiliar)
TLR	*Toll-like receptor*
TNF-α	*Tumor necrosis factor* (Fator de necrose tumoral)-alfa
Treg	Célula T regulatória

SUMÁRIO

1 INTRODUÇÃO

As leishmanioses são doenças infecto-parasitárias que acometem o homem, causadas por diversas espécies de protozoários da ordem Kinetoplastida, família Trypanosomatidae e gênero *Leishmania*. Cinco espécies do subgênero *Viannia* e uma do gênero *Leishmania* estão associadas à doença no Brasil, onde *Leishmania (Viannia) braziliensis* é a principal espécie causadora de leishmaniose tegumentar americana (LTA) (BASANO; CAMARGO, 2004; GONTIJO; CARVALHO, 2003).

O aparecimento das formas clínicas da LTA depende de fatores inerentes ao parasito, como virulência, tropismo e quantidade inoculada. Além disso, características do vetor, o estado imunológico e constituição genética do hospedeiro vertebrado também influenciam o resultado da infecção (ROGERS et al., 2002). Em virtude das diversas manifestações clínicas, o diagnóstico da LTA é realizado através da associação de parâmetros clínicos, epidemiológicos e laboratoriais. O tratamento é baseado na quimioterapia com antimoniais pentavalentes (Sb^{+5}), cuja droga de primeira escolha utilizada no Brasil é o antimoniato de N-metilglucamina (Glucantime®).

A infecção experimental por *L. major* em camundongos tem sido utilizada para examinar aspectos da relação parasito-hospedeiro na leishmaniose. Em contraste, a dificuldade em avaliar a resposta imune celular na infecção experimental por *L. (V.) braziliensis* contribuiu para a realização de estudos com pacientes. Classicamente, a expansão de células T produtoras de citocinas do tipo 1 favorece a eliminação do parasito induzida pela secreção de óxido nítrico (NO), enquanto que o perfil Th2, rico em citocinas inibidoras da produção de NO, ajuda na progressão da doença (De ASSIS SOUZA et al., 2013b). Além desses perfis, o subtipo Th17 têm sido explorado na leishmaniose, sendo suas citocinas relacionadas tanto à cronicidade quanto à resistência ao desenvolvimento da doença (BACELLAR et al., 2009; PITTA et al., 2009). Este parece ter uma relação dicotômica com as células T regulatórias, cuja função principal é garantir que o hospedeiro não seja prejudicado pela própria resposta imune (BETTELLI et al., 2006; RODRIGUES et al., 2013).

Estudos sobre a imunidade mediada por células T na LTA são necessários para o desenvolvimento de novos tratamentos e medidas profiláticas, e a busca por novas moléculas antigênicas pode auxiliar na criação dessas alternativas. Assim, nosso grupo tem avaliado essa resposta frente a antígenos solúvel e insolúvel de *L. (V.) braziliensis* obtidos por ultracentrifugação (BRITO et al., 2000). Em estudo sobre a resposta imune celular frente ao antígeno solúvel, uma resposta proliferativa específica foi observada, além da produção de IL-

10 e IFN-γ antes e após tratamento quimioterápico (REIS et al., 2009). Mais recentemente, foi observada a produção de IL-10 e IL-4 na fase inicial da leishmaniose, com a presença significativa de células T CD4+, sugerindo a sua conexão com a patogênese da doença. Após a cura clínica (pós-quimioterapia ou espontânea), o padrão de resposta imune celular observado foi do tipo 1, o que parece estar associado com a cura ou proteção na LTA (BRELAZ-DE-CASTRO et al., 2012). No entanto, o envolvimento de mediadores dos perfis clássicos (citocinas e NO), além de Th17 e Treg, na resolução ou na progressão da LTA em humanos necessita ser melhor definido.

Nesta tese, foi elaborada uma revisão bibliográfica que abrange desde aspectos gerais da leishmaniose até particularidades da resposta imune desenvolvida contra o parasito. Em seguida, são apresentados os objetivos e a metodologia utilizada para alcançá-los. Os resultados obtidos e a discussão dos mesmos estão divididos em quatro artigos: o primeiro, publicado na revista *Scandinavian Journal of Immunology*, mostra a produção de citocinas frente ao antígeno solúvel de *L. (V.) braziliensis* por células mononucleares do sangue periférico (PBMC) de pacientes com lesão ativa e após cura clínica pós-quimioterapia ou espontânea. O segundo artigo, publicado na *Microbial Pathogenesis*, investiga também os perfis de citocinas produzidas pelos pacientes, dessa vez frente ao antígeno insolúvel de *L. (V.) braziliensis*.

A expressão de genes codificantes para mediadores da resposta imune pode ser um dado relevante para melhor compreender a doença. Assim, o terceiro artigo, submetido à revista *Journal of Interferon & Cytokine Research* utiliza a PCR quantitativa em tempo real para avaliar a expressão gênica de mediadores da resposta imune celular em PBMC de pacientes com LTA ativa, sob estímulo dos antígenos solúvel e insolúvel. Os resultados exibidos neste trabalho pretendem ajudar a melhor compreender o papel de citocinas e outros mediadores na patogênese e resolução da LTA.

2 OBJETIVOS

2.1 Geral

Quantificar mediadores dos perfis Th1, Th2, Th17 e Treg na resposta imune contra Leishmaniose Tegumentar Americana ativa e após cura clínica.

2.2 Específicos

a) Avaliar a produção das citocinas IFN-γ, TNF-α, IL-10, IL-17 e IL-22 nos sobrenadantes de cultura de PBMC de pacientes com lesão ativa e após cura clínica (tratados ou não) após estímulo *in vitro* com os antígenos solúvel e insolúvel de *L.(V.) braziliensis* e com o mitógeno PHA;

b) Verificar a produção de NO nos sobrenadantes de cultura de PBMC após estímulo *in vitro* com os antígenos de *L.(V.) braziliensis*;

c) Caracterizar, nas PBMC dos pacientes com LTA ativa, a expressão do RNA mensageiro para as citocinas IFN-γ, TNF-α, IL-4, IL-10, IL-6, TGF-β, IL-17 e IL22, para os fatores de transcrição RORC e Foxp3 e para a enzima iNOS, frente ao estímulo *in vitro* com os antígenos solúvel e insolúvel de *L. (V.) braziliensis* e com o mitógeno PHA;

3 REVISÃO DA LITERATURA

3.1 Considerações gerais

As leishmanioses representam um complexo de doenças com importante espectro clínico e diversidade epidemiológica. A Organização Mundial da Saúde (OMS) estima que 350 milhões de pessoas estejam expostas ao risco de adquirirem a doença; ao ano, são registrados aproximadamente dois milhões de novos casos das diferentes formas clínicas (BRASIL, 2007). São consideradas doenças negligenciadas por, geralmente, atingirem uma parcela da população que não possui condições de custear seu tratamento e, devido a isto, são colocadas em segundo plano pela indústria de medicamentos e por órgãos governamentais (BRASIL, 2007; ORGANIZAÇÃO MUNDIAL DE SAÚDE, 2013; NATIONAL INSTITUTES OF HEALTH, 2014).

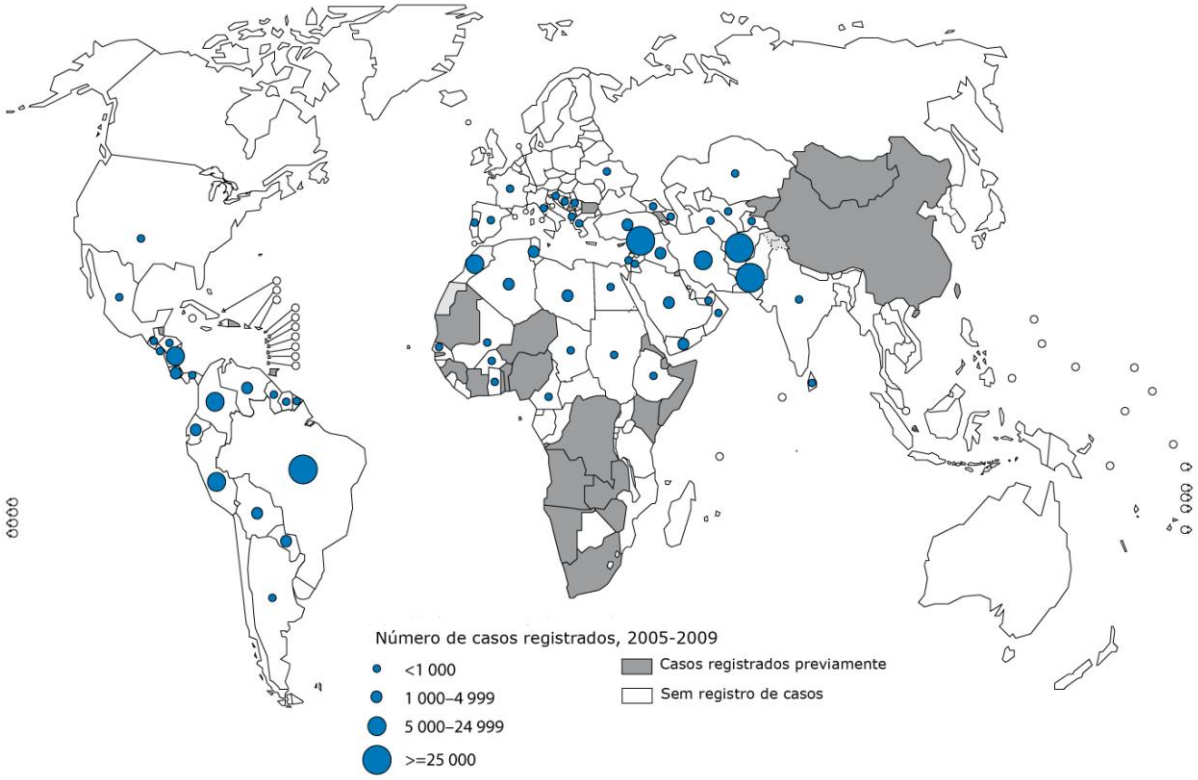
A forma tegumentar da doença constitui um problema de saúde pública em 88 países, distribuídos em quatro continentes (Américas, Europa, África e Ásia), com registro anual de um a 1,5 milhões de casos (Figura 1). Nas Américas, a leishmaniose tegumentar americana (LTA) ocorre desde o Sul dos Estados Unidos ao norte da Argentina, com exceção do Uruguai e do Chile (GONTIJO; CARVALHO, 2003; CENTERS FOR DISEASE CONTROL AND PREVENTION, 2013). É considerada uma zoonose primária de mamíferos silvestres (roedores, marsupiais, edentados e primatas). Dessa forma, o homem adquire a infecção ao entrar em contato com áreas florestais onde existam as enzootias pelas diferentes espécies de *Leishmania*.

A doença está amplamente distribuída no território brasileiro, com casos autóctones em todas as regiões e unidades federadas. Dos anos 2000 a 2008 foram registrados 238.749 casos de LTA no país, com média anual de 26.528 casos novos (BRASIL, 2010). Dentre os casos confirmados nesse período, 75.657 ocorreram na região Nordeste, compreendendo mais de 30% do total (Figura 2).

Em Pernambuco, entre os anos de 2001 a 2010, foram reportados 4.855 casos de LTA, com uma média de 485 casos/ano (BRITO et al., 2012). Embora a maioria dos casos seja registrada na Zona da Mata (BRANDÃO-FILHO et al., 1999), a doença é encontrada em todas as regiões do Estado, e estima-se que a incidência da LTA tenha aumentado cerca de dez vezes nos últimos anos (BRITO et al., 2009). É importante notar que boa parte dos

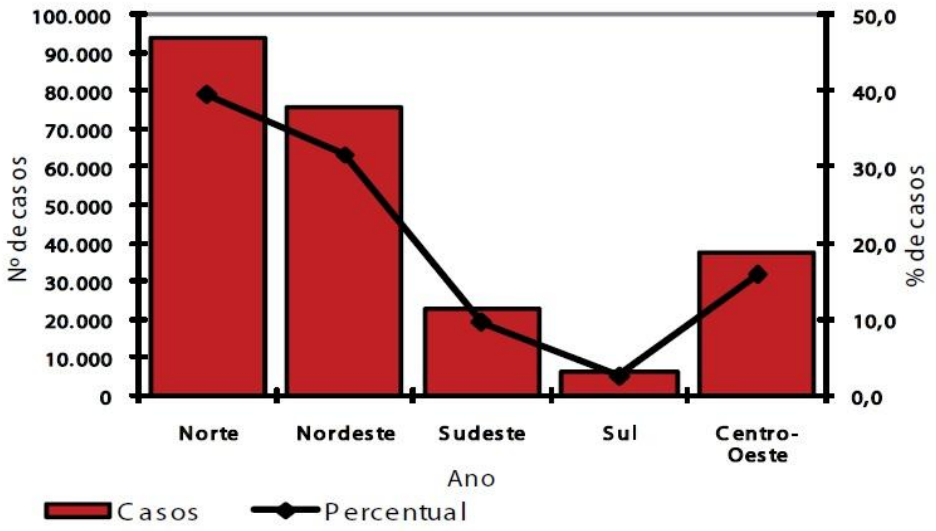
indivíduos afetados reside em regiões pobres e é permanentemente exposta a fatores de risco, frequentemente devido a causas ocupacionais (BRITO et al, 2012).

Figura 1 - Distribuição mundial de casos de leishmaniose cutânea.



Fonte: Adaptado de Organização Mundial de Saúde (Controle de Doenças Tropicais Negligenciadas), 2009.

Figura 2 - Casos de LTA e percentual nas regiões brasileiras. Brasil, 2000-2008.



Fonte: Brasil, 2010.

Os agentes causadores da leishmaniose são espécies de protozoários da ordem Kinetoplastida, família Trypanosomatidae e gênero *Leishmania*. No Brasil, sete espécies estão associadas à doença humana: seis pertencentes ao subgênero *Viannia* (*Leishmania* (*Viannia*) *braziliensis*, *Leishmania* (*Viannia*) *guyanensis*, *Leishmania* (*Viannia*) *lainsoni*, *Leishmania* (*Viannia*) *naiffi*, *Leishmania* (*Viannia*) *shawi*, *Leishmania* (*Viannia*) *lindenberg*) e uma do subgênero *Leishmania* (*Leishmania* (*Leishmania*) *amazonensis*). Em Pernambuco, cepas de *Leishmania* (*Viannia*) *braziliensis* e *Leishmania* (*Viannia*) *shawi* foram isoladas, sendo a primeira espécie a de maior incidência no Estado (BRITO et al., 2009).

A transmissão da LTA acontece pela picada de insetos fêmeas do gênero *Phlebotomus*, no Velho Mundo ou *Lutzomyia* no Novo Mundo. No Brasil, as principais espécies envolvidas na transmissão da LTA são: *L. flaviscutellata*, *L. whitmani*, *L. umbratilis*, *L. intermedia*, *L. wellcomei* e *L. migonei*. (BRASIL, 2010). Mais raramente, a doença pode ser transmitida por outros meios, como acidentes de laboratório ou compartilhamento de seringas (BRITO et al., 2012).

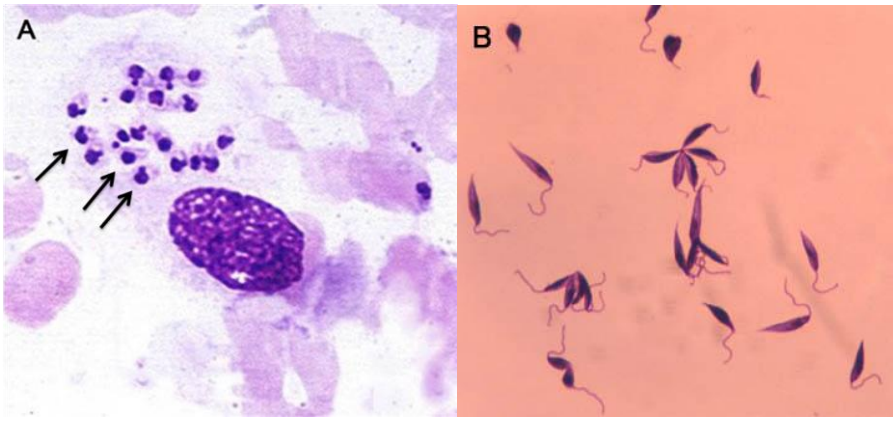
Os reservatórios variam conforme a espécie de *Leishmania*, e incluem animais silvestres como a preguiça (*Choloepus didactilus*), o tamanduá (*Tamandua tetradactyla*), marsupiais (*Didelphis masurpialis*) e roedores. Na Zona da Mata Sul de Pernambuco, área endêmica para LTA, foram isoladas amostras de *L. (V.) braziliensis* em algumas espécies de roedores silvestres como *Bolomys lasiurus*, *Rattus rattus* e *Nectomys squamipes* (BRANDÃO-FILHO et al., 2003).

A *Leishmania* apresenta-se sob as formas amastigota (Figura 3A) e promastigota (Figura 3B) durante o ciclo biológico (Figura 4). A amastigota caracteriza-se por ser imóvel, ovalada e é a forma intracelular obrigatória dos vertebrados. Já a promastigota é alongada, flagelada e móvel, desenvolvendo-se no intestino do inseto vetor. No sub-gênero *Leishmania*, que inclui espécies do complexo *mexicana*, as formas promastigotas multiplicam-se nas regiões média e anterior do intestino do flebótomo. Já no sub-gênero *Viannia*, que inclui espécies do complexo *braziliensis*, as formas promastigotas migram para as regiões anterior, média e posterior do intestino, colonizando o vetor (LAINSON; SHAW, 1998).

A infecção começa quando um inseto infectado executa a hematofagia no hospedeiro vertebrado, inoculando as promastigotas infectantes na pele. Estas são internalizadas por células do sistema fagocitário mononuclear, transformam-se em amastigotas e multiplicam-se por divisão binária, até que as células infectadas se rompem, ficando os parasitos livres na corrente sanguínea e capazes de infectar outras células fagocíticas. Quando o hospedeiro infectado é picado por outro inseto vetor, macrófagos parasitados por amastigotas são

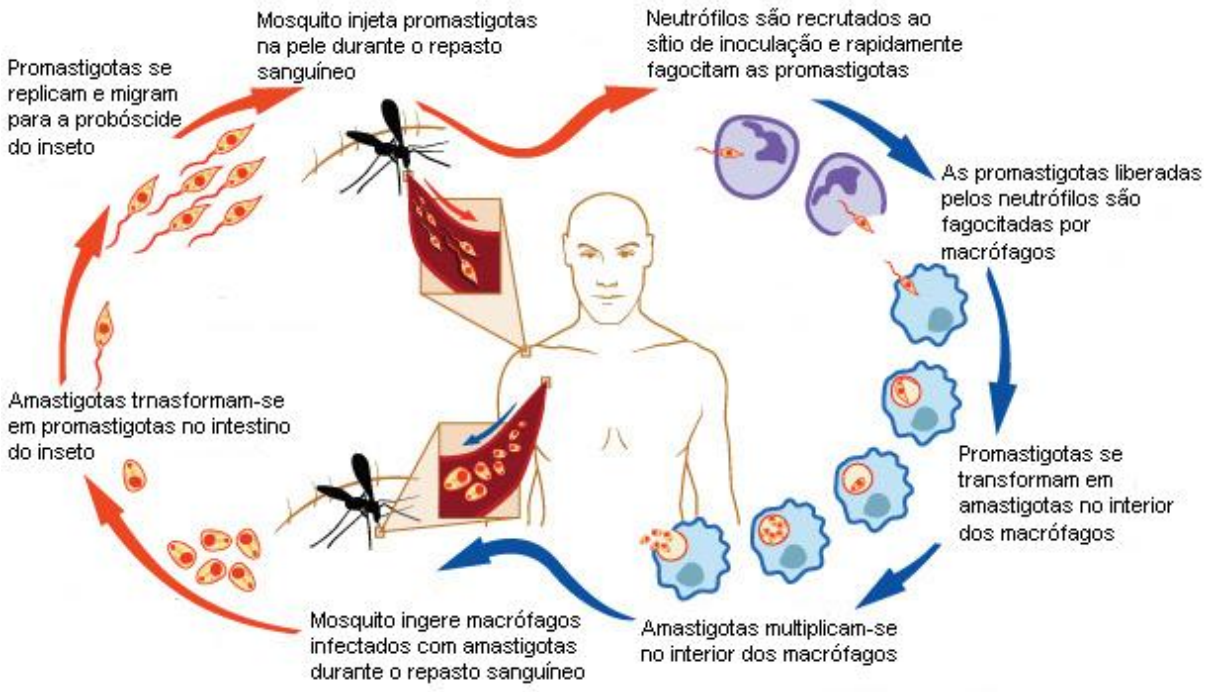
ingeridos. No intestino do inseto, os parasitos se transformam em promastigotas, que alcançam o aparelho bucal, sendo inoculados em um novo hospedeiro, reiniciando assim o ciclo (REY, 2001).

Figura 3 - formas amastigota (A) e promastigota (B).



Fonte: DPDx - Centers for disease Control and Prevention, 2013
(http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Leishmaniasis_il.htm)

Figura 4 - Ciclo biológico da *Leishmania* sp.



Fonte: Adaptado de National Institute of Health, 2008
(http://www.niaid.nih.gov/topics/leishmaniasis/pages/lifecycle.aspx).

3.2 Formas clínicas

O aparecimento das formas clínicas da LTA depende de fatores inerentes ao parasito, como virulência, tropismo e quantidade inoculada. Segundo Rogers et al. (2002), o resultado da infecção é também influenciado por características do vetor, estado imunológico e constituição genética do hospedeiro vertebrado. O homem pode apresentar infecção subclínica ou manifestações que variam desde lesões cutâneas localizadas, disseminadas ou difusas até lesões mucocutâneas agressivas e mutilantes (GOLLOB et al., 2008).

A leishmaniose cutânea (LC; Figura 5A) representa a manifestação clínica mais frequente, cujo período de incubação varia entre dez dias a três meses. De início, nota-se uma pápula ou nódulo no sítio de inoculação pelo mosquito infectado. A partir desta pápula, pode surgir uma lesão ou múltiplas em pequeno número, caracterizando a forma localizada, com tendência à cicatrização. Em casos mais raros, as lesões podem ser numerosas devido a múltiplas picadas do inseto ou por disseminação hematogênica, caracterizando a forma cutânea disseminada. As lesões apresentam aspectos variados e a infecção secundária bacteriana altera este aspecto, tornando-as mais inflamadas, dolorosas e purulentas (BRASIL, 2007; PINHEIRO, 2004).

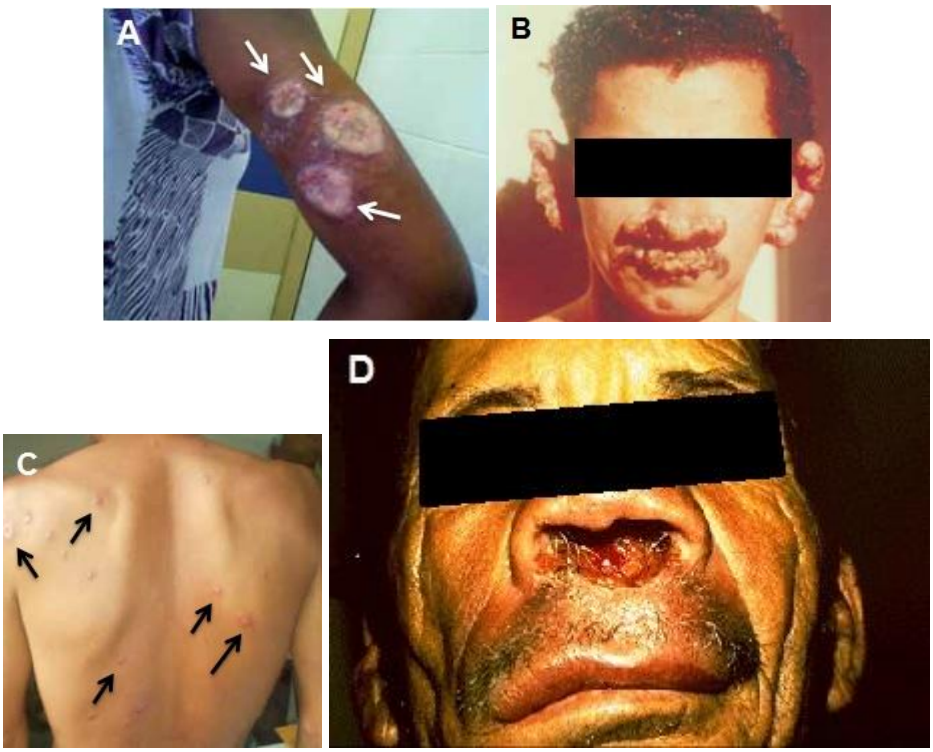
A lesão típica da forma cutânea localizada apresenta-se sob forma habitualmente arredondada ou ovóide, com tamanho variável de alguns milímetros até alguns centímetros. Possui aspecto ulcerado com base infiltrada e firme, bordas bem delimitadas, elevadas e eritematosas, com fundo granuloso e avermelhado. Outras apresentações são encontradas, embora com menor frequência, tais como verrucosa, tuberosa, impetigóide e vegetante. Apresentam pouca quantidade de parasitos e boa resposta ao tratamento habitual ou tendência a regredir espontaneamente (BRASIL, 2007; GONTIJO; CARVALHO, 2003).

A leishmaniose cutânea difusa (LCD; Figura 5B) é caracterizada pelo aparecimento de lesões papulosas ou nodulares por toda a superfície corporal. Associada a *L. (L.) amazonensis*, é considerada a mais rara e severa manifestação clínica, cujas lesões podem persistir indefinidamente. O parasito é encontrado em grandes quantidades nas lesões e a resposta ao tratamento é insatisfatória, sendo este fato relacionado a uma resposta imune deficiente por parte do hospedeiro (BAILEY; LOCKWOOD, 2009).

A LCD não deve ser confundida com a LC disseminada (Figura 5C), que ocorre em indivíduos capazes de montar uma resposta imune celular contra *Leishmania* e que apresentam boa resposta terapêutica (BRASIL, 2007). É caracterizada pelo aparecimento de

múltiplas lesões papulares e de aparência acneiforme que acometem vários segmentos corporais, envolvendo com freqüência a face e o tronco. O número de lesões pode alcançar as centenas. A história natural da doença nestes pacientes inicia com uma ou várias lesões localizadas com as características clássicas de úlceras de fundo granuloso e bordas elevadas. Posteriormente ao desenvolvimento das lesões primárias, acontece um fenômeno provavelmente por disseminação do parasito por vias hematogênica ou linfática, mais ou menos aguda, que se estabelece em poucos dias, às vezes em 24 horas, causando lesões distantes do local da picada (BRASIL, 2007).

Figura 5 - Formas clínicas da LTA.



Fonte: BRASIL (2007).
Nota: A - forma cutânea localizada; B - forma cutânea difusa; C - forma cutânea disseminada; D - forma mucocutânea.

A forma mucocutânea (Figura 5D) é a mais agressiva e mutilante da doença, apresentando lesões infiltrativas, com ulcerações e destruição dos tecidos da cavidade nasal, faringe e laringe. É considerada uma característica peculiar da leishmaniose tegumentar do Novo Mundo por ter sua transmissão associada a *L. (V.) braziliensis*, e pode surgir meses ou anos após o aparecimento de lesão cutânea (AMATO et al., 2008). Com baixa carga parasitária, oferece risco de deformidade permanente se não diagnosticada a tempo (GONTIJO; CARVALHO, 2003). Felizmente, a freqüência desta complicação vem sendo

reduzida, não excedendo a 5% dos casos nas áreas endêmicas. Provavelmente, está relacionada ao diagnóstico e tratamento precoces (BRASIL, 2007).

3.3 Diagnóstico

Atualmente, por não existir um método que detecte a *Leishmania* de maneira rápida, simples e com baixo custo, a doença é confirmada pela associação de técnicas laboratoriais que pode incluir detecção do DNA parasitário, visualização ao microscópio, cultura celular e imunodetecção (KOBETS; GREKOV; LIPODOLVÁ, 2012). Além disso, por ser a LTA uma doença que apresenta manifestações clínicas similares a outras de causas distintas, é necessário que o diagnóstico diferencial seja sempre considerado. Assim, a possibilidade de doenças como hanseníase, câncer de pele, tuberculose e esporotricose, por exemplo, deve ser descartada (BRASIL, 2007).

No exame clínico, é interessante associar as informações coletadas através da anamnese do paciente a dados epidemiológicos. Dentre eles estão a existência de casos de LTA na região, referência de cães ou equinos com lesões residindo nas proximidades, procedência de área endemia e inserção de áreas florestais. Nas lesões cutâneas, os dados epidemiológicos referidos são recentes (em média 2 meses); no caso de lesão mucosa é essencial buscar também a história pregressa de ulceração de pele de longa duração, além da existência de cicatriz e utilização de medicamentos para leishmaniose (FUNDAÇÃO NACIONAL DE SAÚDE, 2000).

Dentre as técnicas laboratoriais, a pesquisa direta é considerada um método padrão desde o início dos estudos em *Leishmania*. O material a ser analisado pode ser obtido da escarificação da borda da lesão, impressão por aposição em lâmina (*imprint*) ou aspirado da lesão, corado pelo Giemsa ou Leishman. Embora a técnica seja específica, pois evidencia as formas amastigotas do parasito, é necessário um profissional treinado para realizá-la, além de apresentar sensibilidade inversamente proporcional à carga parasitária presente na lesão (BAILEY; LOCKWOOD, 2009).

O parasito cresce relativamente bem em meios de cultura enriquecidos, como NNN (McNeal, Novy & Nicole) acrescido de meio Schneider ou LIT (Liver infusion tryptose) com 10% de soro fetal bovino (SFB), à temperatura ambiente (BRASIL, 2007). Assim, a partir de punção aspirativa ou biópsia da lesão pode ser realizada a cultura para isolamento da

Leishmania. Este método é importante para identificar a subespécie envolvida, por análise de zimodemas (isoenzimas) e por sequenciamento de DNA (BAILEY; LOCKWOOD, 2009).

Os testes sorológicos, como a imunofluorescência indireta (IFI), o *Enzyme-linked immunosorbent assay* (ELISA) e o *Western Blot*, são limitados por não relacionar os níveis de anticorpos circulantes com o estágio da doença, além da possibilidade de apresentar reações cruzadas com outros tripanosomatídeos. Pelas limitações dessas técnicas, abordagens imunológicas alternativas vêm sendo empregadas. Uma delas é a citometria de fluxo que permite detectar anticorpos anti-*Leishmania* (ROCHA et al., 2002, 2006). Pereira et al. (2012) e Oliveira et al. (2013) demonstraram que esta técnica pode ser aplicada também para monitoramento da cura pós-tratamento na LTA.

A imunidade celular em resposta à *Leishmania* é avaliada através da intradermo-reação de Montenegro (IDRM), que apresenta uma alta sensibilidade e uma especificidade que varia de acordo com o antígeno utilizado (KOBETS; GREKOV; LIPODOLVÁ, 2012). Embora apresente resultado positivo na maioria dos casos de LTA (90%), o resultado é negativo em lesões recentes, na forma cutânea difusa e em pacientes imunodeprimidos. Em áreas endêmicas, é comum o teste ser positivo devido à ocorrência de infecções subclínicas (VEGA-LÓPEZ, 2003).

Técnicas de diagnóstico molecular baseadas na reação em cadeia da polimerase (PCR) têm sido extensivamente desenvolvidas para a aplicação na LTA. Com alta sensibilidade e especificidade, são particularmente úteis em amostras contendo poucos parasitos, como na forma mucosa, além de permitir o monitoramento da terapia da leishmaniose cutânea. Contudo, o uso rotineiro é ainda impedido por demandar uma infra-estrutura laboratorial adequada, pessoal qualificado e alto custo (REITHINGER; DUJARDIN, 2007).

A utilização de métodos de diagnóstico laboratorial visa não somente a confirmação dos achados clínicos, mas pode fornecer importantes informações epidemiológicas, pela identificação da espécie circulante, orientando quanto às medidas a serem adotadas para o controle do agravo (BRASIL, 2007).

3.4 Tratamento

Há mais de cinco décadas, os compostos utilizados no tratamento da leishmaniose são os antimoniais pentavalentes, que estão disponíveis em duas formulações: stibogluconato de sódio e antimoniato de n-metilglucamina. Este último, de nome comercial Glucantime®, é a formulação de primeira escolha no Brasil, com alta toxicidade e efeitos colaterais diversos (LINDOSO et al., 2012). A administração do medicamento é feita por via intramuscular ou endovenosa, com a dose variando de 10 a 20 mg Sb+5/kg/dia, durante vinte a trinta dias (BRASIL, 2007; ORGANIZAÇÃO MUNDIAL DE SAÚDE, 2010).

O mecanismo de ação dos antimoniais ainda não foi totalmente elucidado. O que se sabe é que, após redução mediada por macrófagos e parasitos, a molécula pentavalente (Sb^{V}) é convertida em antimonial trivalente (Sb^{III}), uma molécula mais tóxica contra a *Leishmania* (FRÉZARD; DEMICHELI; RIBEIRO, 2009). Porém ambas causam a fragmentação do DNA parasitário possivelmente por apoptose, β-oxidação de ácidos graxos e fosforilação de adenosina-difosfato (ADP) (SINGH; KUMAR; SINGH, 2012). Os antimoniais são tóxicos ao coração, rins, fígado e pâncreas, o que representa uma limitação importante no seu uso em gestantes, idosos e indivíduos com doenças cardíacas, renais ou alterações hepáticas (LINDOSO et al., 2012).

No caso de não haver uma boa resposta ao tratamento com Glucantime® ou na impossibilidade de seu uso, a anfotericina B é uma das drogas utilizadas. Considerada como droga de primeira escolha no tratamento de gestantes, o desoxicolato de anfotericina B foi a primeira formulação a ser empregada, e devido aos graves efeitos colaterais que desencadeia, é administrada apenas em ambiente hospitalar (LINDOSO et al., 2012). É dissolvida em 10 mL de água destilada para injeção, sendo a dose diária de 0,2 mg/Kg/dia, gota a gota, por via endovenosa, até no máximo 50 mg/dia, com tempo de infusão de duas a seis horas.

Visando amenizar as reações adversas, uma formulação lipossomal da anfotericina B (AmBisome) com eficiência similar e significativamente menos tóxica foi desenvolvida. No Brasil ela é administrada apenas no tratamento das formas muco-cutânea e visceral da leishmaniose, embora existam evidências de boa eficácia dessa formulação no tratamento da forma cutânea localizada (BROWN et al., 2005; SOLOMON et al., 2010; BUTSCH et al., 2012).

Outra alternativa é a pentamidina, usada na dose de 4 mg/Kg/dia, por via intramuscular, sendo aplicada a cada dois dias. Devido ao medicamento ter ação no

metabolismo da glicose, pode haver hipoglicemia seguida de hiperglicemia quando do seu uso. O paciente deve ser orientado a alimentar-se anteriormente e permanecer em repouso 15 minutos antes e após as injeções. Efeitos adversos como diabetes mellitus, hipoglicemia severa, miocardite e toxicidade renal limitam seu uso (BRASIL, 2007; ORGANIZAÇÃO MUNDIAL DE SAÚDE, 2010).

O critério de cura para LTA é clínico, sendo definido pela reepitelização das lesões ulceradas, regressão total da infiltração e eritema, até três meses após a conclusão do esquema terapêutico. Entretanto, nos casos com evidência de cicatrização progressiva das lesões sem cumprir completamente com os critérios acima, sugere-se o prolongamento da observação até completar seis meses. O paciente deve retornar mensalmente à consulta durante três meses consecutivos após o término do esquema terapêutico para ser avaliada a cura clínica. Uma vez curado, o mesmo deverá ser acompanhado de dois em dois meses até completar 12 meses após o tratamento (BRASIL, 2007).

A leishmaniose é a única doença tropical cujo tratamento é realizado utilizando-se drogas não-específicas para a doença. Além disso, os mecanismos de ação desses medicamentos ainda não são bem conhecidos. Assim, é necessário que a busca por potenciais novos alvos esteja focada principalmente nas vias bioquímicas e metabólicas essenciais para a sobrevivência do parasito (SINGH; KUMAR; SINGH, 2012). Também são importantes pesquisas que visem a consolidação das vias alternativas de administração dos fármacos, como a oral e o uso tópico, objetivando melhorar a qualidade de vida dos pacientes.

Além disso, diante da complexidade apresentada pela interação parasito-vetor-hospedeiro, são relevantes estudos sobre mecanismos imunológicos induzidos em pacientes naturalmente infectados pela *Leishmania*, com o objetivo de desenvolver tratamentos à base de imunoterapia e também de vacinas (GOLLOB et al., 2008).

3.5 Leishmaniose e resposta imune

O desenvolvimento de uma resposta imune frente a *Leishmania* é um processo dinâmico, constituído por uma rede complexa de interações entre células do sistema imune inato e adaptativo. Assim, a participação de fagócitos (macrófagos, neutrófilos, células dendríticas), células NK, linfócitos T ou B, e de moléculas efetoras como quimiocinas, citocinas e anticorpos específicos determinam se a infecção será controlada ou se a doença irá progredir (CARVALHO et al., 2012; DUTHIE et al., 2012).

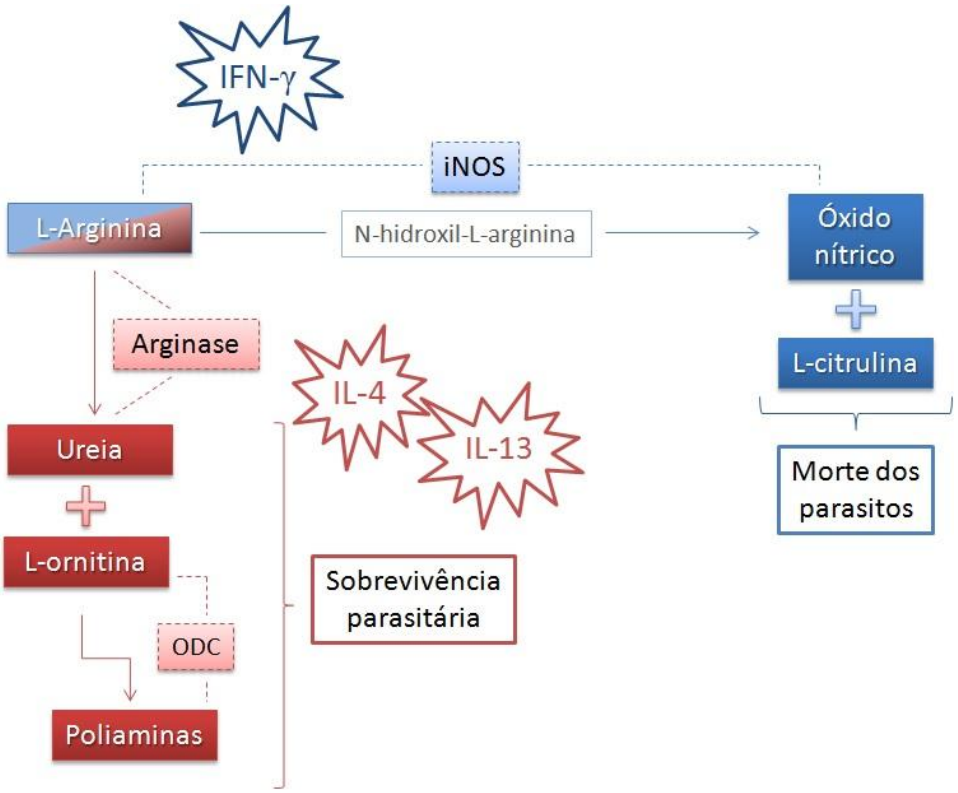
Os neutrófilos contribuem para a primeira linha de defesa contra patógenos invasores. A essas células são atribuídos papéis protetores e promotores da doença. Quanto ativos, eliminam as promastigotas de *Leishmania* via espécies reativas de nitrogênio e oxigênio e constituem um sistema de captura de patógenos no meio extracelular (*Neutrophil Extracellular Traps*, NETs). (RITTER; FRISCHKNECHT; VAN ZANDBERGEN, 2009). Porém, sofrem apoptose após seu curto período de vida, e os restos celulares posteriormente fagocitados podem conter parasitos viáveis, tanto dentro dos neutrófilos quanto no meio extracelular. As promastigotas induzem a produção de TGF-β pelos neutrófilos próximos, o que inibe as funções efetoras antimicrobianas dos fagócitos, contribuindo para a multiplicação parasitária (JOHN & HUNTER, 2008; RITTER; FRISCHKNECHT; VAN ZANDBERGEN, 2009; KAYE & SCOTT, 2011).

As interações complexas entre o parasito e as células apresentadoras de antígeno do hospedeiro têm efeitos no resultado final da infecção. Os macrófagos são as células onde as promastigotas classicamente transformam-se em amastigotas, multiplicando-se no seu interior. Paradoxalmente, os macrófagos são os principais responsáveis pela eliminação dos parasitos. Essa dualidade acontece porque essas células podem ser ativadas por diferentes sinais que levam ao desenvolvimento de subtipos celulares envolvidos com o controle ou progressão da infecção (LIU & UZONNA, 2012).

A ativação macrofágica é geralmente dividida em duas vias funcionalmente distintas, como mostra a Figura 6. Na via clássica (em azul), a presença de IFN-γ estimula macrófagos a produzirem a enzima óxido nítrico sintase induzível (iNOS ou NOS2), que catalisa a conversão de L-arginina em óxido nítrico (NO) e L-citrulina. O NO é uma molécula tóxica que tem um papel importante na eliminação de parasitos intracelulares, incluindo *Leishmania*. Por ser uma molécula instável, decompõe-se entre seis e trinta segundos. A partir daí surgem produtos finais mais estáveis, como o nitrito (NO_2^-). Em contraste, citocinas como IL-4 e IL-

13 inibem a produção de NO, o que caracteriza a via alternativa (em vermelho) de ativação macrofágica. Através da ação da enzima arginase, há a conversão da L-arginina em ureia e L-ornitina. Esta última, sob ação da enzima ornitina descarboxilase (ODC), dá origem a poliaminas, compostos orgânicos que favorecem a sobrevivência da *Leishmania* no organismo do hospedeiro (COLLOTI & ILARI, 2011; LIU & UZONNA, 2012).

Figura 6 - Vias de ativação macrofágica.



Fonte: da autora.
Nota: porção da figura em azul representa a via clássica, benéfica para o hospedeiro.A via alternativa, que favorece a multiplicação do parasito, está ilustrada em vermelho. iNOS = óxido nítrico sintase induzível. ODC = ornitina descarboxilase.

Além dos macrófagos, as células dendríticas têm um papel importante na resposta imune contra a leishmaniose (BRANDONISIO et al., 2004). Conhecidas como potentes APCs, elas são capazes de secretar IL-12 quando infectadas, citocina necessária para estimular uma resposta imune celular do tipo Th1, prejudicial à *Leishmania*. Dos diferentes subtipos de células dendríticas, dois são localizados na derme e um na epiderme (células de Langerhans). É interessante notar que as células dendríticas são diferentemente permissivas à infecção. Essa propriedade parece ser inversamente proporcional à habilidade dessas células em secretar IL-12 quando parasitadas (HENRI et al., 2002).

Junto aos fagócitos, as células *natural killer* (NK) participam do combate à *Leishmania*, sendo rapidamente ativadas. Em camundongos, a atuação dessas células requer estimulação por células dendríticas dependente do TLR (*toll-like receptor*)-9, sendo este evento seguido da produção de IL-12. Embora as células NK pareçam ser essenciais para o controle das leishmanioses e possam exibir funções imunossupressoras como a produção de IL-10 na leishmaniose visceral murina, elas são uma importante fonte de IFN-γ, o que

desencadeia a atividade leishmanicida de macrófagos e ajuda a consolidar uma resposta de células T protetora (BOGDAN, 2012).

O papel da imunidade adaptativa nas leishmanioses tem sido bastante estudado por diversos grupos de pesquisa ao longo dos anos, uma vez que os linfócitos T são cruciais para determinar o tipo de resposta imune e o grau de dano tecidual induzido pela infecção (NYLÉN & EIDSMO, 2012). Uma parte considerável do conhecimento hoje existente teve origem em estudos utilizando camundongos infectados com promastigotas de *L. major*. Aspectos sobre a relação parasito-hospedeiro na leishmaniose, como o controle genético de susceptibilidade e resistência, o papel da resposta imune mediada por células e a interação parasito-macrófago são explorados com frequência nesses trabalhos (SACKS; NOBEN-TRAUTH, 2002; HURDAYAL; BROMBACHER, 2014).

Sabe-se que, nas infecções por *Leishmania*, ocorre uma expansão de células T CD4+, apresentando perfis de citocinas Th1 ou Th2 (PINHEIRO, 2004; REIS et al., 2006). Em linhagens de camundongos resistentes (C57BL/6, CBA, C3H) predomina o perfil Th1, que determina uma doença auto-limitada em resposta à infecção com *L. major* ou *L. tropica*. Há expressão do fator de transcrição T-bet, além de um aumento nos níveis de IL-12, que induz células natural killer (NK) e células T CD4+ e CD8+ a produzirem IFN-γ (ROGERS et al., 2002). Esta atua em sinergia com TNF-α ativando a iNOS a produzir óxido nítrico (NO), resultando na morte intracelular do parasito (BOGDAN; RÖLLINGHOFF; DIEFENBACH, 2000). Além disso, a IL-2, indutora de proliferação celular, está envolvida com o controle da atividade anti-*Leishmania* desempenhada por linfócitos T (BODAS et al., 2006).

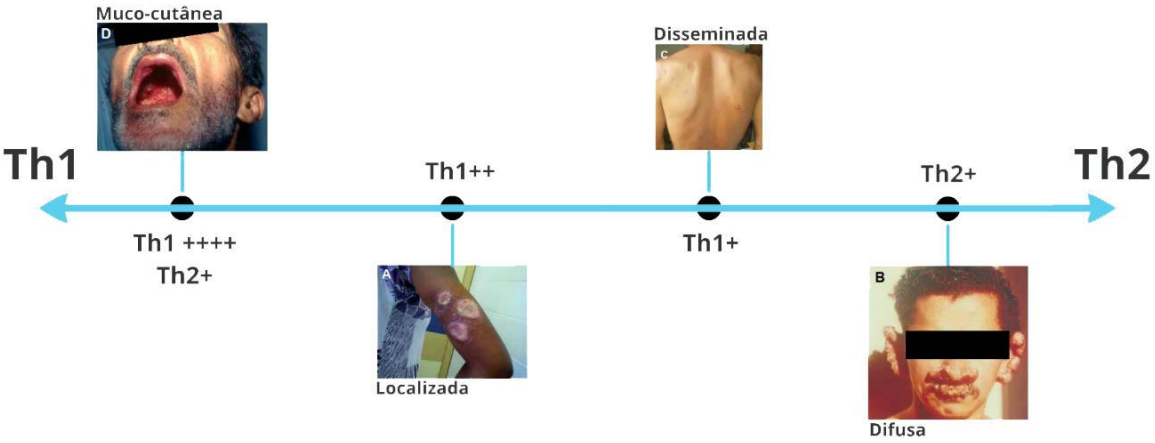
Linhagens susceptíveis, como BALB/c, apresentam o perfil de resposta Th2 e, consequentemente, uma doença progressiva e severa. Há a expressão do fator de transcrição GATA3, aumento na expressão de RNA mensageiro (mRNA) para IL-4 e produção de IL-5, IL-10 e IL-13 (HIMMELRICH et al., 2000; SHARMA et al., 2009). A IL-4 diminui a regulação da expressão da subunidade β dos receptores da IL-12 nas células Th1, suprimindo a produção de IFN-γ e levando ao desenvolvimento da resposta Th2 (SHARMA et al., 2009). A IL-10 desempenha um papel fundamental na inibição da ativação macrofágica e contribui para o crescimento do parasito nas lesões, uma vez que camundongos BALB/c $IL10^{-/-}$ mostraram-se capazes de controlar a progressão da doença durante infecção por *L. major* (KANE; MOSSER, 2001).

Com o objetivo de entender a resposta imunológica frente a outras espécies de *Leishmania*, alguns autores têm estudado os aspectos relacionados à relação parasito-hospedeiro nas infecções causadas por *L. (V.) braziliensis*. Moura et al. (2005) inocularam o

parasito na orelha de camundongos BALB/c, obtendo resultado clínico semelhante ao observado em humanos, especialmente em relação ao aspecto da lesão, à persistência do parasito e à resposta imune desenvolvida. A eliminação da *L. (V.) braziliensis* parece depender inicialmente da interação entre macrófagos e neutrófilos e está associada à presença de TNF e superóxido (NOVAIS et al., 2009). Além disso, as células T $CD8^+$ foram recentemente associadas à patologia severa, uma vez que as perforinas liberadas por essas células parecem agravar a inflamação (NOVAIS et al., 2013).

Embora tenha havido progressos em relação aos modelos experimentais de infecção por *L. (V.) braziliensis*, esta espécie não se desenvolve facilmente no ambiente *in vitro*, uma vez que sua transformação na forma infectante é ineficiente em condições padronizadas de cultura, sendo necessária uma grande carga parasitária para realizar a infecção (LIMA; DEKREY; TITUS, 1999). Como alternativa, estudos imunológicos são mais frequentemente realizados com pacientes portadores de LTA (REIS et al., 2009; BRELAZ et al., 2012; PEREIRA et al., 2012; ASSIS SOUZA et al. 2012; De ASSIS SOUZA et al., 2013a; OLIVEIRA et al., 2013). De forma semelhante ao camundongo, a resposta imune é dependente de linfócitos T, sendo as manifestações clínicas fortemente influenciadas pela expansão dessas células, como mostrado na Figura 7. (De ASSIS SOUZA et al., 2013b).

Figura 7 - Manifestações clínicas da LTA e seus níveis de expansão de células T.



Fonte: da autora.

Na forma cutânea localizada, há uma forte resposta de células T, com citocinas do tipo Th1, como IFN-γ e IL-12, e uma alta freqüência de células B (VIEIRA et al., 2002). Na leishmaniose cutânea disseminada, os níveis de células T CD4+ podem diminuir transitoriamente, retornando ao normal depois do tratamento (CARVALHO et al., 1994).

Durante a infecção, pacientes acometidos pela forma cutânea difusa falham em produzir uma resposta imune mediada por células. Devido a falta da expressão de mRNA para IFN-γ e baixa expressão para IL-2, são incapazes de controlar a multiplicação parasitária e a progressão da doença. Além disso, altos títulos de anticorpos específicos contra o parasito são exibidos, bem como uma resposta quase exclusivamente do tipo Th2, com elevados níveis de IL-4 (SHARMA et al., 2009).

A forma muco-cutânea exibe um aumento de resposta por células T específicas, tanto Th1 como Th2, sendo predominante o perfil Th1. Assim, são produzidos altos níveis de citocinas pró-inflamatórias como TNF-α e IFN-γ, além de IL-4. Com a fraca regulação dessa resposta por IL-10 e pelo fator de crescimento e transformação-beta (TGF-β) e consequente exacerbação do perfil Th1, pode-se perceber que uma resposta inadequada pode levar a uma imunopatogênese exacerbada. Além disso, são encontrados altos títulos de anticorpos (BRASIL, 2007).

Por mais de duas décadas, a maioria dos fenômenos relacionados à imunidade adaptativa foi explicada unicamente pelo paradigma Th1 x Th2. Contudo, um terceiro subtipo de células T, produtor de IL-17, foi descrito como uma linhagem celular que se desenvolve de forma independente (KIMURA & KISHIMOTO, 2010). As células Th17 foram inicialmente relacionadas à indução de doenças autoimunes e inflamação tecidual, sendo capazes de produzir principalmente IL-17A, IL-17F e outras citocinas inflamatórias (BASSO, CHEROUTRE, MUCIDA, 2009).

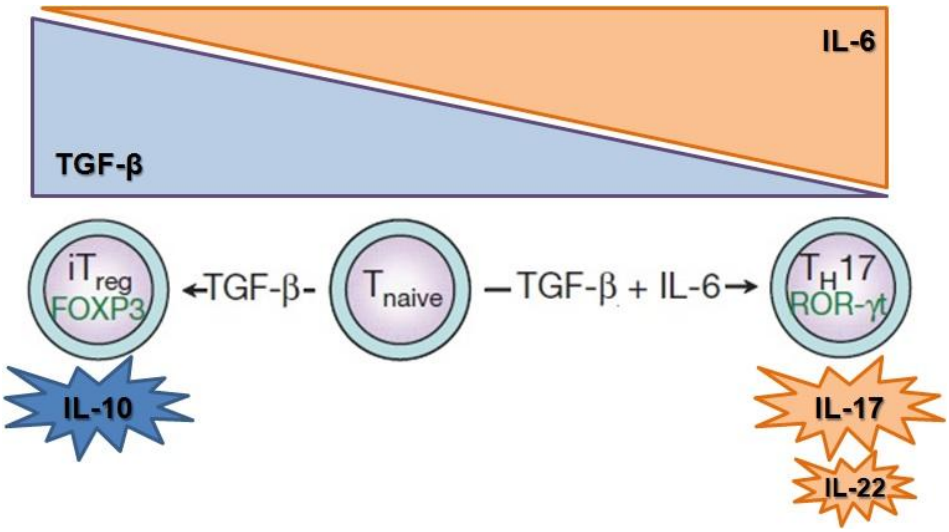
Ao contrário dos perfis Th1 e Th2, cuja diferenciação é dependente de suas citocinas efetoras, a diferenciação de linfócitos Th17 não requer a presença de IL-17. Duas outras citocinas – IL-6 e TGF-β – atuam induzindo sinergicamente a expressão do gene RORC. Este codifica o receptor nuclear órfão RORγt, fator de transcrição considerado principal para a diferenciação do perfil Th17 (MANEL et al., 2008). Outros fatores como RORα, Irf-4, STAT3 e AhR parecem também estar envolvidos nesse processo (CROME et al., 2009a; 2009b). A IL-21 parece ser necessária para a expansão do perfil, enquanto que IL-23 promove sua manutenção (BETTELLI et al., 2008).

Nas leishmanioses, o papel do perfil Th17 ainda não foi totalmente esclarecido. Bacellar et al. (2009) demonstraram *in situ* que a IL-17 é produzida durante a fase ativa da LTA, estando diretamente correlacionada à produção de TNF-α. Além disso, a intensidade do infiltrado inflamatório está associada ao número de células que expressam IL-17, levando os autores a crerem que a citocina pode estar envolvida na patogênese da LTA. Em compensação, Novoa et al. (2011) detectaram IL-17 em indivíduos infectados, mas que não

desenvolveram a doença. Similarmente, IL-22 também já foi associada tanto à proteção quanto ao dano tecidual em infecções. Sonnenberg et al. (2010) sugerem que a ação pró- ou anti-inflamatória dessa citocina seja governada pelos níveis de IL-17 presentes no microambiente. Pitta et al. (2009) observaram células Th17 produtoras de IL-17 e IL-22 em pacientes resistentes ao Calazar, e essas citocinas parecem ter um papel complementar de proteção juntamente com os membros do perfil Th1.

Além do subtipo Th17, as células T regulatórias têm sido estudadas por diversos grupos de pesquisa (CAMPANELLI et al., 2006; CARNEIRO et al., 2009; CHEN et al. 2010; RODRIGUES et al., 2013), sendo a elas atribuídas as funções de manter a tolerância a antígenos próprios e de suprimir respostas imunológicas exacerbadas frente a infecções (SAKAGUCHI et al., 2008). Acredita-se que entre os perfis Treg e Th17 exista uma relação dicotômica, onde a ativação de um ou outro subtipo depende dos níveis de IL-6 e TGF-β existentes no microambiente (Figura 8) (BETTELLI et al., 2006, 2008; BASSO, CHEROUTRE, MUCIDA, 2009). Esta última citocina é determinante na diferenciação das Tregs, que podem ser caracterizadas como naturais (nTregs; desenvolvimento no timo), ou induzidas (iTregs; evoluem na periferia). Belkaid et al. (2003) afirma que em linhagens de camundongos susceptíveis à infecção por *L. major*, a resposta Th2 é suprimida pelas Tregs, enquanto que as mesmas modulam a resposta gerada por células Th1 em camundongos geneticamente resistentes ao parasito. Autores como Campanelli et al (2006) e Rodrigues et al. (2013) detectaram células com fenótipo Treg em lesões de pacientes com LTA causada por *L. braziliensis*.

Figura 8 - Ativação dos perfis Th17 e Treg.



Fonte: da autora.

O fator de transcrição *forkhead box p3* (Foxp3) está presente nas nTregs e em algumas iTregs, e todas as células T regulatórias podem ser fontes de IL-10 (NYLÉN; GAUTAM, 2010). Inicialmente relacionada apenas ao perfil Th2, indutor da desativação macrofágica, IL-10 parece exercer um contrabalanço necessário sobre a resposta imune (ANTONELLI et al., 2004; CAMPANELLI et al., 2006; RODRIGUES et al., 2013). Para isso, os linfócitos produtores de citocinas pró-inflamatórias (IFN-γ e TNF-α) parecem atuar coordenadamente com outros, produtores de IL-10. Em indivíduos infectados com *L. braziliensis*, sugere-se um prognóstigo favorável quando há uma baixa razão IFNγ/IL-10, o que indica que a co-produção dessas citocinas é importante tanto para o controle da patologia quanto para a regulação de uma resposta Th1 exacerbada (GOMES-SILVA et al., 2007). Em compensação, outros autores indicam a relação entre essas duas citocinas pode favorecer a persistência do parasito após a cura (OKWOR; UZONNA, 2008).

Este evento, já demonstrado em modelo murino e em pacientes, parece ser fortemente influenciado pelos níveis de IL-10. Por exemplo, camundongos selvagens tratados com anticorpo monoclonal anti-IL-10R foram capazes de montar uma resposta Th1 suficiente para eliminar completamente os parasitos (BELKAID et al., 2002). Em humanos, a ocorrência de reativação espontânea e a presença de uma resposta imune por um longo tempo após a resolução da doença são evidências que corroboram essa teoria. Através da reação em cadeia de polimerase, foi possível detectar DNA de *Leishmania (Viannia)* em cicatrizes de pacientes. A demonstração da persistência parasitária desperta o interesse sobre pacientes curados e indivíduos com infecção subclínica possivelmente exercerem papel de reservatórios da *Leishmania* (MENDONÇA et al., 2004).

Além disso, existem evidências de que os parasitos remanescentes após a cura podem contribuir para uma estimulação constante do sistema imune, o que confere resistência a novas infecções (BOGDAN et al, 2008). Outros estudos sugerem que estes mesmos parasitos sejam necessários para que o hospedeiro forme uma resposta de memória imunológica (ZAPH et al., 2004; SCOTT et al., 2005). Assim, a persistência parasitária parece ter provido um equilíbrio na relação parasito-hospedeiro, além de vantagens para ambos. Este fenômeno parece representar uma fonte de estimulação antigênica necessária para manter a proteção do hospedeiro e, ao mesmo tempo, permitir a sobrevivência de um pool de parasitos disponível para a transmissão de um hospedeiro a outro (ORKWOR; UZONNA, 2008).

As questões relacionadas à evolução clínica, epidemiologia e controle da leishmaniose têm incentivado o desenvolvimento de estudos sobre a resposta imune celular frente ao parasito, onde técnicas de cultura celular e de dosagem de mediadores específicos são

bastante utilizadas. Diversos antígenos protéicos são utilizados com a finalidade de induzir uma resposta imune benéfica, podendo ser considerados candidatos potenciais ao desenvolvimento de tratamentos baseados em imunoterapia e de vacinas.

O lipofosfoglicano (LPG), uma das moléculas mais estudadas, é um fator de virulência responsável por funções como adesão da *Leishmania* ao intestino do inseto vetor e ao macrófago do hospedeiro, indução da rede extracelular de neutrófilos e modulação da produção de NO (IBRAIM et al., 2013). Além deste, a gp63, presente em ambas as formas evolutivas do parasito, afeta as funções macrofágicas degradando várias proteínas e, consequentemente, favorecendo a sobrevivência da *Leishmania* (OLIVIER et al., 2012). Tanto este quanto o LPG induziram uma proteção em modelo experimental (HABERER et al., 1998).

A proteína P-4 purificada de amastigotas de *Leishmania pifanoi* demonstrou uma resposta celular do tipo Th1 em PBMC de indivíduos infectados por *L. (V.) braziliensis* (HABERER et al., 1998). Em estudos com camundongos, o antígeno PH8, derivado de promastigotas de *L. amazonensis*, levou à produção de níveis elevados de IFN-γ e ausência de IL-4 (MAYRINK et al., 2002). Além dessas proteínas, o extrato total tem sido utilizado para avaliação *in vitro* da resposta imune celular (ANTONELLI et al., 2004; DA-CRUZ et al., 1994; 2002; TELINO et al., 2005; TOLEDO et al., 2001).

Estudos anteriores desenvolvidos por Toledo et al. (2001) e Da Cruz et al. (2002) mostraram que o antígeno total de *L. (V.) braziliensis* promoveu proliferação celular e produção de citocinas em pacientes com LTA ativa e após a quimioterapia. Além disso, os autores sugerem que o estado imunológico do paciente seja importante na resposta ao tratamento.

Em contrapartida, alguns indivíduos alcançam a cura clínica sem necessitar de quimioterapia. Gomes-Silva et al. (2007), também utilizando o antígeno total de *L. (V.) braziliensis*, verificou que esses indivíduos exibiram níveis de IFN-γ menores que aqueles de pacientes com lesão ativa. Em contraste, uma produção significativa de IL-10 contra o mesmo antígeno foi detectada nos pacientes com cura espontânea em relação ao grupo de pacientes com LTA ativa. Ao que parece, os indivíduos curados espontaneamente são capazes de produzir níveis ótimos de IFN-γ e IL-10 com efeitos benéficos no hospedeiro, embora os mecanismos que levam a um equilíbrio adequado entre mediadores pró-inflamatórios e suas moléculas reguladoras ainda não estejam totalmente esclarecidos.

Devido à diversidade protéica existente nas preparações antigênicas totais, a resposta imune gerada pode sofrer discrepâncias. Tendo isto em vista, extratos solúveis e insolúveis de

promastigotas de diferentes espécies de *Leishmania* obtidos através de centrifugações diferenciadas podem promover uma melhor definição da resposta imune. Os primeiros estudos utilizando estes antígenos avaliaram a resposta imune humoral de pacientes com LTA ativa e após cura clínica. Frente ao antígeno solúvel, verificou-se diminuição da reatividade anticórpica ao comparar soros de pacientes obtidos antes e após o tratamento quimioterápico, sendo observado resultado semelhante em pacientes com cura espontânea. Nesses indivíduos também foi observada resposta imune humoral aumentada frente ao antígeno insolúvel (BRITO et al., 2000; 2001).

Os antígenos solúvel e insolúvel vêm sendo estudados por nosso grupo em pacientes com diferentes estados clínicos da doença. REIS et al.(2009) verificaram uma resposta linfoproliferativa específica e produção de IL-10 e IFN-γ frente ao antígeno solúvel antes e após tratamento quimioterápico. Mais recentemente, a produção de IL-10 e IL-4 foi observada na fase inicial da leishmaniose, com a presença significativa de células T CD4+, o que sugere a conexão dessas células com a patogênese da doença. Na cura clínica obtida após o tratamento ou espontaneamente, observou-se uma resposta do tipo Th1, o que parece estar associado com a cura ou proteção na LTA (BRELAZ-DE-CASTRO et al., 2012).

Diante da complexidade das manifestações clínicas da LTA, o envolvimento de mediadores dos perfis clássicos (citocinas e NO), além de Th17 e Treg, na resolução ou na progressão da LTA em humanos necessita ser melhor definido. Dessa forma, entender como funciona a rede de células, suas citocinas e de outros mediadores é fundamental para o desenvolvimento de novas estratégias em relação a tratamento e medidas de profilaxia.

4 METODOLOGIA

4.1 Tipo de estudo

O estudo foi do tipo experimental (não-randomizado). Foram realizadas comparações entre um grupo de participantes sujeitos a tratamento para LTA, outro grupo de participantes que obtiveram cura espontânea da doença e outro formado por sujeitos sem história prévia de LTA, denominado controle. Ambos foram escolhidos a partir de critérios de disponibilidade ou conveniência (ROUQUAYROL; ALMEIDA FILHO, 2003).

4.2 Seleção da população de estudo

Os pacientes do presente estudo foram procedentes de Recife, Moreno, Araçoiaba, Vicência e Chã de Alegria. Para os artigos 1 e 2, foram selecionados 14 pacientes com LTA ativa e 11 pacientes com cura clínica espontânea e, para o terceiro artigo, 23 pacientes com LTA ativa foram recrutados. Ambas as populações foram constituídas por indivíduos de ambos os sexos e idade superior a quinze anos. Para a seleção de pacientes com LTA ativa, foram observados os seguintes critérios: apresentação de uma ou mais lesões cutâneas ativas, ausência de tratamento quimioterápico prévio e resultado positivo em um dos testes laboratoriais. Aqueles menores de quinze anos, com história pregressa de LTA ou que se submeteram à quimioterapia foram excluídos deste grupo.

O histórico de doença pregressa, apresentação de cicatriz característica da lesão (aspecto de pergaminho), IDRM positiva e ausência de quimioterapia foram os critérios para inclusão no grupo de cura espontânea. Como critérios de exclusão foram considerados a ausência de cicatriz, negatividade para a IDRM e tratamento quimioterápico prévio.

Os pacientes foram atendidos no ambulatório do Centro de Pesquisas Aggeu Magalhães, onde realizaram-se os exames clínico-laboratoriais e os procedimentos de coleta de sangue. No grupo de pacientes com LTA ativa, o sangue foi coletado antes da quimioterapia (AT) e um ano após o seu término (PT). Já no grupo dos pacientes curados espontaneamente, a coleta aconteceu apenas em um momento. O critério de cura clínica para os pacientes tratados foi estabelecido considerando-se a completa cicatrização da lesão e a resposta terapêutica. No período de um ano após o final do tratamento, foi realizado o

acompanhamento do processo de cicatrização, além da verificação da ocorrência de efeitos colaterais decorrentes do tratamento.

O tratamento quimioterápico foi realizado nos postos de saúde dos municípios deste estudo, utilizando-se o Glucantime®, droga de primeira escolha, administrado via intramuscular. O tratamento foi feito em ciclos de 20 a 30 dias em doses diárias de 20 mg/Kg com intervalo de 10 dias entre cada série. Os pacientes foram submetidos a nova série do tratamento de acordo com o processo de cicatrização de cada indivíduo.

O grupo controle constituiu-se de voluntários saudáveis, com idade acima de quinze anos, residentes em área não endêmica e sem história prévia de transfusão sanguínea. Nos artigos publicados, nove indivíduos constituíram o grupo, e no artigo submetido foram recrutados seis voluntários. O material coletado foi processado no Centro de Pesquisas Aggeu Magalhães-FIOCRUZ, na cidade de Recife-PE.

4.3 Considerações éticas

Os procedimentos para coleta de sangue foram realizados somente após o indivíduo ou seu responsável concordar em assinar o "Termo de Consentimento Livre e Esclarecido" (TCLE) (apêndices A, B e C) . Os protocolos experimentais foram aprovados pelo Comitê de Ética da Instituição (CAAE n° 0122.0.095.000-08 e n° 00082.0.095.000-09, Anexos A, B e C).

4.4 Obtenção dos antígenos solúvel e insolúvel de *L. (V.) braziliensis*

Formas promastigotas (cepa MHOM/BR/75/M2903) foram expandidas em cultura com meio Schneider's (Sigma) contendo 10% de soro fetal bovino (SFB, Cultilab) e 1% de antibiótico (penicilina 100 UI/ml e estreptomicina 100 mg/ml – Cultilab) até a fase de crescimento exponencial. A massa parasitária foi então submetida a três lavagens com salina tamponada (PBS – pH 7,2) através de centrifugações a 800 x g, por 15 minutos, a 4ºC. O sedimento resultante foi armazenado a –20ºC até o momento do uso.

Para a obtenção dos antígenos, as alíquotas estocadas foram descongeladas, ressuspendidas em água destilada contendo inibidores de proteases (metil-fenil-fluoreto –

PMSF, 0,01mM e ácido etilenodiaminotetracético – EDTA, 2 mM) e pepstatina A, 0,001 M. Em seguida, foram ultrassonicadas e centrifugadas a 10.000 x g durante 10 minutos a 4ºC. O sobrenadante removido foi submetido a uma nova centrifugação a 100.000 x g durante uma hora, à mesma temperatura. O sobrenadante resultante correspondente ao antígeno solúvel e o sedimento, antígeno insolúvel de *L. (V.) braziliensis*, foram submetidos a uma dosagem protéica segundo o método de Bradford (1976) modificado por Read & Northcote (1981), além de eletroforese em gel de poliacrilamida (SDS-PAGE) segundo Laemmli (1970). Após esses procedimentos, os antígenos foram armazenados a –20ºC até a utilização nos ensaios de cultura celular (BRELAZ-DE-CASTRO et al., 2012).

4.5 Obtenção de células mononucleares do sangue periférico

Quarenta mililitros de sangue foram coletados utilizando-se o sistema a vácuo (Vacutainer). O sangue foi diluído em PBS pH 7,2 na proporção 2:1 e transferido para tubos cônicos (BD) contendo Ficoll-Paque (Amersham Biosciences), também na proporção 2:1. Após centrifugação a 900 x g por 30 minutos a 20ºC, a camada de PBMC obtida entre a mistura de Ficoll-Paque e o plasma foi removida e depositada em novos tubos cônicos. Depois de nova centrifugação a 300 x g por 15 minutos a 20ºC, o sobrenadante foi descartado e o sedimento ressuspendido em 10 ml de PBS (pH 7,2) e novamente centrifugado a 300 x g, durante o mesmo tempo e mesma temperatura. Depois do descarte do sobrenadante, o sedimento composto de PBMC foi ressuspendido em meio de cultura RPMI 1640 (*Roswell Park Memorial Institute*, Sigma) suplementado com 10% de SFB. Uma alíquota da suspensão celular foi então removida, diluída 1:20 em azul de trypan (Sigma) e quantificada em câmara de Neubauer.

4.6 Cultura de PBMC

Suspensões celulares foram cultivadas em placas de 24 poços (TPP), na concentração de 10^6 células/poço. O cultivo foi realizado em meio RPMI 1640 contendo 1% de L-glutamina 200 mM, 1% piruvato de sódio 100 mM, 0,2% de bicarbonato de sódio 7,5% e 1% de antibiótico (penicilina 100 UI/ml e estreptomicina 100 mg/ml) suplementado com 10% de soro fetal bovino (Cultilab). As PBMC foram estimuladas com os antígenos solúvel (1,25µg/ml) e insolúvel (2,5µg/ml) de *L. (V.) braziliensis*. Como controle positivo do ensaio, o mitógeno fitohemaglutinina (PHA; Cultilab) foi utilizado na concentração de 2,5µg/ml. Células mantidas apenas com meio de cultura foram os controles negativos do ensaio. As placas foram mantidas em estufa à 37ºC/5% de CO_2 durante 24 horas, 48 horas e 6 dias.

Dados os tempos de incubação, as placas passaram por centrifugação (1800 x g por 10 min, a T.A.) e os sobrenadantes foram coletados e estocados a -80°C para posterior utilização. Da placa incubada por 24 horas, removeu-se também o pellet de PBMC em cada poço através de raspagem, utilizando-se 1 mL de PBS gelado. Em seguida, a suspensão celular resultante foi transferida para microtubos de 1,5 mL, identificados por estímulo. Procedeu-se à centrifugação dos microtubos à 300 Xg por cinco minutos à 4ºC e, em seguida, o sobrenadante foi descartado e o pellet de PBMC ressuspendido em 800 µL de TRIzol (Invitrogen). Após homogenização, as células foram armazenadas à –80ºC até a utilização nos ensaios de extração de RNA.

4.7 Dosagem das citocinas secretadas nos sobrenadantes de cultura

Os níveis de citocinas existentes nos sobrenadantes de cultura foram mensurados utilizando-se kits de ELISA de captura, seguindo as instruções dos fabricantes. TNF-α (BD Biosciences) foi quantificada depois de 48 horas de cultura. As concentrações de IFN-γ (BD Biosciences), IL-17 (R&D Systems), IL-10 (BD Biosciences) e IL-22 (R&D Systems) foram avaliadas no tempo de 6 dias. Os limites de detecção mínimos para as análises foram: 1,95 pg ⁄ml para IFN-γ, TNF-α, IL-17 e IL-10 e 3,9 pg⁄ml para IL-22. As concentrações finais foram obtidas utilizando-se o software Microplate Manager (versão 4.0; Bio-Rad Laboratories, Vienna, Austria).

4.8 Dosagem de óxido nítrico

Para a dosagem do óxido nítrico, foi utilizada a reação colorimétrica de Griess, que consiste na detecção de nitrito (NO_2^-), resultante da oxidação do NO nos sobrenadantes de cultura previamente coletados no tempo de cultivo de 6 dias. Adicionou-se à uma placa de ELISA de 96 poços (Costar half area plate) 25µl do sobrenadante de cultura em duplicata (diluído em RPMI suplementado com SFB 2%, quando necessário), seguido do mesmo volume do reagente de Griess. Este é composto de sulfanilamida 1% diluída em H_3PO_4 2,5% (solução A) e de N-1-naphtylethtylenodiamina, também diluído em solução de H_3PO_4 a 2,5% (solução B). Para a confecção de uma curva-padrão, uma solução de nitrito de sódio na concentração inicial de 200µM passou por diluições seriadas (fator 2) em RPMI suplementado com 2% de SFB. Após incubação de 10 minutos ao abrigo da luz, a leitura no espectrofotômetro foi realizada a 450 nm. A absorbância das diferentes amostras foram comparadas com a curva-padrão, e os resultados obtidos expressos como a média da duplicata ± erro padrão, utilizando-se o software Microplate Manager 4.0 (BioRad).

4.9 Análises de expressão gênica

4.9.1 Extração de RNA

As células armazenadas foram descongeladas até atingirem a temperatura ambiente. Em seguida, foram adicionados a cada microtubo 200µl de clorofórmio P.A. Após centrifugação a 10000 X g por dez minutos a 4ºC, a fase polar (DNA e RNA) foi separada da fase apolar (resíduos celulares, proteínas). O sobrenadante contendo RNA/DNA foi então transferido para novos microtubos, onde foram adicionados 500µl de isopropanol, que promove a precipitação do RNA. Após leve agitação manual e incubação por dez minutos à T. A., as amostras foram novamente centrifugadas a 10000 X g por dez minutos a 4ºC. O sobrenadante foi descartado e o pellet foi submetido a duas centrifugações a 7500 X g por cinco minutos a 4ºC na presença de álcool 75%. O sobrenadante resultante foi então descartado e o pellet de RNA foi submetido a secagem à T. A., por cinco minutos. Após esta etapa, foram adicionados aos microtubos 30ul de água livre de RNAse. O RNA foi então

submetido a quantificação por espectrofotometria. Em seguida as amostras foram estocadas a –80ºC até o momento da utilização em ensaios de transcrição reversa.

4.9.2 Transcrição reversa

Para a transcrição reversa foi utilizado o kit *TaqMan® Reverse Transcription Reagents* (Applied Biosystems), segundo protocolo do fabricante. O RNA obtido a partir de PBMC pelo método do TRIzol foi submetido a conversão em cDNA em um termociclador (Eppendorf mastercycler gradient). A RT-PCR foi realizada em volume final de 50µl, contendo TaqMan RT Buffer 1x, $MgCl_2$ 5,5mM, Mix de dNTPs 500µM, hexâmeros randômicos 2,5µM, inibidor de RNAse 0,4 U/µL, a enzima multiscribe reverse transcriptase 3,125 U/µL e 12,375µL de amostra contendo uma quantidade normalizada de RNA (100ng). A reação consistiu de um ciclo, sendo este dividido em dez minutos a 25ºC, trinta minutos a 48ºC e cinco minutos a 95ºC. Após esta etapa, as amostras foram armazenadas a -20ºC até a utilização nos ensaios de PCR em tempo real.

4.9.3 PCR quantitativa em tempo real

A expressão de mRNA para IFN-γ (Assay ID: Hs99999041_m1), TNF-α (Hs00174128_m1), iNOS (Hs00167257_m1), IL-10 (Hs99999035_m1), IL-4 (Hs99999030_m1), IL-6 (Hs00985639_m1), TGF-β (Hs99999918_m1), IL-17 (Hs00174383_m1), IL-22 (Hs01574154_m1), Foxp3 (Hs00203958_m1) e RORC (Hs01076122_m1) (genes alvo) foi avaliada através da reação de PCR em tempo real, utilizando-se o sistema de detecção de sequências ABI PRISM 7500 (Applied Biosystems). A reação foi confeccionada em *singleplex*, num volume final de 20µL, contendo TaqMan Universal PCR Master Mix (2x), TaqMan Gene Expression Assays para os genes alvo, constituído de primers e sonda específicos (fluoróforo FAM), água livre de RNAse e 1µL de amostra. Em paralelo, como gene de referência foi utilizado RNA ribossomal 18S, cuja reação foi preparada com TaqMan Universal PCR Master Mix, primers forward e reverse para RNA ribossomal 18S, sonda para RNA ribossomal 18S (fluoróforo VIC), água livre de RNAse e 1µL de amostra. A expressão de mRNA para os alvos foi calculada pelo método do Ct

comparativo, sendo os valores obtidos normalizados com o gene de referência (ΔCt). Este cálculo foi feito da seguinte maneira:

$$\Delta Ct = Ct\ (Alvo) - Ct\ (RNA\ 18S),$$

onde Ct corresponde ao ponto no gráfico onde a amplificação se torna exponencial. Após a normalização dos valores do gene alvo, o ΔCt deste foi subtraído do ΔCt de um elemento calibrador (ΔΔCt) que, neste caso, foi o grupo controle. Foi utilizada a fórmula:

$$\Delta\Delta Ct = \Delta Ct\ (amostra) - \Delta Ct\ (calibrador),$$

onde ΔCt (amostra) corresponde a um parâmetro de interesse a ser relativizado. Obtido o ΔΔCt, este é aplicado na fórmula:

$$RQ = 2^{-\Delta\Delta Ct},$$

obtendo-se assim a quantidade relativa de cada mediador expresso em determinada amostra.

4.10 Análise estatística

A análise estatística foi realizada empregando-se testes não-paramétricos. A análise para medir a variabilidade intra-grupo foi realizada pelo teste dos postos sinalizados de Wilcoxon. Já entre os grupos o teste U de Mann-Whitney foi utilizado. Todas as conclusões foram tomadas no nível de significância de 5%. Os softwares utilizados foram o Excel 2007 e o Graphpad Prism 5.0.

5 RESULTADOS E DISCUSSÃO

5.1 Artigo 1. *"American Tegumentary Leishmaniasis: cytokines and nitric oxide in active disease and after clinical cure, with or without chemotherapy"* (publicado na revista *Scandinavian Journal of Immunology* n. 76, p. 175-180, 2012)

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American Tegumentary Leishmaniasis: Cytokines and Nitric Oxide in Active Disease and After Clinical Cure, With or Without Chemotherapy

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RESUMO

A influência da resposta immune no tratamento da leishmaniose tegumentar americana é apontada por diversos autores, e a existência de imunidade protetora em pacientes curados espontaneamente (SH) é também sugerida. Assim, a produção de interferon-gama (IFN-γ), fator de necrose tumoral-alfa (TNF-α), interleucinas (IL-) 10, IL-17, IL-22 e óxido nítrico (NO) foi determinada em sobrenadantes de cultura de PBMC de pacientes com doença ativa (AD) e após quimioterapia (AT), pacientes SH e indivíduos sadios, em resposta ao antígeno solúvel de *Leishmania (Viannia) braziliensis*. Foi demonstrado que, durante a doença ativa, há uma predominância de IFN-γ e TNF-α, indicando uma fase pró-inflamatória da doença; IL-17 também é evidenciada nesse estado clínico. Além disso, TNF-α esteve levemente aumentada nos pacientes após o tratamento. A secreção de NO foi observada nos indivíduos SH, enquanto que IL-17 apareceu em baixos níveis nesses pacientes e parece ser regulada pelo NO. A presença de IL-10 foi observada em todos os grupos de pacientes. A partir deste estudo, podemos sugerir que na doença ativa e após a cura clínica, com ou sem quimioterapia, uma resposta immune cellular específica atua contra a *Leishmania*, existindo algumas similaridades entre os estados clínicos. Assim, isto indica que os mediadores aqui descritos são necessários para que a cura ocorra.

ABSTRACT

The influence of immune response on the treatment of American tegumentary leishmaniasis is pointed by several authors, and the existence of protective immunity in self-healed patients (SH) is also suggested. Thus, interferon-gamma (IFN-γ, tumour necrosis factor-alpha (TNF-α), interleukin (IL-) 10, IL-17, IL-22 and nitric oxide (NO) production was determined in PBMC culture supernatants from patients with active disease (AD) and after therapy, SH patients and healthy subjects, in response to the soluble antigen of *Leishmania (Viannia) braziliensis*. It was demonstrated that, during the active disease, there is a predominance of IFN-γ and TNF-α, indicating a proinflammatory phase of the response; IL-17 is also highlighted at this clinical state. Also, TNF-α was slightly increased in patients after therapy. NO secretion was noticed in SH individuals, while IL-17 appeared in low levels in these patients and seems to be regulated by NO. The presence of IL-10 was observed in all groups of patients. From this study, we can suggest that in the active disease and after clinical cure, with or without chemotherapy, specific cellular immunity takes part against *Leishmania*, but with some similarities between the clinical states. Thus, it indicates that the mediators herein described are necessary for the cure to occur.

Keywords: cutaneous leishmaniasis; immune response; chemotherapy; self-healing.

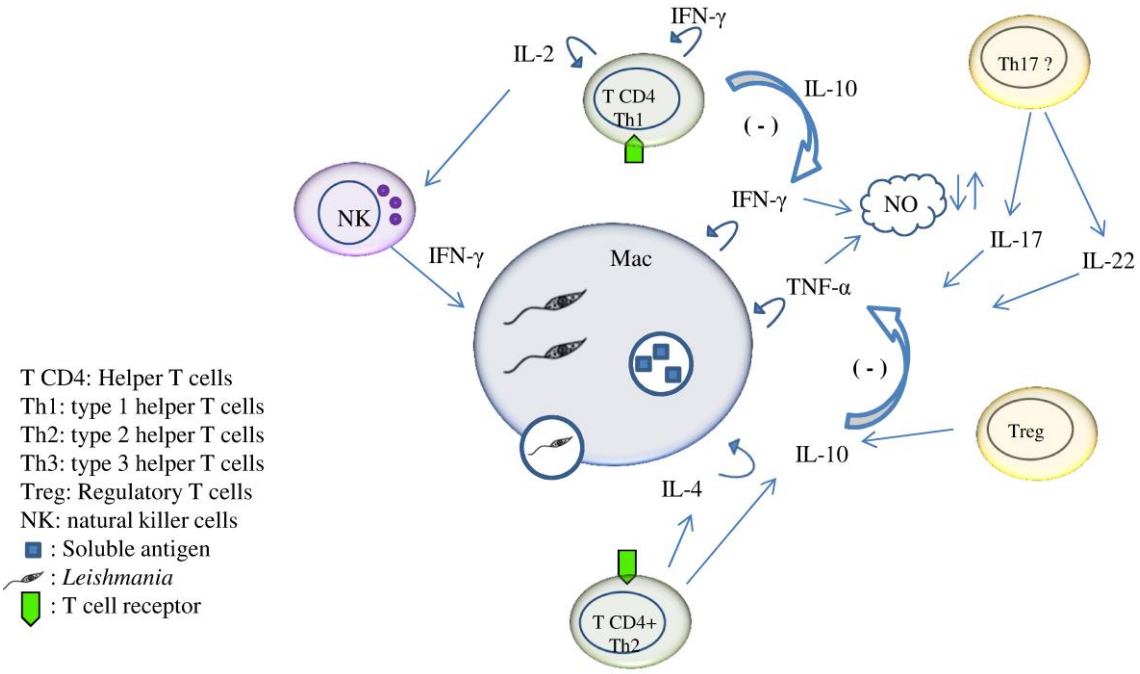
1 INTRODUCTION

American tegumentary leishmaniasis (ATL) is a vectorially transmitted anthropozoonosis, which is caused by several species of *Leishmania*. *Leishmania (Viannia) braziliensis* is the major species that causes ATL in Brazil, and clinical outcomes of the disease depend on factors inherent to the parasite, the vector and the host [1,2,3]. In humans the infection can be subclinical, or they can present manifestations ranging from localized, disseminated or diffuse skin lesions to aggressive and mutilating mucocutaneous lesions [1]. For all clinical forms the treatment is executed through chemotherapy with pentavalent antimonials, and the first line drug is meglumine antimoniate (Glucantime®).

As shown in Figure 1, an expansion characterized by T CD4+ cells occurs in all clinical outcomes of the disease, with Th1 or Th2 cytokines profiles [4]. The resistance and elimination of the parasites are due to the presence of interferon-gamma (IFN-γ), tumor

necrosis factor alpha (TNF- α), from the Th1 profile, while Th2 cytokines, like interleukin (IL-) 4 and IL-10 are linked to susceptibility to infections by *Leishmania* [5,6]. The diversity in clinical manifestations is strongly influenced by the host immune response [7].

Figure 1. Immune response developed against *Leishmania* infections. The presence of Th1 and Th2 cytokines as a consequence of the expansion of T CD4+ cells is well-established, whereas the role of Th17 subtype is not yet clarified.



In addition to Th1 and Th2 profiles, recent studies suggest the involvement of IL-17, predominantly produced by Th17 cells, in processes that lead to chronicity of the disease [8,9]. Also, IL-22, another Th17-produced cytokine, and to a lesser extent by Th1 and NK cells [10], is said to be involved in immunity at the epithelium and mucosal surfaces. Both cytokines were produced in human Kala-Azar caused by *Leishmania donovani*, and were also associated to the resistance to infection [11]. Furthermore, IL-10 production, initially related only to the improved survival of the parasite in the host, leading to macrophage deactivation, may represent a necessary counterbalance to the resolution of the disease [12].

Previous studies demonstrate that cellular immune response is involved with the healing process after the treatment with antimonials. Authors such as Toledo et al. [13] and Da Cruz et al. [14] evaluated the cellular immunity of patients with ATL using total antigen of *L. (V.) braziliensis*, prior and post chemotherapy, trying to detect possible parameters in the immune response associated with healing after treatment.

Previously, our group evaluated the cellular immune response of patients with active ATL before and after the chemotherapy against the soluble antigen of *L. (V.) braziliensis* [15], once the search for immunogenic fractions of *Leishmania* is necessary for vaccine synthesis and development of prognostic tests. Not least relevant, other authors related the importance of cellular immunity in patients who have spontaneously achieved clinical cure (without being through treatment), which suggests the development of a protective immune response [16,17]. Considering that few studies were developed around the self-healing process, that cytokines and NO are crucial in the host defense against *Leishmania*, and that the role of IL-17 and IL-22 in the immunity against *L. (V.) braziliensis* is not well defined, this paper evaluated IFN-γ, TNF-α, IL-10, IL-17, IL-22 and nitric oxide (NO) production of patients with active ATL and post-therapy or spontaneous clinical cure against the soluble antigen of *L. (V.) braziliensis*.

2 MATERIALS AND METHODS

2.1 Study population

Individuals of both gender and older than 15 years old were selected from the municipalities of Moreno, Araçoiaba, Amaraji, Vicência and Chã de Alegria, endemic areas for ATL. Fourteen patients with active disease (AD) were chosen based on criteria such as: presence of cutaneous lesions, confirmed diagnosis by the Reference Service in leishmaniasis of CPqAM and no previous chemotherapy treatment. The history of previous ATL, presence of characteristic scars, positive Montenegro skin test (MST) and absence of chemotherapy were the considered criteria to select eleven self-healed patients (SH). The ones with active disease were submitted to blood collection prior to chemotherapy treatment with Glucantime® and then 12 months after the end of treatment. Therapeutic scheme was made of doses of 20 mg/kg/day by subcutaneous injections during 20 and 30 days (1 cycle). After finishing treatment, patients (AT) were followed up for a period of 12 months to confirm clinical cure and to avoid the appearance of new lesions and relapse. At the end of this period, all treated individuals were submitted to a new blood collection. In the self healing group the blood was collected in only one moment. Nine healthy individuals represented the control group (CT) from non-endemic areas and without previous ATL infection. All individuals signed the "Term of Free and Informed Consent" and CPqAM/Fiocruz Research Ethics Committee (Protocol No. 123/08) approved the experimental protocols.

2.2 Soluble antigen of L. (V.) braziliensis

As described by Reis et al. [15], promastigote forms of *L. (V.) braziliensis* (MHOM/BR/75/M2903), cultured in vitro, were expanded in Schneider's medium (Sigma, St. Louis, MO) supplemented with 10% of fetal calf serum (Cultilab) and 1% of antibiotics (100UI/ml penicillin and 100 mg/ml streptomycin; Sigma) until they reached the exponential phase. Afterwards, they were sedimented by centrifugation at 800 x g for 15min at 4ºC and three times washed with phosphate-buffered saline (PBS; pH 7.2). Proteases inhibitors such as 0.1mM methyl-phenyl-fluoride and 2mM ethylenediaminotetraacetic acid (Sigma), pepstatine A 0.001M (Sigma) were added and, right after, the parasites were ultrasonicated. The parasitic suspension was centrifuged at 10.000 x g for 10min at 4ºC. The resultant supernatant was removed and submitted to a new centrifugation at 100.000 x g for 1 hr at the same temperature. Protein concentration was determined [18] from the new resultant supernatant, the soluble antigen (AgSol). The antigens were stored at -20ºC for further use.

2.3 Cell Culture

PBMC obtained from venous blood (10^6 cells/ml) were cultured (37ºC/5% CO_2) in 24-well plates (TPP, Switzerland) with AgSol (1,25 μg/ml) of *L. (V.) braziliensis*, as well as with phytohemagglutinin mitogen (PHA; 5,0 μg/ml), during 48h and 6 days. Cells only in the presence of the culture medium were used as the negative control. After incubation, the plates were centrifuged (1800 x g for 10 minutes, at RT) and the culture supernatants were collected and stored at -70ºC.

2.4 Cytokine determination in culture supernatants

Cytokines in the supernatants of cultures were assayed with capture ELISA kits according to the manufacturer's instructions. IFN-γ (BD Biosciences), IL-17 (R&D Systems), IL-10 (BD Biosciences) and IL-22 (R&D Systems) levels were measured at 6 days. TNF-α (BD Biosciences) was quantified at 48 hours. The lower limits of detection for the ELISA analyses were as follows: 1,95 pg/ml for IFN-γ, TNF-α, IL-17, IL-10 and 3,9 pg/ml for IL-22. The final concentrations were expressed in pg/ml using the Microplate Manager Version 4.0 software (Bio-Rad Laboratories, Vienna, VA).

2.5 Nitrite detection by Griess method

ELISA plates (96-well-Costar half-area plate) were filled with 25µl of culture supernatants (two replicates), followed by the Griess reagent in the same volume. A standard curve was made using sodium nitrite at 200 µM, and submitted to serial dilution (factor 2) in RPMI medium supplemented with 2% of fetal calf serum (Cultilab, Brazil). After incubation for 10 min in the dark, the reading in the spectrophotometer was carried out at 450 nm. The absorbances were compared to the standard curve (threshold set in 0,19 µM), and the results were expressed as the replicate means ± standard error, using the Microplate Manager Version 4.0 software (Bio-Rad Laboratories).

2.6 Statistical Analysis

The data were analyzed using nonparametric tests. For intragroup comparative analysis (AD x AT), the Wilcoxon test was used and to detect differences between groups the Mann-Whitney U-test was used. The results were considered significant when $P < 0,05$.

3 RESULTS

Before treatment, the AD patients presented ulcerated skin lesions with raised borders and granulomatous bottom, distributed mostly by uncovered areas of the body. The disease evolution time, calculated from the lesion appearance until the patient visits the health surveillance service in the municipal districts, varied from eight days to three months. After treatment with Glucantime® and subsequent monitoring of the patients for until a year, all patients showed complete healing of the lesions. All the SH patients presented typical scars indicating previous disease at the time of clinical evaluation. The period between the emergence of the lesion and the scar formation varied from fifteen days to nine months. MST result above 5mm was observed in all patients. As shown in Tables 1 and 2, in both groups gender proportion was statistically similar ($P > 0,05$).

PBMC from all groups were efficiently induced to secrete cytokines in response to PHA, and the non-stimulated cultures secreted minimal or any levels of all mediators (data not shown). As shown in Figure 2, a significant difference in the levels of IFN-γ was observed in AD patients (4551 ± 6018, $P = 0,006$) and in SH (2722 ± 5366, $P = 0,01$) after stimulation with the antigen, when compared to the control group (CT, $80 \pm 98,63$). A decrease in IFN-γ

concentration after treatment was observed (2323 ± 2954), although without statistical significance in comparison to AD.

The antigen significantly induced the production of TNF-α in AD (109,8 ± 130,9, P = 0,008) and AT (136,6 ± 191,6, P = 0,0003) comparing to CT (7,88 ± 16,20). The levels of TNF-α produced by treated patients showed a tendency to be greater than the AD and SH patients (P > 0,05).

All groups presented higher levels of IL-10 after antigenic stimulation in comparison to CT, although without statistical significance. AD patients produced IL-17 in significant concentrations (47,82 ± 15,62) in relation to the healthy subjects (2,66 ± 1,72; P = 0,04) and to the self-healed patients (0,66 ± 0,47, Ξ: P = 0,02). IL-22 was exhibited in higher levels by all groups in relation to CT, although without statistical difference among them (P > 0,05).

In relation to nitric oxide production, significant levels were observed in SH patients (39,6 ± 37,56) in comparison to AD (Ξ: 10,83 ± 16,44, P = 0,023), AT (Ξ: 4,35 ± 10,66, P = 0,001) and CT (0,1 ± 0,3, P = 0,0007).

Table 1. Demographic, clinical and therapeutic data of the patients with ATL.

Patient	Age	Gender	Location	Clinical form	N° lesions	Evolution	Local lesions	Lesion size(mm^2)	Cicles
01	53	F	Amaraji	LCL	01	03 months	R leg	12,25	02
02	47	M	Amaraji	LCL	01	03 months	L leg	21	02
03	21	F	Moreno	LCL	01	02 months	Body	32	02
04*	25	M	Moreno	LCL	08	01 month	R and L legs/ L hand	2/ 24,5	04
05	41	F	Moreno	LCL	02	08 days	R leg	0,25/ 5	02
06	39	M	Moreno	LCL	01	01 month	L leg	36	02
07	33	F	Moreno	LCL	01	01 months	R leg	24/ 27,5	02
08	52	M	Moreno	LCL	02	02 months	Body/ Shoulder	24,75/ 15	01
09	58	F	Moreno	LCL	01	01 month	R leg	18	01
10	20	M	Chã de Alegria	LCL	02	02 months	R leg	8/ 7,5	02
11	56	M	Araçoiaba	LCL	01	03 months	R leg	39	03
12	28	F	Moreno	LCL	01	15 days	R foot	NI	02
13	26	F	Moreno	LCL	01	02 months	R leg	36	02
14	34	M	Moreno	LCL	01	03 months	L leg	6	02

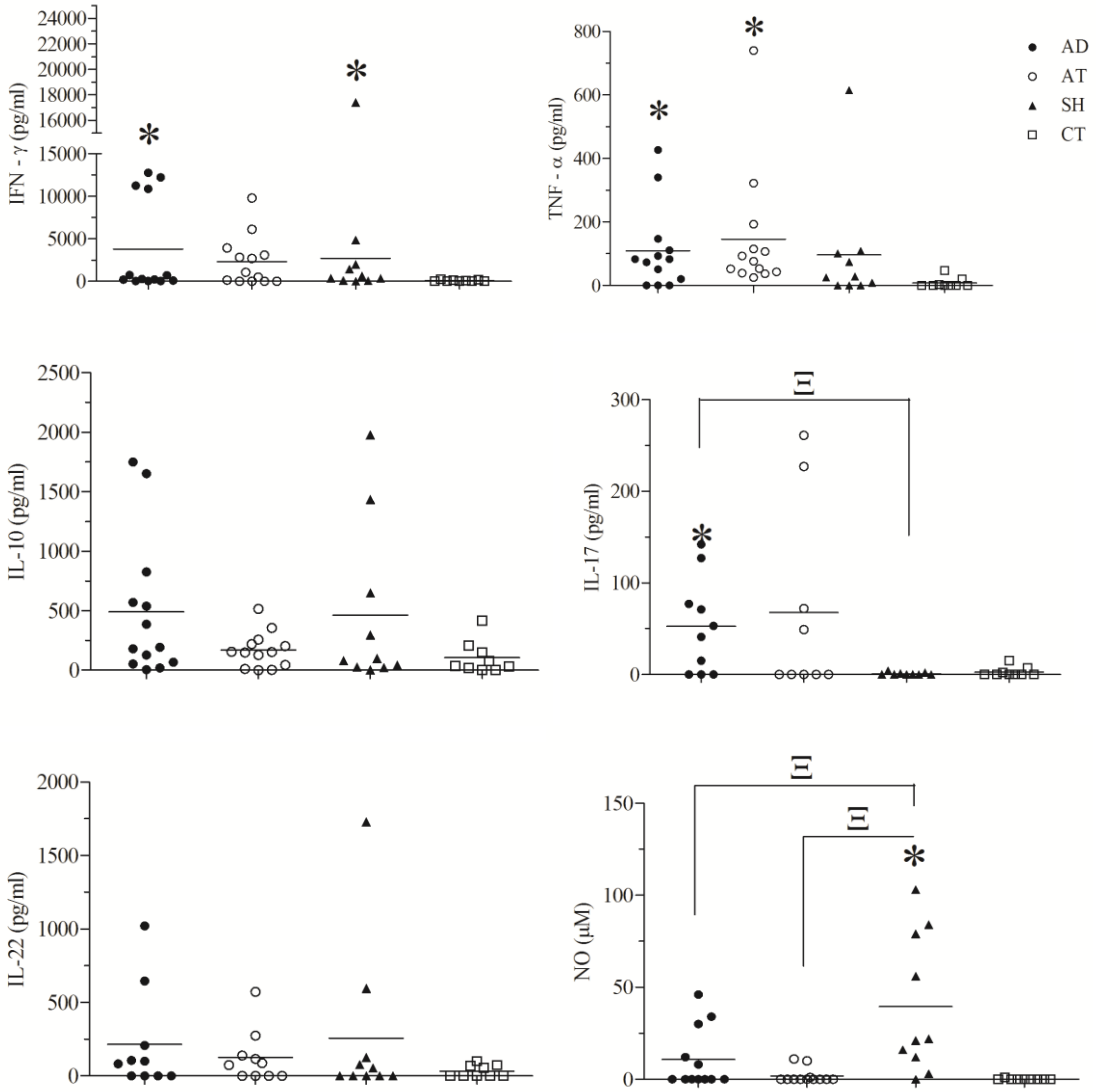
NI = not informed; F = female; M = male; CL = localized cutaneous leishmaniasis; L = left; R = right
* Patient presented 01 lesion measuring 24,5 mm^2, and seven others measuring 2mm^2.

Table 2. Demographic and clinical data of the self-healed (SH) patients.

Patient	Age	Gender	Location	Nº scars	Local of scars	Healing time	MST (mm)
01	77	M	Moreno	01	L leg	3 months	12
02	71	M	Moreno	01	L forearm	NI	10
03	81	M	Moreno	01	L leg	15 days	10
04	28	M	Moreno	01	L thigh	3 months	12
05	NI	F	Amaraji	01	L foot	1 month	05
06	47	F	Vicência	04	Neck, L shoulder, R thigh, L leg	9 months	12
07	17	F	Vicência	01	L leg	NI	08
08	30	M	Vicência	01	R foot	4 months	10
09	39	F	Vicência	01	R leg	8 months	07
10	60	F	Moreno	01	L ankle	3 months	10
11	57	F	Moreno	01	L leg	2 months	15

NI = not informed; F = female; M = male; L = left; R = right; MST = Montenegro Skin Test.

Figure 2: **Cytokine and nitric oxide production among patients with active disease (AD), after treatment (AT), spontaneously healed patients (SH) and control group, in response to soluble antigen.** The asterisk represents the significant differences between patients and control group, and the greek letter Ξ (ksi) shows the significances between the groups of patients (P<0,05). The horizontal bars represent the mean of the groups.



4 DISCUSSION

Investigations addressing the immune response in ATL have been performed in patients with active disease, as well as after chemotherapy [13,15,19]. However, few data is known about what makes the difference in those individuals who develop the disease and achieve clinical cure without drug intervention [16,17]. Considering the importance of the cellular immune response in ATL, and also that few studies on self-healed individuals were developed, the aim of this study was to evaluate cytokine and nitric oxide production in patients with active and post-healed American tegumentary leishmaniasis, with or without chemotherapy.

The immune response in cutaneous leishmaniasis has been assessed by many authors using different antigens [5,8,12,13,14]. Herein, the soluble antigen of *L. (V.) braziliensis* was used for PBMC stimulation in an attempt to evaluate a more specific immune response and possibly indicate an immunogenic fraction for further studies toward other tools for treatment and prevention of disease development.

From the high IFN-γ production observed herein, we can suggest that the patients have established an immune response in order to eliminate the parasite. In addition, during the course of chemotherapy, gradual parasite destruction by macrophages is expected and, as a consequence, there is a depletion of parasite load. This event may justify the decreased levels of IFN-γ under antigenic stimulation in the patients after treatment, as seen by Reis et al. [15] as well as in self-healed patients, comparing to AD. Although in SH patients the chemotherapy has not been administered, we can suggest that their immune response underwent a modulation, reaching clinical cure [17].

As well as IFN-γ, TNF-α seems to be important to the disease control. In the present study, under antigenic stimulation, PBMC from all groups of patients were able to produce the cytokine, and in the patients post-treatment, the levels of TNF-α were slightly increased in relation to the other groups. This event is maybe due to the possible influence of antimonial treatment on the secretion of TNF-α, which may not occur in self-healed individuals[20,21].

Concerning NO secretion, some stable and measurable products come from its decomposition process, like nitrite (NO2-). In response to the antigen, SH patients presented significant amounts of nitrite in relation to the other groups, as well as a considerable production of pro-inflammatory cytokines. Thus, it is reasonable to suggest that IFN-γ and TNF-α may be necessary to NO production [22,23,24]. Nevertheless, nitrite levels exhibited

after therapy were rare, despite the great concentration of IFN-γ and TNF-α secreted by these patients. We believe that this mechanism occurs by influence of the pentavalent antimonials treatment.

Besides the benefits of Th1 response, Gollob et al. [25] suggest that IL-10 production may be critical for the disease control. Thus, it is postulated that IL-10 levels presented by the patients in this study regulate the production of pro-inflammatory cytokines, consequently leading to the clinical resolution of the disease.

Regarding IL-17, it has been increasingly studied in bacterial, fungal and protozoal infections in mice [26], but little is known about the role of this cytokine in human infections, especially in leishmaniasis. Herein, the significant levels of IL-17 observed in AD patients in comparison to SH and CT could be explained by the proinflammatory role of this cytokine. This was previously verified by Bacellar et al. [8] that observed in lesions of cutaneous and mucosal leishmaniasis patients the intensity of the inflammatory infiltration and its direct correlation to the number of cells expressing this cytokine. In addition, low levels of IL-17 observed in SH patients under antigenic stimulus may be, at least in part, due to a suppressive effect exerted by NO levels, which impair the polarization and the stabilization of IL-17-producing cells [27].

Another cytokine evaluated in this study was IL-22, which belongs to the IL-10 family [28]. In this study, a higher but not significant production of IL-22 was observed in patients in relation to the control group. IL-22 production was observed in SH patients, but not IL-17. As it happens to IL-17, little is known about the role of IL-22 in *Leishmania* infections, although protective and harmful roles are attributed for this cytokine. Sonnenberg et al. [29] suggests that these mechanisms may occur under influence of IL-17, which absence or presence seems to govern the proinflammatory versus tissue-protective properties of IL-22. On the other hand, Pitta et al. [11] observed that IL-22 and also IL-17 production was higher in patients who presented resistance to kala azar and, along with Th1 cytokines, IL-22 and IL-17 are supposed to play complementary roles in human protection against the disease. According to the authors, the production of these cytokines may point the presence of Th17 profile in the defense against the parasite. However, more investigation about the cytokines and their producing cells from this profile is needed to better understand its role in the pathogenesis of leishmaniasis.

From the results in this study, we can suggest that the patients with active disease and after clinical cure, with or without chemotherapy, exhibit specific cellular immune response profiles, but with some similarities between them. IFN-γ production by the three groups of

patients suggests an influent inflammatory response against the parasite, as well as in relation to TNF-α. The secretion of TNF-α showed a tendency to be higher after treatment, maybe by influence of pentavalent antimonials, and NO production may be increased by these two cytokines acting synergically. Moreover, IL-17 and IL-22 may also contribute to mount an effective response against the parasite. The former seems to be regulated by NO levels, which is known to be regulated by cytokines, and this mechanism may be important to enable the self-healing process. None the less, the presence of IL-10 suggests the induction of immunomodulatory mechanisms necessary for the establishment of an effective immune response against *Leishmania*.

From the results herein described, we can suggest that in active disease and after clinical cure, with or without chemotherapy, specific cellular immunity takes part against *Leishmania*, but with some similarities between the clinical states. Thus, it indicates that the mediators herein described are necessary for the cure to occur.

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Cytokines and NO in American tegumentary leishmaniasis patients: Profiles in active disease, after therapy and in self-healed individuals

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RESUMO

Estudos sugerem a influência da resposta imune no tratamento bem sucedido da leishmaniose tegumentar Americana (LTA), e indicam a existência de imunidade protetora em pacientes curados espontaneamente. Assim, o objetivo deste trabalho foi quantificar interferon-gama (IFN-γ), fator de necrose tumoral-alfa (TNF-α), interleucinas (IL-) 10, IL-17, IL-22 e óxido nítrico (NO) em sobrenadantes de cultura de PBMC de pacientes com doença ativa (AD), após o tratamento (AT), curados espontaneamente (SH) e de indivíduos sadios (CT) em resposta ao antígeno insolúvel de *Leishmania (Viannia) braziliensis* (AgIns). Todos os grupos de pacientes produziram IFN- γ, indicando um perfil pró-inflamatório predominante. Os pacientes AD e AT apresentaram níveis de TNF-α, com um leve aumento depois da terapia, enquanto que a citocia foi fracamente quantificada nos SH. Interessantemente, a secreção de NO foi significativa nesses indivíduos, enquanto que IL-17 apareceu em baixos níveis e parece ser regulada pelo NO.Embora IL-22 tenha sido detectada em AD, seu papel é questionável. A presença de IL-10 em todos os grupos de pacientes sugere que a citocina desempenhe diferentes papeis na doença. Esses resultados indicam que uma imunidade celular específica age contra a *Leishmania*, porém com alguma similaridade entre os estados clínicos aqui descritos; estes mediadores parecem ser necessários para que ocorra a cura.

ABSTRACT

Studies suggest the influence of immune response on the successful treatment of American tegumentary leishmaniasis (ATL), and indicate the existence of protective immunity in self-healed patients. Thus, the aim of this work was to quantify interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin (IL-) 10, IL-17, IL-22 and nitric oxide (NO) in culture supernatants of PBMC from patients with active disease (AD), after treatment (AT), and from self-healed (SH) and healthy subjects (CT), in response to *Leishmania (Viannia) braziliensis* insoluble antigen (AgIns). All groups of patients produced IFN- γ, indicating a predominant proinflammatory profile. AD and AT patients presented TNF-α levels, with a slight increase after therapy, whereas it was weakly quantified in SH. Interestingly, NO secretion was significant in these individuals, whereas IL-17 appeared in low levels and seems to be regulated by NO. Although IL-22 was detected in AD, its role is still questionable. The presence of IL-10 in all groups of patients suggests that the cytokine plays distinct roles in the disease. These results indicate that specific cellular immunity takes part against *Leishmania*, but with some similarities between the different clinical states herein described; these mediators seem to be necessary for the cure to occur.
Keywords: cytokines; cellular immunity; *Leishmania braziliensis*; leishmaniasis; nitric oxide.

1 INTRODUCTION

American tegumentary leishmaniasis (ATL) is an anthropozoonosis caused by several species of *Leishmania*. It is considered an endemic disease in Brazil, where the main causative agent is *Leishmania (Viannia) braziliensis*. The emergence of the diverse clinical forms of ATL depends on characteristics of the parasite and the vector, in addition to the immune status and genetic constitution of the vertebrate host [1-3]. In humans, the infection can be subclinical or it can present manifestations ranging from localized, disseminated or diffuse skin lesions to aggressive and mutilating mucocutaneous lesions [1]. The treatment is executed through chemotherapy for all clinical forms, and the first line drug used is meglumine antimoniate (Glucantime®).

The diversity of clinical manifestations in human ATL is strongly influenced by the host immune response [4]. In all clinical forms, there is an expansion characterized by T $CD4^+$ cells, presenting Th1 or Th2 cytokines profiles [5]. Interferon-gamma (IFN-γ) and

tumor necrosis factor (TNF) -α and -β, from the Th1 profile, are known to be involved in the resistance and elimination of the parasites, while Th2 cytokines such as IL-4 and IL-10 are linked to susceptibility to infections by *Leishmania* [6,7].

In addition to these cytokines, recent studies suggest that IL-17 is involved in processes that lead to chronicity of the disease [8,9]. Moreover, IL-22 is known to be involved in immunity at the epithelium and mucosal surfaces [10]. Both cytokines were produced in human Kala-Azar caused by *Leishmania donovani*, and were also associated to the resistance to infection [11]. Furthermore, IL-10 may develop a wider role in leishmaniasis, once it is related not only to the improved survival of the parasite in the host, leading to macrophage deactivation, but also to counterbalance mechanisms necessary to the resolution of the disease [12].

As previously demonstrated by some authors, cellular immune response is involved with the healing process after the treatment with antimonials. In attempt to detect possible parameters in the immune response associated with healing after treatment, the cellular immunity of patients with ATL was previously evaluated using total antigen of *L. (V.) braziliensis*, before and after the chemotherapy [13, 14].

Once the search for immunogenic fractions of *Leishmania* is necessary for vaccine synthesis and development of prognostic tests, our group previously assessed the cellular immune response of patients with active disease and after clinical cure, with or without chemotherapy (self-healed individuals), in response to the soluble antigen of *L. (V.) braziliensis* [15]. The results demonstrated a specific immune response developed by the patients, with some similarities in cytokine production among the different groups. Considering that this response may be diverse in the presence of different fractions, this work evaluated IFN-γ, TNF-α, IL-10, IL-17, IL-22 and nitric oxide (NO) production of patients with active ATL and post-therapy or spontaneous clinical cure against the insoluble (particulate) antigen of *L. (V.) braziliensis*.

2 MATERIALS AND METHODS

2.1 Study population

Individuals of both gender and older than 15 years old were selected from the municipalities of Moreno, Araçoiaba, Amaraji, Vicência and Chã de Alegria, endemic areas for ATL. Fourteen patients with active disease (AD) were chosen based on criteria such as:

presence of cutaneous lesions, confirmed diagnosis by the Reference Service in leishmaniasis of CPqAM and no previous chemotherapy treatment. The history of previous ATL, presence of characteristic scars, positive Montenegro skin test (MST) and absence of chemotherapy were the considered criteria to select eleven self-healed patients (SH). The ones with active disease were submitted to blood collection prior to chemotherapy treatment with Glucantime® and then 12 months after the end of treatment. Therapeutic scheme was composed by doses of 20 mg/kg/day by subcutaneous injections during 20 and 30 days (1 cycle). After the end of treatment, patients (AT) were followed up for a period of 12 months to confirm clinical cure and to avoid the appearance of new lesions and relapse. At the end of this period, all treated individuals were submitted to a new blood collection. In the self healing group the blood was collected in only one moment. Nine healthy individuals represented the control group (CT) from non-endemic areas and without previous ATL infection were selected. All individuals signed the "Term of Free and Informed Consent" and CPqAM/Fiocruz Research Ethics Committee (Protocol No. 123/08) approved the experimental protocols.

2.2 Insoluble antigen of L. (V.) braziliensis

As described by Brito et al. [16], promastigote forms of *L. (V.) braziliensis* (MHOM/BR/75/M2903), cultured in vitro, were expanded in Schneider's medium (Sigma) supplemented with 10 % of fetal calf serum (Cultilab) and 1 % of antibiotics (100 UI/ml penicillin and 100 mg/ml streptomycin; Sigma) until they reached the exponential phase. Afterwards, they were sedimented by centrifugation at 800 x g for 15 minutes at 4 ºC and three times washed with phosphate-buffered saline (PBS; pH 7.2). Proteases inhibitors such as 0.1 mM methyl-phenyl-fluoride and 2 mM ethylenediaminotetraacetic acid (Sigma), pepstatine A 0.001 M (Sigma) were added and, right after, the parasites were ultrasonicated. The parasitic suspension was centrifuged at 10.000 x g for 10 minutes at 4 ºC. The resultant supernatant was removed and submitted to a new centrifugation at 100.000 x g for 1 hour at the same temperature. Protein concentration was determined [17] from the pellet, the insoluble antigen (AgIns), which was stored at -20 ºC for further use.

2.3 Cell Culture

PBMC obtained from venous blood (10^6 cells/ml) were cultured (37 ºC / 5 % CO_2) in 24-well plates (TPP) with RPMI medium (Sigma) in the presence of AgIns (2.5 μg/ml) of *L. (V.) braziliensis*. Wells containing phytohemagglutinin mitogen (PHA; 5.0 μg/ml) were the positive control of the assay, and cells only in the presence of the culture medium were used as the negative control. After incubation during 48h and 6 days, the plates were centrifuged (1800 x g for 10 minutes, at RT) and the culture supernatants were collected and stored at -70 ºC.

2.4 Cytokine determination in culture supernatants

Cytokines in the supernatants of cultures were assayed with capture ELISA kits according to the manufacturer's instructions. IFN-γ (BD Biosciences), IL-17 (R&D Systems), IL-10 (BD Biosciences) and IL-22 (R&D Systems) levels were measured at 6 days. TNF-α (BD Biosciences) was quantified at 48 hours. The lower limits of detection for the ELISA analyses were as follows: 1.95 pg/ml for IFN-γ, TNF-α, IL-17, IL-10 and 3.9 pg/ml for IL-22. The final concentrations were expressed in pg/ml using the Microplate Manager Version 4.0 software (Bio-Rad Laboratories).

2.5 Nitrite detection by Griess method

ELISA plates (96-well-Costar half-area plate) were filled with 25 μl of culture supernatants (two replicates), followed by the Griess reagent in the same volume. A standard curve was made using sodium nitrite at 200 μM, and submitted to serial dilution (factor 2) in RPMI medium (Sigma) supplemented with 2 % of fetal calf serum (Cultilab). After incubation for 10 min in the dark, the reading in the spectrophotometer was carried out at 450 nm. The absorbances were compared to the standard curve (threshold set in 0.19 μM), and the results were expressed as the replicate means ± standard error, using the Microplate Manager Version 4.0 software (Bio-Rad Laboratories).

2.6 Statistical Analysis

The data were analyzed using nonparametric tests. For intragroup comparative analysis (AD x AT), the Wilcoxon test was used and to detect differences between groups the Mann-Whitney U-test was used. The results were considered significant when $P < 0.05$.

3 RESULTS

Before treatment, the AD patients presented ulcerated skin lesions with raised borders and granulomatous bottom, distributed mostly by uncovered areas of the body. The disease evolution time, calculated from the lesion appearance until the patient visits the health surveillance service in the municipal districts, varied from eight days to three months. After treatment with Glucantime® and subsequent monitoring of the patients for until a year, all patients showed complete healing of the lesions. All the SH patients presented typical scars indicating previous disease at the time of clinical evaluation. The period between the emergence of the lesion and the scar formation varied from fifteen days to nine months. MST result above 5mm was observed in all patients. As shown in Tables 1 and 2, in both groups gender proportion was statistically similar ($P > 0.05$).

PBMC from all groups were efficiently induced to secrete cytokines and NO in response to PHA, and the non-stimulated cultures secreted minimal or any levels of all mediators (data not shown). In relation to IFN- γ, PBMC of AD (7742 ± 7884, $P = 0.003$), AT (8129 ± 7934, $P = 0.01$) and SH (9317 ± 10290, $P = 0.001$) presented significant levels of the cytokine in comparison to CT (167.3 ± 196.2). On the other hand, it was not observed statistic difference when comparing patients prior, post treatment and self-healed (Figure 1).

TNF-α production in active disease (310 ± 362.1; $P = 0.007$) and after therapy (358.4 ± 406.1; $P = 0.003$) was significant in comparison to the healthy donors (48.67 ± 77.18). The AT patients ($P = 0.035$) exhibited significant levels of this cytokine when compared to the self-healed group (124.6 ± 197.3).

All groups of patients produced IL-10 in response to AgIns, and significant levels of the cytokine were observed in AD (261.9 ± 220) when comparing with AT (139.1 ± 98.11; $P = 0.03$). In contrast, significant levels of IL-17 were observed after therapy (139.9 ± 54.36) in comparison to the control group (1.77 ± 0.89; $P = 0.04$), as well as in AD patients (37 ± 13.78) in comparison to SH group (5.5 ± 5.5; $P = 0.04$). Concerning IL-22, significant concentrations of the cytokine were observed in AD group (571.8 ± 219.7) in comparison to the healthy subjects (71.4 ± 34.1; $P = 0.02$).

AD patients exhibited higher and significant levels of NO (10.29 ± 15.2, $P = 0.048$) in comparison to AT (1.429 ± 3.48). Both SH (16.36 ± 21.85, $P = 0.04$) and AD groups (10.29 ± 15.72, $P = 0.012$) produced NO in greater levels in relation to CT (1.4 ± 4.3), with statistical difference between groups. SH produced NO with statistical significance ($P = 0.008$) when comparing with AT (Figure 1).

Table 1. Demographic, clinical and therapeutic data of the patients with ATL.

Patient	Age	Gender	Location	Clinical form	N° lesions	Evolution	Local lesions	Lesion size(mm^2)	Cicles
01	53	F	Amaraji	LCL	01	03 months	R leg	12,25	02
02	47	M	Amaraji	LCL	01	03 months	L leg	21	02
03	21	F	Moreno	LCL	01	02 months	Body	32	02
04[a]	25	M	Moreno	LCL	08	01 month	R and L legs/ L hand	2/ 24,5	04
05	41	F	Moreno	LCL	02	08 days	R leg	0,25/ 5	02
06	39	M	Moreno	LCL	01	01 month	L leg	36	02
07	33	F	Moreno	LCL	01	01 months	R leg	24/ 27,5	02
08	52	M	Moreno	LCL	02	02 months	Body/ Shoulder	24,75/ 15	01
09	58	F	Moreno	LCL	01	01 month	R leg	18	01
10	20	M	Chã de Alegria	LCL	02	02 months	R leg	8/ 7,5	02
11	56	M	Araçoiaba	LCL	01	03 months	R leg	39	03
12	28	F	Moreno	LCL	01	15 days	R foot	NI	02
13	26	F	Moreno	LCL	01	02 months	R leg	36	02
14	34	M	Moreno	LCL	01	03 months	L leg	6	02

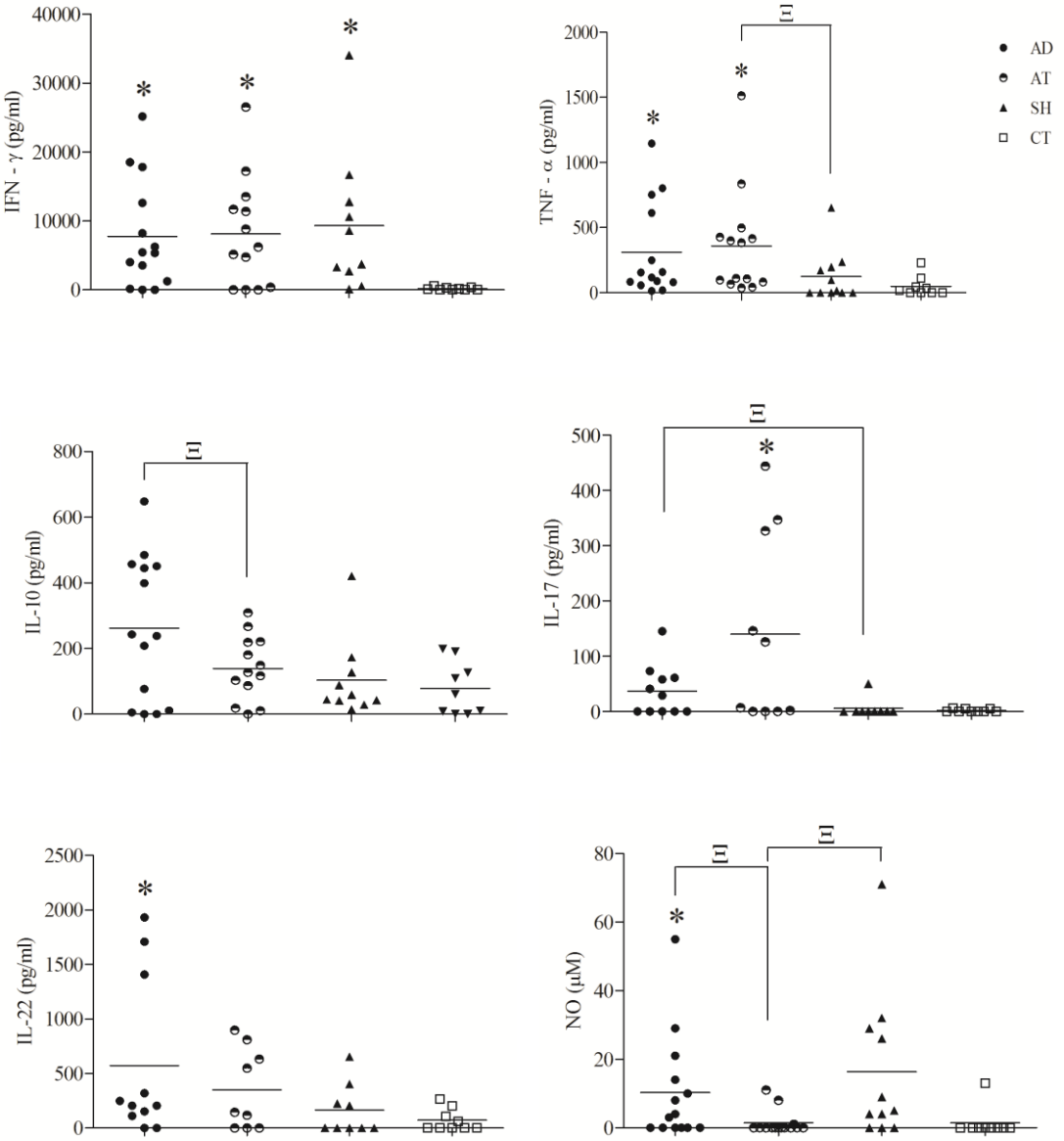
NI = not informed; F = female; M = male; CL = localized cutaneous leishmaniasis; L = left; R = right
[a]Patient presented 01 lesion measuring 24,5 mm^2, and seven others measuring 2mm^2.

Table 2. Demographic and clinical data of the self-healed (SH) patients.

Patient	Age	Gender	Location	Nº scars	Local of scars	Healing time	MST (mm)
01	77	M	Moreno	01	L leg	3 months	12
02	71	M	Moreno	01	L forearm	NI	10
03	81	M	Moreno	01	L leg	15 days	10
04	28	M	Moreno	01	L thigh	3 months	12
05	NI	F	Amaraji	01	L foot	1 month	05
06	47	F	Vicência	04	Neck, L shoulder, R thigh, L leg	9 months	12
07	17	F	Vicência	01	L leg	NI	08
08	30	M	Vicência	01	R foot	4 months	10
09	39	F	Vicência	01	R leg	8 months	07
10	60	F	Moreno	01	L ankle	3 months	10
11	57	F	Moreno	01	L leg	2 months	15

NI = not informed; F = female; M = male; L = left; R = right; MST = Montenegro Skin Test.

Figure 1 :Cytokine and nitric oxide production among patients with active disease (AD), after treatment (AT), spontaneously healed patients (SH) and control group, in response to insoluble antigen. The asterisk represents the significant differences between patients and control group, and the greek letter Ξ (ksi) shows the significances between the groups of patients (P<0,05). The horizontal bars represent the mean of the groups.



4 DISCUSSION

American tegumentary leishmaniasis presents a spectrum of manifestations that ranges from localized skin lesions to the impairment of mucous regions. [18, 19]. In addition, the occurrence of self healing in patients with cutaneous leishmaniasis has been documented in endemic areas of *L. (V.) braziliensis* infection [16, 20, 21]. Considering the importance of the cellular immune response in ATL, and also that few studies on self-healed individuals were developed, this work evaluated cytokine and nitric oxide production in patients with active and post-healed American tegumentary leishmaniasis, with or without chemotherapy.

The immune response in cutaneous leishmaniasis has been assessed by many authors using different antigens [6, 8, 12-14, 40]. In an attempt to evaluate a more specific immune response, we have previously used the soluble antigen of *L. (V.) braziliensis* for PBMC stimulation [15]. We demonstrated that in active disease and after clinical cure, with or without chemotherapy, specific cellular immunity takes part against *Leishmania*, but with some similarities between the clinical states. Sequentially, in this work, we proceeded to evaluate the cellular immunity of these patients after exposure to the insoluble antigen of *L. (V.) braziliensis*, once particulate fractions are known to be constituted by membrane fractions of the parasite [22].

From the increased IFN-γ production observed herein, we can suggest that the patients have established an immune response in order to eliminate the parasite. The levels of the cytokine found after treatment and in self-healed patients tended to be higher than in the active disease, in contrast with the results previously presented by our group [15]. There are evidences that the decreasing in parasite load after the use of antimonials may contribute to the development of a Th1 response, once it is no longer suppressed by the presence of *Leishmania* parasites [23]. Thus, IFN-γ production is maintained post chemotherapy [6, 14, 24]. In addition, although in SH patients the chemotherapy has not been administered, we can suggest that their immune response underwent a modulation, reaching clinical cure [21].

The same increasing IFN-γ levels after clinical cure were previously observed, although the total antigen of *L. (V.) braziliensis* was used for PBMC stimulation [13, 14]. Once this antigen is not submitted to differentiated centrifugation process to separate the membrane components from the cytoplasmic ones, we can suggest that the immunogenicity of the total fraction arises from the existence of portions of membrane of the parasite antigenic preparation [22].

In addition to IFN-γ, TNF-α levels were also measured in this study. Under antigenic stimulation, the treated patients produced significant levels of TNF-α in relation to the spontaneously-healed ones. These results could be explained by the possible influence of antimonial treatment on the secretion of pro-inflammatory cytokines [25, 26]. Furthermore, the different levels of TNF- α observed in these groups may exist due to the genetic constitution of the host. It is known that the presence of polymorphisms in tumor necrosis factor genes contributes to functional differences in the cytokine levels, as it was shown by Cabrera et al. [27]. According to the authors, susceptibility to the mucocutaneous form of disease may be directly associated with regulatory polymorphisms affecting TNF- α production.

In relation to NO, some stable, measurable products come from its decomposition process, such as nitrite (NO_2-). In response to AgIns, the self-healed individuals exhibited significant nitrite levels in relation to the treated patients, as well as a considerable production of pro-inflammatory cytokines. Thus, it is reasonable to suggest that IFN-γ and TNF-α may be necessary to NO production [28, 29, 30]. Nevertheless, nitrite levels exhibited after therapy were rare, despite the great concentration of IFN-γ and TNF-α secreted by these patients. Once the nitric oxide production tends to be elevated by influence of antimonials [31], we believe that the reduction observed after treatment occurs by influence of the decreased parasite load, as well as due to the period of re-evaluation of the patients, which occurred a year after the end of the treatment.

Besides the benefits of Th1 response, Gollob et al. [32] suggest that IL-10 production may be critical for the disease control. Thus, it is postulated that IL-10 levels presented by the patients in this study regulate the production of pro-inflammatory cytokines, consequently leading to the clinical resolution of the disease. However, studies point to the reminiscence of parasites after the wound healing [33], and IL-10 seems to play a role in this event [34]. Thus, it is constantly discussed whether a patient who has achieved clinical cure remains healthy due to a memory immune response or by the existence of constant stimulation of the immune system by *Leishmania* antigens present in the individual (concomitant immunity). Amato et al. [35], who observed the persistence of T lymphocytes in lesions of healed patients, suggest that this event may work as a defense mechanism against reinfection in regions endemic for leishmaniasis.

Concerning IL-17, this cytokine has been increasingly studied in protozoal, fungal and bacterial infections in mice [36], but the role of this cytokine in human infections, especially in leishmaniasis, is still undefined. Herein, the significant levels of IL-17 observed in AD

patients in comparison to SH could be explained by the proinflammatory role of this cytokine. In a previous study, Bacellar et al. [8] verified that in lesions of cutaneous and mucosal leishmaniasis patients, the intensity of the inflammatory infiltration is directly correlated to the number of cells expressing this cytokine. In addition, a decreased quantity of IL-17 exhibited by SH patients under antigenic stimulus may be, at least in part, due to a suppressive effect exerted by NO levels, which impair the polarization and the stabilization of IL-17-producing cells [37].

The inverse situation seems to occur with the treated patients, which exhibited a considerable quantity of IL-17 and low levels of NO. Similar behavior was observed in the production of TNF-α in these groups of patients, although it was not possible to evaluate the existence of a direct correlation between the secretion of IL-17 and TNF-α. In contrast, Bacellar et al. [8] observed that the production of these two cytokines were directly proportional in response to *Leishmania* infection. Due to the different counterbalance among TNF-α, IL-17 and NO observed after therapy and in self-healed patients, both resulting in clinical cure, we can suggest that the chemotherapy process can exert some influence in the immune response.

Another cytokine evaluated in this study was IL-22, which belongs to the IL-10 family [38]. IL-22 levels were observed in all groups of patients, being significant in the AD group in comparison to the healthy donors. As previously seen [15], the cytokine was observed in SH patients, but not IL-17. As it happens to IL-17, little is known about the role of IL-22 in *Leishmania* infections, although protective and harmful roles are attributed for this cytokine. These mechanisms may occur under influence of IL-17, which absence or presence seems to govern the proinflammatory versus tissue-protective properties of IL-22 [39]. In contrast, Pitta et al. [11] verified an increasing in IL-22 and also IL-17 production in patients who presented resistance to kala azar. According to the authors, IL-22 and IL-17, along with Th1 cytokines, are supposed to play complementary roles in human protection against the disease. Moreover, the production of these cytokines may point the presence of Th17 profile in the defense against the parasite. However, more investigation about the cytokines and their producing cells from this profile is needed to better understand its role in the pathogenesis of leishmaniasis.

From the data herein demonstrated, we can postulate that the patients with active disease and after clinical cure, with or without chemotherapy, exhibit specific cellular immune response profiles, but with some similarities between them, as it previously occurred using other antigen [15]. IFN-γ production by the three groups of patients suggests an influent

inflammatory response against the parasite, as well as in relation to TNF-α. The secretion of TNF-α showed a tendency to be higher after treatment, maybe by influence of pentavalent antimonials. Moreover, IL-17 and IL-22 may also contribute to mount an effective response against the parasite. The former seems to be regulated by NO levels, which is known to be regulated by cytokines, and this mechanism may be important to enable the self-healing process. None the less, the presence of IL-10 suggests the induction of immunomodulatory mechanisms necessary for the establishment of an effective immune response against *Leishmania*.

Thus, we can suggest that specific cellular immunity takes part against *Leishmania*, but with some similarities between the different clinical states herein described. Thus, the results suggest that the mediators assessed are necessary for the cure to occur.

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5.3 Artigo 3. *"Th1 and Treg mediators are predominantly expressed in patients with recent active American tegumentary leishmaniasis"* (submetido à revista *Journal of Interferon & Cytokine Research*)

Th1 and Treg mediators are predominantly expressed in patients with recent active American tegumentary leishmaniasis

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Running title: Th1 and Treg responses predominate in active ATL.

ABSTRACT

Besides the Th1 x Th2 paradigm, Treg and Th17 cytokines may play a role in the response to American tegumentary leishmaniasis. Considering the sensitivity and accuracy of qPCR and the lack of studies using this approach, we evaluated mRNA expression for IFN-γ, TNF-α, IL-4, IL-10, IL-6, IL-17A, IL-22, TGF-β, iNOS, Foxp3 and RORC in peripheral blood mononuclear cells (PBMC) from patients with active disease, after stimulation with *L. (V.) braziliensis* soluble or insoluble fractions. Our results show that both antigens promoted specific mRNA expression related to the immune response in patients with ATL, with a major presence of transcripts for IFN-γ. The pro-inflammatory response was also fueled by TNF-α, probably due to the recent evolution of the disease. IL-4, in certain way, seems to regulate this response along with IL-10 that may be produced by Treg cells, which are supposedly present in the patients' samples due the evidenced expression of Foxp3, in the presence of AgIns. In

contrast, down-regulated RORC suggests that the significant levels of IL-6 expressed in response to AgSol were not able to induce an expressive Th17 profile along with TGF-β, which might have predominantly contributed to the development of a regulatory profile in the active disease.

1 INTRODUCTION

Leishmaniasis represents a complex of diseases with important clinical manifestations and epidemiological diversity. American tegumentary leishmaniasis (ATL) is widely distributed in Brasil, and 30% of the registered cases occurred in the Northeast region from 2000 to 2008 (Brasil 2010). ATL is endemic in Pernambuco state, where 60% of the cases take place at areas of forest (Zona da Mata), and it is estimated that the number of affected people has increased in the last ten years (Brito and others 2009; 2012).

In humans, the spectrum of the disease may vary from subclinical infection to manifestations such as localized, disseminated or diffuse cutaneous lesions, or even the involvement of the nasal mucosa, pharynx and larynx. In all these possibilities, the expansion of T helper cells exerts a fundamental influence (Gollob and others 2008). Classically, a Th1 profile is responsible for producing interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), which activates the inducible nitric-oxide synthase (iNOS) to catalyze NO secretion by macrophages, eliminating the parasite. On the other hand, cytokines such as IL-4 and IL-10, secreted by Th2 cells, are known to favor the survival of the parasite (Reis and others 2013; Assis Souza and others 2012).

For more than two decades, the Th1 x Th2 paradigm is used to justify the majority of the events related to adaptive immunity. However, the role of other cytokines in leishmaniasis is being currently explored. IL-17 and IL-22, mainly produced by Th17 cells, were detected both in active ATL and after clinical cure (Assis Souza and others 2012; De Assis Souza and others 2013), as well as in resistant individuals to Kala-Azar (Pitta and others 2009). The production of these cytokines may suggest the presence of Th17 cells, which differentiate under influence of interleukin (IL-) 6 and transforming growth factor-beta (TGF-β). Together, these two cytokines activates the related orphan receptor C (RORC) transcription factor, a keystone for Th17 development (Kimura and Kishimoto 2010).

In addition to Th17, studies suggest that the regulatory T cells (T_{reg}) are supposed to maintain the immunological balance during the infection (Campanelli and others 2006; Carneiro and others 2009; Chen and others 2010; Rodrigues and others 2013). It seems that there is a dichotomy between these subpopulations, where the activation of one or another

depends on the levels of IL-6 and TGF-β in the microenvironment (Bettelli and others 2006; 2008; Basso and others 2009). The latter cytokine is determinant for T_{reg} differentiation. The transcription factor forkhead box p3 (Foxp3) is present in natural T_{reg} cells (nT_{reg}) and in some induced T_{reg} cells (iT_{reg}), and all of them are able to produce IL-10 (Nylén and Gautam 2010). Initially related to Th2 profile, IL-10 seems also to exert a necessary counterbalance on the immune response (Carneiro and others, 2009; Rodrigues and others, 2013; Antonelli and others, 2004; Costa and others, 2013).

Studies on T cell-mediated immunity against ATL are necessary due to the need of new approaches concerning treatment and prophylaxis, and the search for new antigenic molecules may enable the development of such alternatives. Thus, this response has been evaluated by our group in the presence of antigenic fractions of *L (V.) braziliensis* by cytokine and NO measurement and cell immunophenotyping and in patients with different clinical states (Assis Souza and others, 2012; De Assis Souza and others, 2013; Brelaz de Castro and others, 2012). However, gene expression analysis by quantitative polymerase chain reaction concerning the immunological mediators involved in human ATL is not frequently performed, and even less regarding Th17 and Treg components. Considering the sensitivity and accuracy of qPCR and the lack of studies using this approach, we study evaluated mRNA expression for IFN-γ, TNF-α, iNOS, IL-4, IL-10, IL-6, TGF-β, Foxp3, IL-17A, IL-22 and RORC in peripheral blood mononuclear cells (PBMC) from patients with active disease, after stimulation with *L. (V.) braziliensis* soluble or insoluble antigens.

2 MATERIAL AND METHODS

2.1 Study population

Individuals of both gender and older than 12 years old were selected from the municipality of Moreno, an endemic area for ATL. Twenty-three patients with active disease (AD) were chosen based on criteria such as: presence of cutaneous lesions, confirmed diagnosis and no previous chemotherapy treatment. Six healthy individuals represented the control group (CT) from non-endemic areas and without previous ATL infection. All individuals signed the "Term of Free and Informed Consent" and CPqAM/Fiocruz Research Ethics Committee (Protocol No. 123/08) approved the experimental protocols.

2.2 Soluble and Insoluble antigen of L. (V.) braziliensis

As described by Brito and others (2000), promastigote forms of *L. (V.) braziliensis* (MHOM/BR/75/M2903), cultured in vitro, were expanded in Schneider's medium (Sigma, St. Louis, MO) supplemented with 10% of fetal calf serum (Cultilab) and 1% of antibiotics (100UI/ml penicillin and 100 mg/ml streptomycin; Sigma) until they reached the exponential phase. Afterwards, they were sedimented by centrifugation at 800 x g for 15min at 4ºC and three times washed with phosphate-buffered saline (PBS; pH 7.2). Proteases inhibitors such as 0.1mM methyl-phenyl-fluoride and 2mM ethylenediaminotetraacetic acid (Sigma), pepstatine A 0.001M (Sigma) were added and, right after, the parasites were ultrasonicated. The parasitic suspension was centrifuged at 10.000 x g for 10min at 4ºC. The resultant supernatant was removed and submitted to a new centrifugation at 100.000 x g for 1 hr at the same temperature. Protein concentration was determined from the new supernatant (soluble antigen, AgSol) and the resultant pellet (insoluble antigen, AgIns), which were lately stored at -20ºC for further use.

2.3 Cell Culture

PBMC obtained from venous blood (10^6 cells/ml) were cultured (37ºC/5% CO_2) for 24 hours in 24-well plates (TPP, Switzerland) containing RPMI medium enriched with 1% L-glutamin 200 mM, 1% sodium piruvate 100 mM, 0,2% de sodium bicarbonate 7,5% and 1% antibiotics (penicilin 100 UI/ml e streptomycin 100 mg/ml) and supplemented with com 10% fetal calf serum (Cultilab). AgSol (1,25 μg/ml) or AgIns (2,5 μg/ml) of *L. (V.) braziliensis* was added to wells. Cells cultivated with with phytohemagglutinin mitogen (PHA; 5,0 μg/ml) were the positive control of the assay. PBMC only in the presence of the culture medium were used as the negative control. After incubation, the plates were centrifuged (1800 x g for 10 minutes, at RT) and the cells were stored in TRIzol solution (Life Technologies) at -80ºC.

2.4 RNA isolation and cDNA synthesis

Total RNA was isolated from PBMC according to TRIzol manufacturer's recommendations. RNA purity was verified by agarose gel electrophoresis followed by ethidium bromide staining. $OD_{260/280}$ determination (above 1.8) and RNA was quantified using a NanoDrop™ Spectrophotometer (Thermo Scientific, Waltham, Massachusetts) and the RNA was kept at -80°C. The quantities of the samples were adjusted to 100ng before performing the Reverse Transcription PCR reaction (RT) with the "TaqMan® Reverse Transcription Reagents" kit (Applied Biosystems, Foster City, CA). The reactions were

carried out in a final volume of 50µl with TaqMan RT Buffer 1x, $MgCl_2$ 5.5mM, dNTPs mix 500µM, randomic hexamers 2.5µM, RNAse inhibitor 0.4 U/µL, *multiscribe reverse transcriptase* enzyme 3.125 U/µL and 12.375µL of the sample. The cycling conditions were 10 minutes at 25ºC, 30 minutes at 48 ºC and 5 minutes at 95 ºC. Negative controls were included in each run. After this stage, samples were stored at -20 ºC until use in real time quantitative PCR assays (qPCR).

2.5 Gene expression of immunological mediators

mRNA expression for the target genes IFN-γ (Hs99999041_m1), IL-10 (Hs99999035_m1), TNF (Hs00174128_m1), IL-4 (Hs99999030_m1), iNOS (Hs00167257_m1), IL-6 (Hs00985639_m1), TGF-β (Hs99999918_m1), IL-17 (Hs00174383_m1), IL-22 (Hs01574154_m1), Foxp3 (Hs00203958_m1), and RORC (Hs01076122_m1) was performed using ABI PRISM 7500 Sequence Detection System (Applied Biosystems). Singleplex reactions were made in a final volume of 20µL, with TaqMan Universal PCR Master Mix (2x), TaqMan Gene Expression Assay for the target genes (constituted of specific primers and probes labeled with FAM), RNAse free water and 1µL of sample. At the same time, 18S ribosomal RNA was the reference gene, with the reaction prepared with TaqMan Universal PCR Master Mix, forward and reverse specific primers, specific probe labeled with VIC-MGB, RNAse free water and1µL of sample. mRNA expression for the target genes in response to antigenic stimulation was calculated by the comparative Ct method ($2^{-\Delta\Delta Ct}$).

2.6 Statistical analysis

The data (ΔCt) were analyzed using nonparametric tests. To detect differences between groups the Mann-Whitney U-test was used. The results were considered significant when $P < 0,05$.

3 RESULTS

All patients presented ulcerated skin lesions with raised borders and granulomatous bottom, distributed mostly by uncovered areas of the body. The disease evolution time, calculated from the lesion appearance until the patient visits the health surveillance service in the municipal districts, varied from ten days to three months, as shown in Table 1.

PBMC from both groups successfully expressed similar levels of mRNA for all the mediators in response to PHA with exception of iNOS, which mRNA was not detected; cells maintained only in medium showed a basal mRNA expression for the mediators (data not shown). In response to the soluble antigen (Figure 1), we observed that IFN-γ (RQ = 25.6; P = 0.017), TNF-α (RQ = 4.4; P = 0.0306), IL-10 (RQ = 7.13; P = 0.0119) and IL-6 (RQ = 3.13; P = 0.0189) were significantly more expressed in patients. They also expressed more mRNA for Foxp3 (RQ = 7.11), TGF-β (RQ = 2.35) and IL-22 (RQ = 2.81) in relation to control group, although without statistical significance (P > 0.05). IL-4 expression was very similar between the groups (RQ = 0.17; P > 0.05). mRNA levels for iNOS and IL-17 were not detected and RORC expression was slightly under-expressed in patients (RQ = -1.09; P > 0.05).

In relation to the insoluble antigen (Figure 2), we observed that IFN-γ was more than 80-fold expressed in patients (P = 0,0004). The expression of TNF-α (RQ = 8,58; P = 0,027), IL-10 (RQ = 8.26; P = 0.0114), and Foxp3 (RQ = 14.2; P = 0.0208) was also significantly higher in individuals with active disease than in the control group. IL-4 (RQ = 25.0), IL-6 (RQ = 1.56), TGF-β (RQ = 2.06), IL-17 (RQ = 3.66) and IL-22 (RQ = 4.58) were also more expressed by the patients, but there was not statistical significance between the groups (P > 0.05). In contrast, RORC expression was also under-expressed in patients (RQ = -1.66; P > 0.05).

Table 1. Demographic, clinical and therapeutic data of the patients with ATL.

Patient	Age	Gender	Location	Clinical form	N° lesions	Progression	Local lesions	Lesion size(mm^2)
01	42	M	Moreno	LCL	02	1 month	L knee; R arm	18; 4
02	28	F	Moreno	LCL	01	1 month	R ankle	15
03	32	F	Moreno	LCL	01	2 months	R leg	60
04	12	M	Moreno	LCL	03	10 days	R elbow; L leg; L foot	10.5; 64; 10.5
05	66	F	Moreno	LCL	01	1 month	R leg	50
06	73	M	Moreno	LCL	01	1 month	L ankle	4
07	74	M	Moreno	LCL	01	3 months	R arm	80
08	57	F	Moreno	LCL	01	1 month	R foot	15
09	19	F	Moreno	LCL	01	1 month	R supraorbital region	6
10	35	M	Moreno	LCL	02	2 months	R and L legs	39; 9
11	24	F	Moreno	LCL	02	1 month	L leg	7; 15
12	47	M	Moreno	LCL	01	2 months	L elbow	13.5
13	21	M	Moreno	LCL	01	2 months	L leg	35
14	16	M	Moreno	LCL	02	3 months	Face; L arm	4; NI
15	20	M	Moreno	LCL	02	1.5 month	R thigh	5; 4
16	43	F	Recife	LCL	01	2 months	L ankle	12
17	26	F	Moreno	LCL	01	2 months	L leg 4x3 cm	12
18	13	F	Moreno	LCL	01	3 months	L knee	15
19	21	F	Moreno	LCL	01	1.5month	L thigh	25
20	28	F	Moreno	LCL	01	1 month	R ankle	3
21	22	F	Moreno	LCL	01	2 months	Forearm	12
22	15	F	Moreno	LCL	04	1 month	R arm; L leg	5; 8
23	43	F	J. Guararapes	LCL	02	0.5 month	L fist; L arm	4.5; 3

Notes: NI = not informed; F = female; M = male; LCL = localized cutaneous leishmaniasis; L = left; R = right

Figure 1: mRNA expression for Th1, Th2, Th17 and Treg mediators in response to *L. (V.) braziliensis* soluble antigen. Relative quantity values (RQ) obtained after normalization with the reference gene and subsequent application of the formula $2^{-\Delta\Delta Ct}$, with the control group as the calibrator. * = statistical significance.

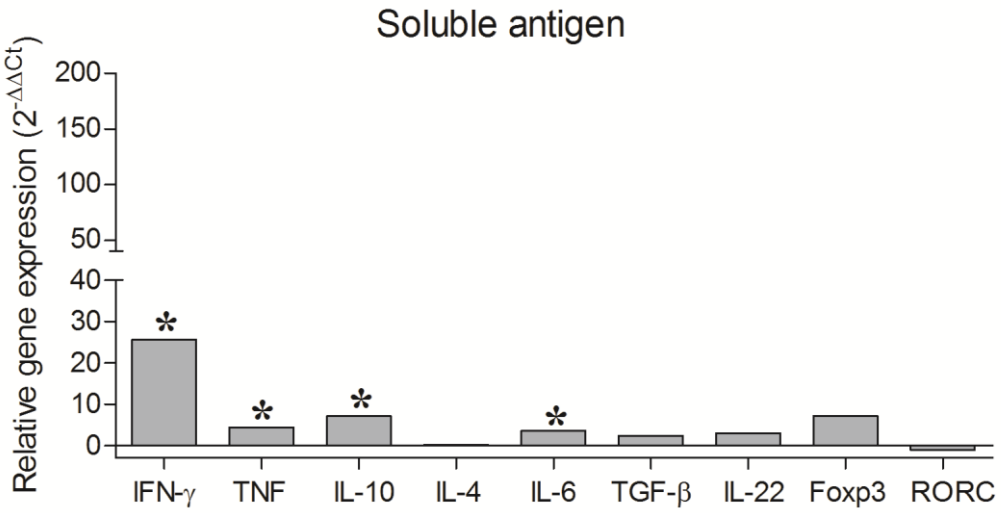
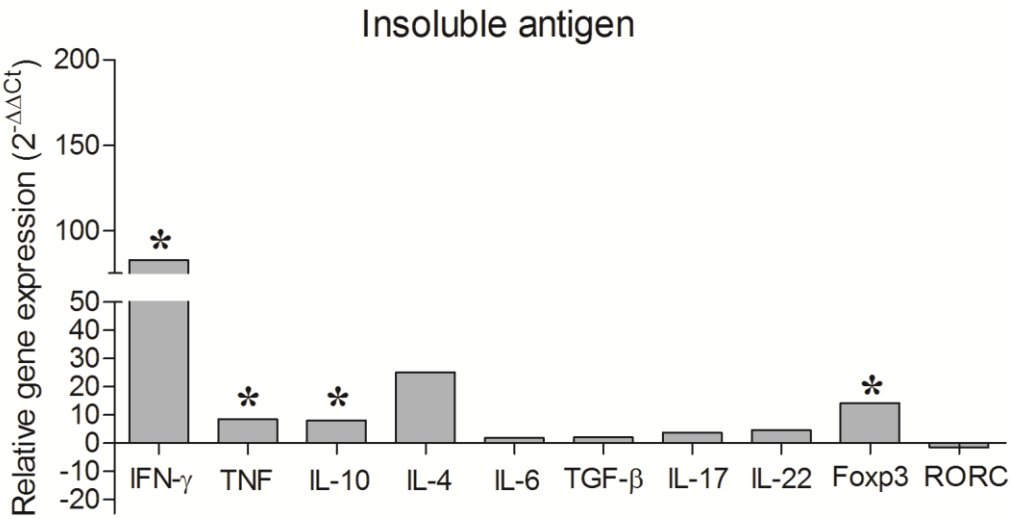


Figure 2: mRNA expression for Th1, Th2, Th17 and Treg mediators in response to *L. (V.) braziliensis* insoluble antigen. Relative quantity values (RQ) obtained after normalization with the reference gene and subsequent application of the formula $2^{-\Delta\Delta Ct}$, with the control group as the calibrator. * = statistical significance.



4 DISCUSSION

In American tegumentary leishmaniasis the involvement of cellular immunity is critical, where Th1 and Th2 cytokines are the main actors (Reis and others, 2006). However, T cells subtypes such as Th17 and Treg are suggested to take part in this event, although the exact role of each is not well established (Campanelli and others, 2006; Rodrigues and others, 2013; Bacellar and others, 2009). Taken these aspects, we evaluated mRNA expression of Th1, Th2, Th17 and Treg mediators in PBMC from patients with active ATL in response to *L. (V.) braziliensis* soluble and insoluble antigen.

The comprehension of how the host responds to the parasite is a critical step to create alternative treatments, such as immunotherapeutic or prophylactic schemes. For this purpose, total antigens from the promastigote forms of *L. (V.) braziliensis* are used to induce cellular immune response *in vitro* (Antonelli and others, 2004). However, due to the protein diversity in this antigenic preparation, it may generate discrepant responses. Thus, here and in previous studies of our group, we utilized the soluble and insoluble antigens of *L. (V.) braziliensis* obtained by ultra-sonication (Assis Souza and others, 2012; De Assis Souza and others, 2013; Brelaz-de-Castro and others, 2012; Brelaz and others, 2012) in an attempt to generate a more specific response.

In *Leishmania* infections, the presence of IFN-γ is critical to establish and maintain a protective Th1 response that controls infection (Reis and others, 2006). In this study, IFN-γ expression was significantly up-regulated in the patients evaluated in response to the antigens. The majority of these individuals presented lesions less than 60 days old, which suggests a pro-inflammatory expression profile during the initial phase of the disease. Similarly, high IFN-γ secreted levels were verified using capture ELISA in recent active disease (Assis Souza and others, 2012; De Assis Souza and others, 2013), and its mRNA was also expressed in biopsy samples of active LCL lesions (Cáceres-Dittmar and others, 1993). In contrast, our group previously observed by flow cytometry low IFN-γ levels and a predominance of IL-10 and IL-4 after 48h of PBMC incubation during the first 60 days of active disease (Brelaz and others, 2012). The different period of PBMC incubation in the present study (24h) may contribute to obtain different results. Also, as qPCR quantifies transcribed mRNAs but not their cellular origin (Sachdeva and Ashtana, 2009), we may probably be detecting mRNAs from lymphocytes and other immune cells, such as monocytes, which are known to contribute to IFN-γ expression (Carvalho and others, 2012).

The Th1 response seen in active ATL is also ensured by TNF-α, which plays an essential role during LCL development. Herein, the antigens promoted significant TNF-α expression in the patients in relation to the control group. We previously observed significant production of TNF-α in PBMC supernatants from patients with recent disease using capture ELISA (Assis Souza and others, 2012; De Assis Souza and others, 2013). Also according to these previous works, this cytokine seems to contribute to the achievement of clinical cure after chemotherapy, and it was also suggested by Brelaz and others (2012). Conversely, the involvement of TNF-α in longer-lasting lesions was also documented (Bacellar and others, 2009), what suggests that the different levels of TNF-α observed here and in other studies may exist due to the genetic constitution of the host. According to Cabrera and others (1995), susceptibility to the severe mucocutaneous form of the disease may be directly associated with regulatory polymorphisms affecting TNF-α production.

Along with IFN-γ, TNF-α promotes nitric oxide synthesis via catalysis by the inducible nitric oxide synthase (iNOS). Strikingly, mRNA for this enzyme was not detected in the samples herein analyzed. According to Bogdan (2001), a negative-feedback event caused by high NO concentrations in the culture may be responsible for the non-expression of iNOS, and that may be useful to impair the exacerbation of Th1 response.

Aiming its survival in the host, *Leishmania* is capable of polarizing the immunity to a Th2 axis (Reis and others, 2009). In this context, IL-4 works as the main cytokine produced in response to the parasite, and promotes the differentiation of Th2 cells by activation of GATA-3 transcription factor (Souza and others, 2013). Here, we observed an up-regulation of IL-4 transcripts in patients in response to AgIns, but even so, they appeared to express a predominant Th1 profile at the moment of the evaluation. In contrast, IL-4 secretion was shown to be dominant in the first 60 days of lesion existence (Brelaz-de-Castro and others, 2012; Brelaz and others, 2012; Maurer and others, 2009). It seems that, at the moment of the evaluation, lower mRNA levels were detected due to the rapid processing of this material, including its translation into actual IL-4 cytokine.

When evaluating IL-10 expression in response to the antigens, the patients presented more mRNA than the healthy individuals. The initial role of IL-10 was to favor Th2 proliferation during *Leishmania* infection. However, the role of this cytokine has grown in complexity, once it may represent a necessary counterbalance to achieve the resolution of the disease (Gollob and others, 2008; Belkaid and others, 2006). In this context, regulatory T cells are critical, once they were previously related to immunomodulatory mechanisms in active leishmaniasis through IL-10 secretion (Campanelli and others, 2006; Rodrigues and others,

2013, Salhi and others, 2010). Thus, in order to endorse these informations, we also measured mRNA for Foxp3, the transcription factor to Treg cells (Campanelli and others, 2006; Souza and others, 2013). We observed a expression of this mediator in patients, being statistically significant in response to AgIns, and this result suggests that they play a role in active disease, which may include IL-10 production in response to antigenic stimulus.

In addition to IL-10, the functions of regulatory T cells are also mediated by TGF-β (Sakaguchi, 2010; O'Garra and Vieira, 2004), which expression was also measured in this study. The mRNA levels presented by the patients against both antigens were higher than the healthy individuals, although statistically similar, as shown in Figure 1. TGF-β may promote parasite survival within macrophages by suppressing the cells' oxidative function, thereby possibly playing a role in the immunopathogenesis of chronic cutaneous leishmaniasis (Reed, 1999). mRNA for TGF-β was once detected in late lesions (>120 days of evolution) of patients with ATL (Melby and others, 1994). Recently, Rodrigues and others (2013) used immunohistochemistry to detect TGF-β in lesions of active ATL patients, and the levels of the cytokine tended to be higher in the later phase of infection (>60 days of lesion duration). Considering this, we suggest that the incidence of recent lesions in our patients may be the reason why we did not observe a high relative quantity for TGF-β. Even so, we reiterate the possible acting of Treg cells in the immune response of our patients, given the significant levels of Foxp3 herein found.

Some authors describe a reciprocal relationship between Treg and Th17 cells (Bettelli and others, 2008; Crome and others, 1994; Ganjalikhani Hakemi and others, 2011). The latter subtype, initially related to autoimmune disorders and allergen-specific responses, is now being studied in *Leishmania* infections (Pitta and others, 2009; Bacellar and others, 2009; Boaventura and others, 2010). Interestingly, our results seem to confirm such reciprocity, once RORC (the human orthologue of RORγt), needed for Th17 activation, was found to be slightly down-regulated in patients. According to Jetten (2009), the balance between the expression of RORC and Foxp3 plays a critical role in determining whether uncommitted CD4+ T helper cells differentiate into Treg or Th17 cells. Also, the balance between TGF-β and IL-6 is important in this point. Together, they are known to be responsible for the activation of RORC (Kimura and Kishimoto, 2010). However, even being detected in our patients and significantly expressed in response to AgSol, the levels of IL-6 appeared not to be sufficient to convert the immune response into a Th17 predominant profile. This is likely to be due the presence of IL-10, which is capable of suppressing Th17 differentiation and the expression of RORC (Gu and others, 2008).

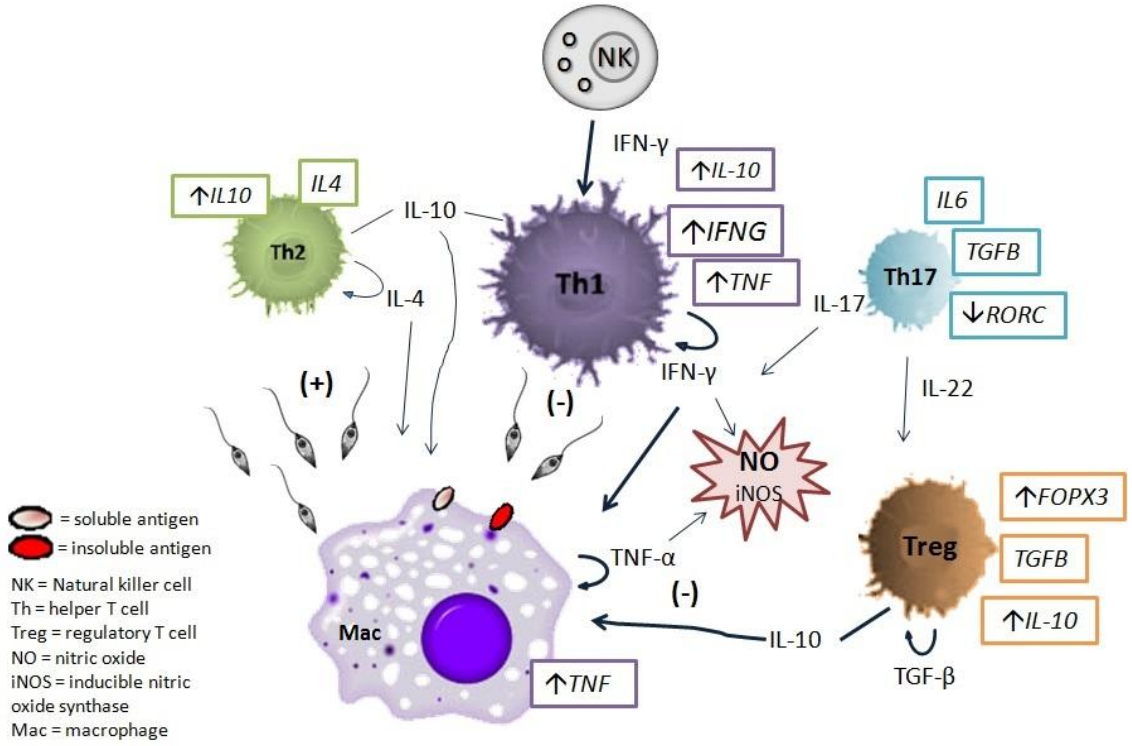
The Th17 profile has IL-17A (or IL-17) as the main effector cytokine, which has been largely studied in protozoal, fungal and bacterial infections in mice (Matsuzaki and Umemura, 2007). In humans, it has been related to host defense to bacteria, fungi and viruses, and a possible pathogenic role in rheumatoid arthritis is also suggested (Melby and others, 1994). Concerning leishmaniasis, the exact role of IL-17 is also a matter of debate. Herein, the expression of the cytokine in response to AgIns was slightly up-regulated in patients, without statistical difference in relation to the control. This may be due the presence of IL-10 in the microenvironment, which is capable of impairing IL-17 expression (Boaventura and others, 2010). Previous study of our laboratory verified secreted levels IL-17 in PBMC from ATL patients stimulated by both antigens, but at the same time, IL-10 was produced (Assis Souza and others, 2012), reiterating the possible influence of immunomodulation by IL-10. In contrast, Bacellar and others (2009) verified that in lesions of cutaneous and mucosal leishmaniasis patients, the intensity of the inflammatory infiltration is directly correlated to the number of cells expressing IL-17. Boaventura et al (2010) also detected high levels of IL-17 in mucosal lesions secreted by CD4+, CD8+ and CD14+ cells, suggesting that they participate in the inflammatory response in mucocutaneous leishmaniasis.

In this study, the expression of IL-22 was also evaluated, and we observed an increased expression of the cytokine in ATL patients, but without statistical difference in relation to the control group. Similar to IL-17, the role of IL-22 in leishmaniasis is still undefined. Harmful and protective roles have been attributed to this cytokine, and it is suggested that this variation is influenced by the presence or absence of IL-17 in the microenvironment (Sonnenberg and others, 2010). Pitta and others (2009) verified an increasing in IL-22 and also IL-17 production in patients who presented resistance to kala-azar. According to the authors, IL-17, IL-22 and Th1 cytokines are supposed to play complementary roles in human protection against the disease.

From the observations herein presented, it is possible to conclude that the soluble and insoluble antigen specifically stimulated mRNA expression related to the cellular immune response in individuals with ATL. As shown in Figure 3, we observed a major presence of transcripts for IFN-γ. The pro-inflammatory response was also fueled by TNF-α, but in lower levels probably due to the recent evolution of the disease. IL-4, in certain way, seems to regulate this response along with IL-10 that may be produced by Treg cells, which are supposedly present in the patients' samples due the evidenced expression of Foxp3. In contrast, the down-regulation of RORC suggests that the levels of IL-6 were not able to

induce an expressive Th17 profile along with TGF-β, which might have predominantly contributed to the development of a regulatory profile.

Figure 3: gene expression of mediators involved in the immune response of patients with active ATL elicited by *L. (V.) braziliensis* soluble and insoluble antigens.



The informations provided in this study are useful to better understand the immune response in active disease, and further analyses will be performed including patients in different clinical status. In addition, given the specific immune response elicited by *L. (V.) braziliensis* soluble and insoluble antigens, they should be an option to the search of antigenic molecules to be used in immunological therapy and prophylaxis.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist

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6 CONCLUSÕES

a) Na LTA ativa e após cura clínica, com ou sem quimioterapia, há a participação da imunidade celular específica, embora com algumas similaridades entre os estados clínicos estudados;

b) A secreção de IFN-γ sugere a atuação de uma resposta inflamatória contra o parasito, e isto é também apoiado pela presença de TNF-α. Esta última parece ser importante no processo de cura após o tratamento. Na LTA ativa, a expressão de ambas as citocinas reforça este resultado;

c) Os baixos níveis de óxido nítrico encontrados em pacientes tratados sugerem a presença de um mecanismo de compensação em favor do equilíbrio imunológico, que parece ser necessário para alcançar a cura clínica. Mecanismo similar parece acontecer na LTA ativa, onde a ausência de mRNA para a óxido nítrico sintase-induzível parece ser devido à presença de NO secretado, o que pode contribuir para evitar uma excessiva resposta pró-inflamatória;

d) A presença de IL-10 sugere a indução de mecanismos imunomodulatórios necessários para o estabelecimento de uma resposta imune efetiva contra a *Leishmania*. Isto é reforçado pela presença de transcritos de mRNA para a citocina. Além disso, a expressão de Foxp3 indica a atuação de células T regulatórias na resposta de pacientes com lesão ativa;

e) IL-17 e IL-22 parecem ser importantes no estabelecimento de uma resposta imune efetiva na LTA. IL-17 parece ser regulada pelo NO, e este mecanismo pode ser importante no processo de cura espontânea. Porém, os níveis de mRNA para essas citocinas não foram expressivos;

f) A expressão não significativa de RORC sugere que os níveis de IL-6 expressos pelos pacientes com lesão ativa parecem não ter sido suficientes para induzir significativamente um perfil Th17 juntamente com TGF-β. Esta última pode ter contribuído para a regulação da resposta imune pelas células T regulatórias, o que reforça a suposta relação de antagonismo entre os perfis Th17 e Treg.

g) A expressão/secreção desses mediadores endossa o envolvimento da resposta imune celular contra a LTA ativa ou após cura clínica, e traz informações potencialmente úteis para o desenvolvimento de esquemas imunoterapêuticos e vacinas.

7 PERSPECTIVAS

a) Diante da influência da resposta imune do hospedeiro sobre o resultado da infecção, os subtipos Th1, Th2, Th17 e Treg e seus mediadores serão avaliados em pacientes com diferentes estados clínicos (cura espontânea, pós-quimioterapia, resistente ao tratamento, infecção assintomática) utilizando diferentes abordagens metodológicas (citometria de fluxo, ELISA, qPCR). Além disso, as investigações em torno da possível dicotomia existente entre os perfis Th17 e Treg serão aprofundadas;

b) Uma vez que a regulação pós-transcricional realizada por MicroRNAs é um importante alvo de patógenos, é valido questionar se este evento acontece na leishmaniose. Portanto, outra perspectiva será avaliar a possível influência de *L (V.) braziliensis* sobre a expressão de MicroRNAs na infecção humana.

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APÊNDICE A - TCLE Grupo Paciente

Fundação Oswaldo Cruz, Ministério da Saúde

Centro de Pesquisas
AGGEU MAGALHÃES

FIOCRUZ

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – Grupo Paciente

Projeto: "Caracterização da resposta imune celular em pacientes portadores de Leishmaniose Tegumentar Americana ativa e após a cura clínica".

Eu, ..., concordo em participar como voluntário neste projeto que será desenvolvido no Centro de Pesquisas Aggeu Magalhães da Fundação Oswaldo Cruz (CPqAM/FIOCRUZ). Fui informado que o objetivo principal do referido projeto é a investigação da resposta imunológica dos pacientes com leishmaniose tegumentar ativa, e após a cura clínica espontânea ou após tratamento quimioterápico.

Como faço parte do grupo de pacientes, serei submetido ao teste de Montenegro, que é uma injeção sob a pele e que poderá causar ou não, após dois dias, o aparecimento de um "caroço" no local. Passarei também por exames como pesquisa direta, punção aspirativa e biópsia da borda da lesão, cuja coleta do material para realizá-los pode causar um pouco de dor ou desconforto. Serei então submetido a coleta de sangue venoso em volume equivalente a quatro colheres de sopa antes e após tratamento quimioterápico ou após cura clínica espontânea. Todo procedimento será realizado com material estéril descartável e por profissionais de saúde de reconhecida capacidade para executar os procedimentos, podendo ser considerado isento de riscos. Vale ressaltar que todas as informações e os detalhes dos exames que serão realizados serão esclarecidos para mim.

Esse trabalho trará grande benefício, pois indicará se componentes do sistema imunológico poderão ser usados como marcadores da resposta terapêutica e se outras células do sistema imunológico participam na evolução clínica de pacientes com leishmaniose tegumentar americana em Pernambuco. Antes de minha participação no referido projeto, fui incentivado a pedir esclarecimento adicional que julgasse necessário, esclarecido por um participante do projeto. Estou ciente que poderei recusar ou retirar meu consentimento, em qualquer momento da investigação, sem qualquer punição ou prejuízo.

Autorizo a Fundação Oswaldo Cruz (CPqAM/FIOCRUZ) a utilização das informações obtidas através dos resultados dos procedimentos em reuniões, congressos e publicações científicas preservando, neste caso, a minha identidade. Autorizo, também que o CPqAM/FIOCRUZ poderá estocar amostra biológica para posteriores estudos.

Estou ciente que este documento é feito em duas vias, ficando uma em posse do participante e a outra com a equipe.

_____ _____

Assinatura do paciente **data**

_____ _____

Assinatura do responsável **data**

_____ _____

Assinatura do médico responsável – CPqAM/FIOCRUZ **data**

Endereço profissional: Ambulatório de Dermatologia do Hospital das Clínicas da Universidade Federal de Pernambuco (UFPE), Av. Moraes Rêgo, s/n°, Recife, fone: (81) 34215003.

Departamento de Imunologia, Centro de Pesquisas Aggeu Magalhães, FIOCRUZ, Av. Moraes Rego, s/n, Cidade Universitária, Cx. Postal 7472, CEP: 50670-420, Recife – PE, Brasil. Tel.: (081) 3301 2500; Fax: (081) 3453 2449; http://www.cpqam.fiocruz.

APÊNDICE B - TCLE Grupo paciente (menor de 18 anos)

Fundação Oswaldo Cruz, Ministério da Saúde

Centro de Pesquisas
AGGEU MAGALHÃES

FIOCRUZ

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – Grupo Paciente menor de 18 anos

Projeto: "Caracterização da resposta imune celular em pacientes portadores de Leishmaniose Tegumentar Americana ativa e após a cura clínica".

Eu, ..., concordo que meu(minha) filho(a) ..... participe voluntariamente neste projeto que será desenvolvido no Centro de Pesquisas Aggeu Magalhães da Fundação Oswaldo Cruz (CPqAM/FIOCRUZ). Fui informado que o objetivo principal do referido projeto é a investigação da resposta imunológica dos pacientes com leishmaniose tegumentar ativa, e após a cura clínica espontânea ou após tratamento quimioterápico.

Como meu(minha) filho(a) faz parte do grupo de pacientes, será submetido(a) ao teste de Montenegro, que é uma injeção sob a pele e que poderá causar ou não, após dois dias, o aparecimento de um "caroço" no local. Passará também por exames como pesquisa direta, punção aspirativa e biópsia da borda da lesão, cuja coleta do material para realizá-los pode causar um pouco de dor ou desconforto. Por fim, será então submetido(a) a coleta de sangue venoso em volume equivalente a quatro colheres de sopa, antes e após tratamento quimioterápico ou após cura clínica espontânea. Todo procedimento será realizado com material estéril descartável e por profissionais de saúde de reconhecida capacidade para executar os procedimentos, podendo ser considerado isento de riscos. Vale ressaltar que todas as informações e os detalhes dos exames que serão realizados serão esclarecidos para mim e meu filho(a).

Esse trabalho trará grande benefício, pois indicará se componentes do sistema imunológico poderão ser usadas como marcadores da resposta terapêutica e se outras células do sistema imunológico participam na evolução clínica de pacientes com leishmaniose tegumentar americana em Pernambuco. Antes da participação de meu(minha) filho(a) no referido projeto, fui incentivado a pedir esclarecimento adicional que julgasse necessário, esclarecido por um participante do projeto. Estou ciente que poderei recusar ou retirar meu consentimento, em qualquer momento da investigação, sem qualquer punição ou prejuízo.

Autorizo a Fundação Oswaldo Cruz (CPqAM/FIOCRUZ) a utilização das informações obtidas através dos resultados dos procedimentos em reuniões, congressos e publicações científicas preservando, neste caso, a identidade de meu (minha) filho(a). Autorizo, também que o CPqAM/FIOCRUZ poderá estocar amostra biológica para posteriores estudos.

Estou ciente que este documento é feito em duas vias, ficando uma em posse do participante e a outra com a equipe.

_____ _____

Assinatura do menor de 18 anos **data**

_____ _____

Assinatura do responsável **data**

_____ _____

Assinatura do médico responsável – CPqAM/FIOCRUZ **data**

Endereço profissional: Ambulatório de Dermatologia do Hospital das Clínicas da Universidade Federal de Pernambuco (UFPE), Av. Moraes Rêgo, s/n°, Recife, fone: (81) 34215003.

Departamento de Imunologia, Centro de Pesquisas Aggeu Magalhães, FIOCRUZ, Av. Moraes Rego, s/n, Cidade Universitária, Cx. Postal 7472, CEP: 50670-420, Recife – PE, Brasil. Tel.: (081) 3301 2500; Fax: (081) 3453 2449; http://www.cpqam.fiocruz.

APÊNDICE C - TCLE Grupo Controle

Fundação Oswaldo Cruz, Ministério da Saúde

Centro de Pesquisas
AGGEU MAGALHÃES

FIOCRUZ

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – Grupo Controle

Projeto: "Caracterização da resposta imune celular em pacientes portadores de Leishmaniose Tegumentar Americana ativa e após a cura clínica".

Eu, ... concordo em participar voluntariamente neste projeto que será desenvolvido no Centro de Pesquisas Aggeu Magalhães da Fundação Oswaldo Cruz (CPqAM/FIOCRUZ). O objetivo principal do referido projeto é a investigação da resposta imunológica dos pacientes com leishmaniose tegumentar ativa, e após a cura clínica espontânea ou após tratamento quimioterápico. Eu farei parte do grupo controle, ou seja, grupo de indivíduos que não apresentam a doença e que servirão de comparação com os indivíduos doentes. Serei submetido a coleta de sangue venoso em volume equivalente a quatro colheres de sopa. Todo procedimento será realizado com material estéril descartável e por profissionais de saúde de reconhecida capacidade para executar os procedimentos, podendo ser considerado isento de riscos. Vale ressaltar que todas as informações e os detalhes do procedimento serão esclarecidos para mim.

Esse trabalho trará grande benefício, pois indicará se componentes do sistema imunológico poderão ser usadas como marcadores da resposta terapêutica e se outras células do sistema imunológico participam na evolução clínica de pacientes com leishmaniose tegumentar americana em Pernambuco.

Antes de minha participação no referido projeto, fui incentivado a pedir esclarecimento adicional que julgasse necessário, esclarecido por um participante do projeto, sobretudo em relação a importância do grupo controle. Estou ciente que poderei recusar ou retirar meu consentimento, em qualquer momento da investigação, sem qualquer punição ou prejuízo. Autorizo a Fundação Oswaldo Cruz (CPqAM/FIOCRUZ) a utilização das informações obtidas através dos resultados dos procedimentos em reuniões, congressos e publicações científicas preservando, neste caso, a minha identidade. Autorizo, também que o CPqAM/FIOCRUZ poderá estocar amostra biológica para posteriores estudos.

Estou ciente que este documento é feito em duas vias, ficando uma em posse do participante e a outra com a equipe.

Assinatura do voluntário	**data**
Assinatura do responsável	**data**
Assinatura do médico responsável – CPqAM/FIOCRUZ	**data**

Endereço profissional: Ambulatório de Dermatologia do Hospital das Clínicas da Universidade Federal de Pernambuco (UFPE), Av. Moraes Rêgo, s/n°, Recife, fone: (81) 34215003.

Departamento de Imunologia, Centro de Pesquisas Aggeu Magalhães, FIOCRUZ, Av. Moraes Rego, s/n, Cidade Universitária, Cx. Postal 7472, CEP: 50670-420, Recife – PE, Brasil. Tel.: (081) 3301 2500; Fax: (081) 3453 2449; http://www.cpqam.fiocruz.

ANEXO A- Parecer do Comitê de Ética

Centro de Pesquisas AGGEU MAGALHÃES

FIOCRUZ Ministério da Saúde

COMITÊ DE ÉTICA EM PESQUISA DO CPqAM/FIOCRUZ

Título do Projeto: "Caracterização da resposta imune celular em portadores de Leishmaniose Tegumentar Americana ativa e após cura clínica."
Pesquisador responsável: Valéria Rego Alves Pereira
Instituição onde se realizará o projeto: CPqAM/FIOCRUZ
Data de apresentação ao CEP: 21/11/2005
Registro no CEP/CPqAM/FIOCRUZ: 60/05
Registro no CAEE: 0757.0.095.000-05

PARECER

O Comitê avaliou as modificações introduzidas e considera que os procedimentos metodológicos do Projeto em questão estão condizentes com a conduta ética que deve nortear pesquisas envolvendo seres humanos, de acordo com o Código de Ética, resolução CNS 196/96, e complementares.

O projeto está aprovado para ser realizado em sua última formatação apresentada ao CEP, bem como o modelo do Termo de Consentimento Livre e Esclarecido-TCLE apresentado. Este parecer tem validade até 08 de março de 2009 e em caso de necessidade de renovação do Parecer, encaminhar relatório e atualização do projeto.

Recife, 08 de março de 2006

Ana Maria A. Santos

Drª Ana Maria Aguiar dos Santos
Médica
Coordenação
CEP/CPqAM/FIOCRUZ

Observação:

- O referido parecer foi extraído do projeto, de modo que a análise dos pareceristas baseou-se nos fatos nele apresentados. Assim, **quaisquer modificações realizada** pelo Pesquisador responsável por esta pesquisa, devidamente identificado nas Folhas de Rosto e no Corpo do Projeto **e não submetida à nova avaliação a este Comitê de Ética em Pesquisa (CEP/CPqAM), estará assumindo total responsabilidade pelo descumprimento da Legislação vigente** que trata do assunto Ética em Pesquisa Envolvendo Seres Humanos, e demais códigos Civis e Penais que garantam a proteção a vida humana e a cidadania daqueles que se sentirem lesados em quaisquer um de seus direitos.
- O pesquisador responsável deverá enviar ao CEP um relatório anual sobre o andamento do projeto.

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ANEXO B - Renovação do Parecer do Comitê de Ética

CEP
Comitê de Ética em Pesquisa

DECLARAÇÃO

Declaro para devidos fins que o **projeto 60/05 – "Caracterização da resposta imune celular em portadores de leishmaniose tegumentar americana ativa após cura clínica"**, aprovado neste comitê em março de 2006, tem a sua solicitação de prorrogação do Parecer de Aprovação concedida por mais três anos.

Recife, 08 de setembro de 2009.

Gisélle Campozana Gouveia
Farmacêutica
Coordenadora
Mat. SIAPE 0463576
CPqAM / FIOCRUZ

Campus da UFPE - Av. Moraes Rego, s/n
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ANEXO C - Parecer CEP - Projeto Treg

Comitê de Ética em Pesquisa

Título do Projeto: "Estudo do papel de células T reguladoras $CD4^+$ $CD25^+$ na Leishmaniose Tegumentar".

Pesquisador responsável: Valéria Rêgo Alves Pereira.

Instituição onde será realizado o projeto: CPqAM/FIOCRUZ

Data de apresentação ao CEP: 23/12/2009

Registro no CEP/CPqAM/FIOCRUZ: 01/10

Registro no CAAE: 00082.0.095.000-09

PARECER Nº 15/2011

O Comitê avaliou as modificações introduzidas e considera que os procedimentos metodológicos do Projeto em questão estão condizentes com a conduta ética que deve nortear pesquisas envolvendo seres humanos, de acordo com o Código de Ética, Resolução CNS 196/96, e complementares.

O projeto está aprovado para ser realizado em sua última formatação apresentada ao CEP e este parecer tem validade até 04 de maio de 2014. Em caso de necessidade de renovação do Parecer, encaminhar relatório e atualização do projeto.

Recife, 04 de maio de 2011.

Gisalle Campozana Gouveia
Farmacêutica
Coordenadora
Mat. SIAPE 0463376
CPqAm / FIOCRUZ

Observação:
Anexos:
- Orientações ao pesquisador para projetos aprovados;
- Modelo de relatório anual com 1º prazo de entrega para 04/05/2012.

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Centro de Pesquisas AGGEU MAGALHÃES
FIOCRUZ Ministério da Saúde

ANEXO D – Protocolo de submissão do Artigo 3 ao *Journal of Interferon and Cytokine Research*

Journal of Interferon & Cytokine Research

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Manuscript ID:	JICR-2014-0027
Title:	Th1 and Treg mediators are predominantly expressed in patients with recent active American tegumentary leishmaniasis
Authors:	Souza, Marina Almeida, Thays Castro, Maria Carolina Oliveira, Andresa Almeida, Amanda Oliveira, Beatriz Rocha, Lucas Medeiros, Angela Brito, Maria Edileuza Dessein, Alain Pereira, Valéria
Date Submitted:	06-Feb-2014
Payment method:	Waived 06-Feb-2014

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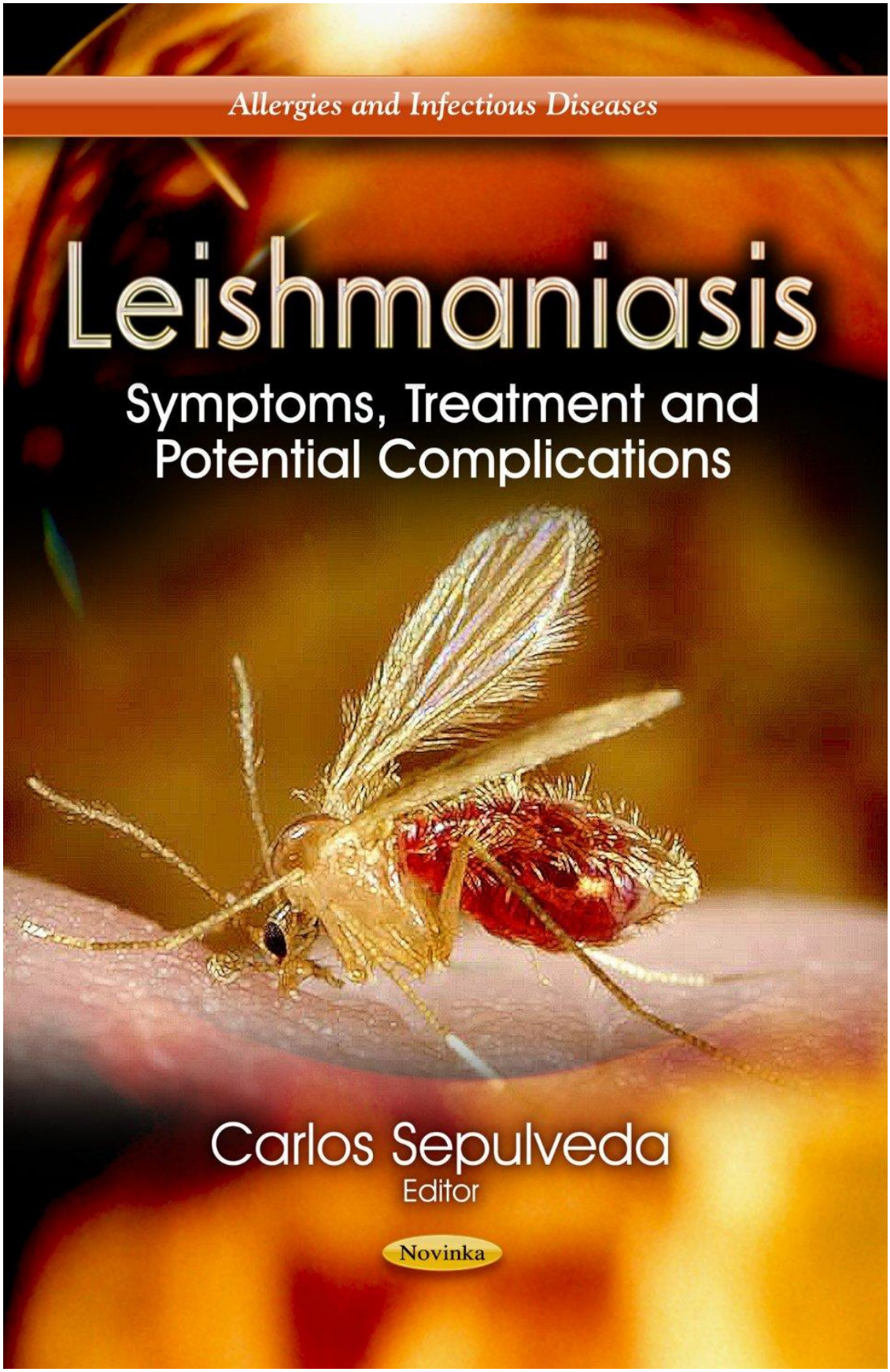
ANEXO E - Capítulo de livro: *Immunity against Leishmaniasis*



Chapter

LEISHMANIASIS: SYMPTOMS, TREATMENT AND POTENTIAL COMPLICATIONS. IMMUNITY AGAINST LEISHMANIASIS

Marina de Assis Souza[1], Maria Carolina Accioly Brelaz de Castro[1], Andresa Pereira de Oliveira[1], Beatriz Coutinho de Oliveira[1], Amanda Ferreira de Almeida[1], Thays Miranda de Almeida[1] and Valéria Rêgo Alves Pereira[1]•

[1]Laboratory of Immunogenetics, Department of Immunology, Aggeu Magalhães Research Center, Oswaldo Cruz Foundation (CPqAM/FIOCRUZ), Recife, PE, Brazil

ABSTRACT

Leishmaniasis is an anthropozoonotic, vectorially transmitted disease, which is caused by different *Leishmania* species. It is estimated that 350 million people worldwide are at risk of acquiring the disease, which has an annual incidence of 2 million cases. Under the influence of characteristics of the vector, vertebrate host and parasite, leishmaniasis can appear in the cutaneous (localized, disseminated and diffuse), mucocutaneous and visceral forms. In all clinical manifestations, the immune response plays an important role, contributing to the clinical cure or disease progression. Components of innate and acquired immunity act dynamically attempting to control the infection, so the host can achieve clinical cure.

Considering these aspects, this chapter describes the functions of some important elements in innate and acquired responses against *Leishmania* (i. e. chemokines, co stimulatory molecules, receptors, cytokines and cells) in the different clinical forms of leishmaniasis.

Keywords: leishmaniasis, innate immune response, cellular immune response.

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1. Introduction

Leishmaniasis is an anthropozoonotic, vectorially transmitted disease, which is caused by different *Leishmania* species. It is estimated that 350 million people worldwide are at risk of acquiring the disease, which has an annual incidence of 2 million cases. Depending on some features of the parasite, vector and the vertebrate host, including immunological state, the development of the disease can happen under a spectrum of clinical forms [1]. Localized cutaneous leishmaniasis is the most frequent outcome, being characterized by the presence of one or more ulcerated lesions which tend to self-healing. In rare cases, the lesions can be numerous due to multiple sand-fly bites or parasite dissemination by blood [2]. In diffuse leishmaniasis, there are several popular or nodular lesions throughout the body surface that can persist indefinitely. The mucocutaneous form is the most aggressive, presenting infiltrative lesions, with ulceration and tissue destruction in the nasal cavity, pharynx and larynx [3]. The appearance of different clinical manifestations is influenced by the host immune response. Thus, the presence of immune effector cells such as macrophages, natural killer cels, CD4+ and CD8+ T cells, cytokines, effector molecules and specific antibodies are critical components to the control of leishmaniasis [4,5,6]. Considering these aspects, this chapter describes the functions of some important elements in innate and acquired responses against *Leishmania* in the different clinical forms of leishmaniasis.

2. Innate Immune Response

Innate responses develop after the initial sensing of invading microbes, leading to the production of effector molecules that contribute to contain initial infection and to mount the subsequent adaptive immune response [5,7]. There is growing evidence that the innate immune response mechanisms are also important to the antiparasitic response and infection control[4,7]. We will discuss the aspects of the innate immune response in Leishmaniasis with more details below.

2.1. Contributing Cells

Leishmania life cycle inside the host is dependent upon internalization by phagocytic cells either resident or recruited to the wound site [7]. *Leishmania* spp. has been considered an obligate intracellular pathogen of macrophages, but the parasite also has adapted to live within different host cells than those previously described [8,9].

Neutrophils rapidly infiltrate the skin after *Leishmania* spp. infection, in cutaneous and visceral leishmaniasis, and are present in early lesions being the most immediate responders [9,10]. Both host protective and disease promoting roles for neutrophils have been reported. The protective role of neutrophils is associated with rapid recruitment to sites of tissue damage and pathogen entry, and the subsequent clearance of these recruited neutrophils by macrophage/monocyte populations [10,11]. Active neutrophils kill promastigotes via reactive oxygen and reactive nitrogen species as wells as neutrophils extracellular traps [4,9,11]. However neutrophils are short-lived and undergo apoptosis, and when their corpses are

phagocytosed by macrophages it allows silent entry of the parasites into macrophages through direct ingestion of the parasite or through ingestion of parasites that hide outside the dead neutrophils [4,9,12]. These apoptotic neutrophils at infection site may also suppress macrophages functions with the release of anti-inflammatory cytokines such as TGF-β and can cause immune mediated tissue pathology [4,8,9,10,12]. Passage through neutrophils is believed to be temporary, a way of camouflage. Parasites usually can establish infections in macrophages, differentiating into amastigotes that replicate inside parasitophorous vacuole [7,11]. However reports showed that in human visceral leishmaniasis neutrophils can harbor parasites during active disease [11]. *Leishmania* amastigotes, the intracellular form of the parasite, are able to multiply within macrophages, dendritic cells (DC) and neutrophils [13]. However, it is within mononuclear phagocytes that there is the best evidence for replication and long-term survival of *Leishmania* spp [8]. The resolution of infection with *Leishmania* is associated with presentation of *Leishmania* antigens by macrophages and dendritic cells (DCs) and priming of CD4+ and CD8+ T lymphocytes. Ultimately, induction of nitric oxide synthase (iNOS) and interferon-gamma (IFN-γ) leads to nitric oxide (NO) production, reactive oxygen species (ROS), and parasite killing by macrophages [4]. The central irony of leishmaniasis is that the macrophage is both the principal immune effector cell charged with killing *Leishmania* amastigotes and also the principal site of parasite proliferation and dissemination [4]. A complex network of immune cells within the skin—dendritic cells, macrophages and Langerhans cells—have a prominent role in cutaneous leishmaniasis, as a bridge from innate to adaptive immune responses [12,13,14]. DCs not only play a key role in the development of a protective immune response to *Leishmania*, but also act as a host cell for the parasites [15]. Resident dermal macrophages are also rapidly infected, and they become the dominant infected population after 24 hours allowing differentiation, growth of *Leishmania spp* [8,11]. These antigen presenting cells engage pathogens and then acquire a mature phenotype, increase their expression of co-stimulatory molecules and then travel along lymphatics to the nearest lymph node, where T cell responses are developed to control infection. In general, accumulation of DCs bearing protein antigen in lymph nodes is found to peak around 24h after inoculation [12,13,14]. Together with phagocytes, NK cells represent the first line of defense against pathogens, working by two principal mechanisms: cytolytic destruction of infected cells and secretion of proinflammatory cytokines. K cells can be identified at the site of infection as early as 24 hours after *Leishmania* infection [14]. In patients, the amount of NK cells and activity has mainly been related with protection against or healing of disease, and reports from patients with active leishmaniasis (cutaneous and visceral) show that they have a reduction in the frequency of peripheral NK cells [11]. The activation of NK cells in visceral and most likely also in cutaneous leishmaniasis results from the intimate interaction of these cells with dendritic cells, which are triggered by *Leishmania* parasites for the production of IL-12 in a TLR 9 dependent fashion [5].

2.2. Chemokines

Chemokines and chemokine receptors have been shown to have different roles in determining the outcome of leishmaniasis. Chemokines are chemotactic cytokines that coordinate recruitment of leukocytes involved in homeostasis as well as in innate and

adaptive immune responses [6,14]. Infection with *Leishmania* induces the expression of a number of chemokine genes in the host. This could potentially be beneficial to the parasite through recruitment of host cells it can infect, survive in and proliferate. In addition to mediating cellular recruitment, chemokines can activate various cell populations, participate in cell mediated immunity and possess anti-leishmanial properties, having roles in adaptive immunity, in macrophage activation and parasite killing [6,14].

Chemokines produced at the site of an infection are critical in determining the composition of infiltrating cells and defining the eventual outcome of the disease [14]. Patients with visceral leishmaniasis show elevated concentrations of CXCL9 and CXCL10 in their serum during active infection and it has been suggested that these chemokines along with IFN-γ play an important immunopathogenic role in the disease [6]. In localized cutaneous leishmaniasis (LCL) a Th1 chemokine profile is observed in the lesions, consisting of CCL2 (monocyte chemotactic protein-1), CXCL9 and CXCL10- associated with a concentrated dermal infiltrate comprising of macrophages and large numbers of CD4 positive cells. In contrast, the chemokine profile of lesions of chronic diffuse cutaneous leishmaniasis (DCL) is Th2 associated, dominated by the expression of CCL3 (macrophage inflammatory protein 1-α), and the dermal infiltrate is more diffuse with fewer CD4 positive cells [9,16].

2.3. Effector Molecules

The key antileishmanial effector molecules in experimental cutaneous and visceral leishmaniasis are reactive nitrogen intermediates (NO and NO-derived metabolites) and reactive oxygen intermediates ($O2^-$ and subsequent metabolites) [5]. While the production of NO is required for the leishmanicidal activity against *L.major* and *L. braziliensis* in the skin of infected mice, it is dispensable in the spleen and mildly important in the lymph node [7].

The entry of *Leishmania* parasite into host macrophages results in the onset of respiratory burst, characterized by the increased production of reactive oxygen species (ROS), like superoxide (O2 -) and hydrogen peroxide (H2O2), which is required for the killing of the parasites. These O2 - are generated by activities of a multi component enzyme complex i.e., nicotinamide adenine dinucleotide phosphate (NADPH)/NADH oxidase. Moreover, in later stages of infection, reactive nitrogen intermediates (RNI) like nitric oxide groups (NOg) are also produced by the activity of inducible nitric oxide (iNOS), which further contribute to innate immunity and parasitic elimination. However, in leishmanial infections, the microbicidal activities of macrophage are severely hampered, leading to the survival and proliferation of parasites inside the macrophages [19].

2.4. Toll-Like Receptors (TLRs)

The TLR family is highly relevant to immunity against *Leishmania* and other parasites, as they recognize pathogen-associated molecules and participate in innate responses to infections [4,7,19,22]. TLR activation induces innate responses in multiple ways, leading to the production of effector molecules such as nitric oxide, inflammatory cytokines, chemokines, and other anti-microbial products that can directly destroy the pathogens. TLRs are known to participate in the control of *Leishmania* infection by inducing Th1 responses. A

few *Leishmania*-derived molecules have been reported to activate TLRs, and the majority of the studies to date focused on the activation of TLR2, TLR4, and TLR9 [7,13,19,22].

Evidences indicate that TLR4 contributes most significantly to control the growth of *Leishmania* spp. in both phases of the immune response. The TLR4 has been found to be a strong regulator of inducible nitric oxide synthase (iNOS, a marker of innate immunity) leading to the death of parasites. In addition to TLR4, TLR2 and 9 have been detected in the skin of patients with cutaneous leishmaniasis [22].

Lipophosphoglycans (LPG) on the *Leishmania* cell surface have been implicated as agonists of TLR 2, 3, 6 and have also been associated with NK cell activation in *L. major* infection [4]. Purified *L. major* lipophosphoglycan induced the upregulation and stimulation of TLR2 on human NK cells, with additional enhancement of TNF-α and IFN-γ. LPGs of *L. major*, *L. mexicana*, *L.aethiopica*, and *L. tropica* were defined as TLR2 ligands in studies using murine macrophages, although the stimulation with *L. tropica* LPG was only marginal. More recently, it was shown that LPG stimulates cytokine production by human peripheral blood mononuclear cells via TLR2 as well. Those findings assign a protective role for TLR2 which seems required to mount an effective Th1 response [7].

2.5. Complement System

The complement system is a complex set of serum proteins that forms a controlled sequence for the generation of activated molecules. The role of the activated molecules is to increase inflammatory reactions mediated by antibodies.

In addition, generation of the membrane attack complex C5b–C9 leads to the lysis of "unwanted" cells. The complement receptor system is directed against mediators generated by the host right after parasite contact. In *Leishmania* infections the parasites interact with serum and activate complement in both the classical and the alternative pathways. Opsonization of *Leishmania* promastigotes with complement is fast, with lysis by the membrane attack complex beginning seconds after serum contact, resulting in the elimination of more than 90% of the inoculated parasites within a few minutes [13].

2.6. Modulation of Infection in Innate Immune Response

Leishmania parasites are capable of using different components of the host defense innate mechanisms to avoid their elimination from the host before an infection is established. Some of the parasites surface molecules are capable of activating the complement system, resulting in the binding of C3bi and C3b to the surface of the parasite. *Leishmania* parasites smartly use this opsonization to escape from the hostile environment by promoting phagocytosis via complement receptors in cells such as in macrophages, neutrophils and erythrocytes [9,11,13]. They can also entry macrophages using the engagement of non-triggering receptors such the phosphatidyl serine (PS) receptor. *Leishmania* can also evade effector mechanisms of the immune system by direct inhibiting macrophage function through interference with NFB transcription and IL-12 production, disturbing macrophage phagosomal maturation and killing functions.

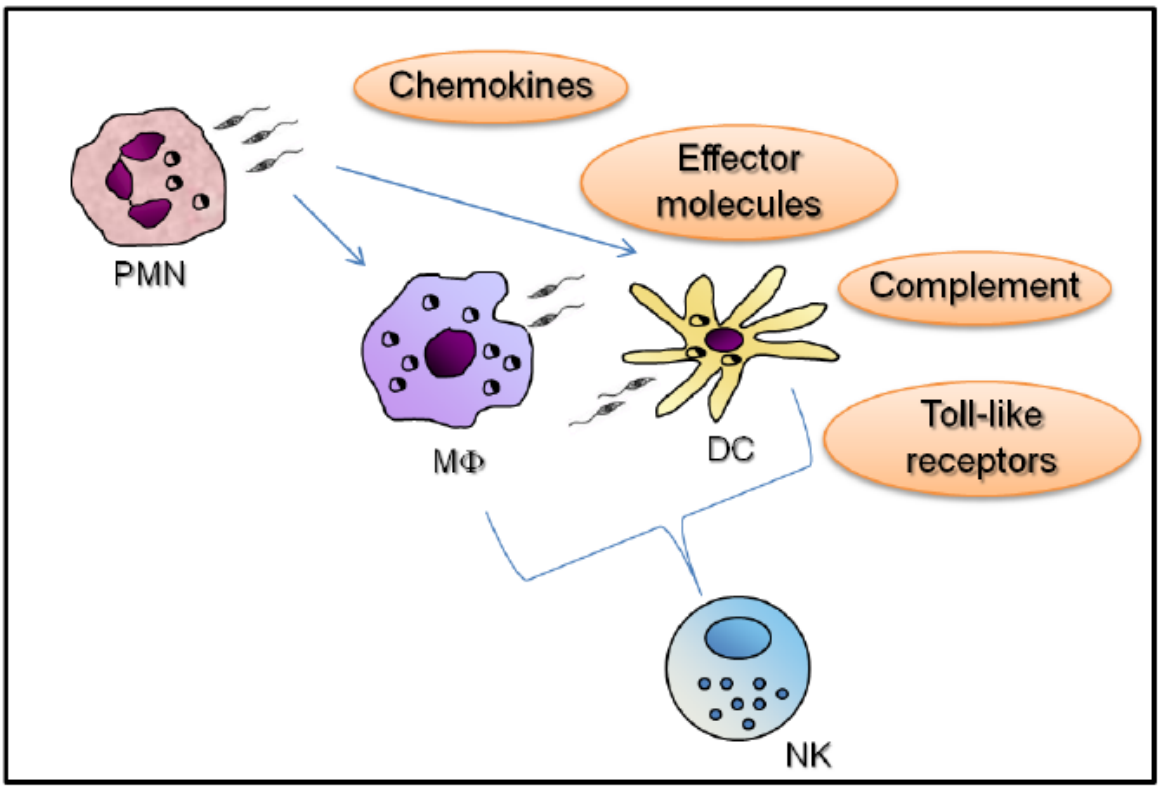

Figure 1. The main components of the innate immune response against Leishmaniasis include some important cells and chemical mediators. Neutrophils are the most immediate responders, which rapidly infiltrate skin after parasite infection. However, it is within mononuclear phagocytes that there is the best evidence for replication and long-term survival of *Leishmania* spp.. Ironically, the macrophage is both the principal immune effector cell charged with killing amastigotes and also the main site of parasite proliferation and dissemination. Within the skin, DCs not only play a key role in the development of a protective immune response to *Leishmania*, but also act as a host cell for the parasites. Together with phagocytes, NK cells represent the first line of defense against pathogens, making cytolytic destruction of infected cells and secreting inflammatory cytokines. The composition of inflitrating cells and the definition of the eventual outcome of the disease are critically determined by chemokines produced at the site of infection. Also, depending on the site of infection, antileishmanial effector molecules play an important role in innate immunity. Composed by a complex set of serum proteins, the complement system is activated in both the classical and alternative ways in leishmaniasis. However, *Leishmania* parasites are capable of using different components of innate defense to avoid their elimination from the host before an infection is established.

They can additionally down regulate MHC class II expression; promote the production of regulatory cytokines like IL-10 and TGFβ and can inhibit dendritic cell maturation and chemotaxis [4,11,15].

3. Acquired Immune Response

In human and experimental leishmaniasis, immunity is predominantly mediated by T lymphocytes. T cells play a major role in generating specific and memory T-cells responses to intracellular parasitic infection and these have been extensively characterized in *Leishmania* infection [25]. In addition, T lymphocytes play critical role in shaping the host immune

response by secreting cytokines, which may act both synergistically and antagonistically through complex signaling pathways to direct both protective and non-protective immunities against intracellular parasites [24].

Although the immune response induced by infection with *Leishmania* has been the subject of many investigations, the mechanisms that underlie host resistance and pathogenesis in leishmaniasis are not entirely understood. During the late 80s and early 90s, the discovery of two distinct subpopulations of CD4+ T helper cells based on their cytokine production, Th1 and Th2 [26], finally explained resistance and susceptibility to *L. major* in the murine model [27].

Early studies using mouse models of experimental cutaneous leishmaniasis (CL) have revealed a clear dichotomy between Th1-associated cytokines mediating protection and Th2-associated cytokines mediating susceptibility [23,24,28,31]. Failure to mount an efficient anti-*Leishmania* Th1 response was shown to cause progressive disease and absence of lesion resolution [29,31]. In resistant C57BL/6 mice, resolution of the disease is mediated as a consequence of IFN-γ release by Th1 cells and upregulation of NO in macrophages the harbor parasites [30,31]. Conversely, persistence of lesions in BALB/c mice is due to Th2 –type $CD4^+$ T cell differentiation and production of IL-4, which suppresses macrophage activation, resulting in parasite survival [29,31]. On the other hand, during VL, Th2 response and cytokines such as IL-4 and IL-13 seem to be necessary for immunity and efficient response to antileishmanial chemotherapy [31,32].

In the murine model of *L. major* infection, the predominant $CD4^+$ T cell subpopulation resulting from infection greatly influences the outcome of disease [34,35,36]. Interleukin-12 (IL-12) produced by macrophages and dendritics cells and interferon-gamma (IFN–γ) produced by natural killer cells (NK), and previously activated T cells, promote the development of Th1 cells, whereas IL-4 induces the development of Th2 cells. The Th1 subpopulation, important for induction of leishmaniasis resistance, produce IFN-γ and tumor necrosis factor – alpha (TNF-α) which play an important role in cellular immune responses against intracellular pathogens by activating macrophages for intracellular killing of pathogens[36,37]. On the other hand, Th2 cells produce IL-4, IL-5, IL-10, IL-13 and TGF-β, and are associated with leishmaniasis susceptibility in *L. major* infection murine models [36,38,39,40].

Most data point to the fact that same or similar Th1 dependent mechanisms are involved in control of human disease. Self-healing forms of leishmaniasis and cure of VL is typically accompanied by parasites specific proliferation and IFN-γ production. Human macrophages are activated to kill intracellular parasites by IFN-γ and exogenous IFN-γ can promote cure of human CL [41,45]. Though Th2 responses can act in favor of the parasite, polarized Th2 response has never been able to explain non-curative or visceralizing human disease. Th2 independent disease progression is also supported by studies on non-healing disease in Th1 phenotypic B6 mice [42,45]. In this context it can also be noted that in patients with VL the effect of IFN-γ administration was limited [43,45] and in human LC, IFN-γ production by CD4+ cells, alone, in response to *Leishmania* antigens is not predictive of protection or disease development [44,45]. This indicates that other mechanisms acting in synergy with IFN-γ or counteracting the effects of IFN-γ as important. Thus, the Th1/Th2 dichotomy as an indicator of resistance and susceptibility might be a generalization and is far more complex than what we currently know and understand [31,45].

Of particular interest in this context is the differentiation of Naïve $CD4^+$ Th cells into various effector lineages orchestrating different immune responses. Naïve $CD4^+$ Th cells can differentiate into IFN-γ producing Th1 cells; into Th2 cell secreting IL-4, IL-5, IL-13, and IL-10; or into the recently described Th17 cells. In addition, Naïve $CD4^+$ Th cells can differentiate into IL-10-secreting regulatory T cells like regulatory type 1 T cells, IL-10, and TGF-β producing Th3 cells or into Foxp3-expressing regulatory T cells [46]. Some cytokines are described in the following section.

3.1. Th1 and Th2 Cytokines

To control leishmaniasis infections, activation of macrophages dependent of CD4+ T cell, IFN-γ and tumor necrosis factor (TNF) are usually required. These effector molecules and cells are typically present in a cell mediated immune response. This leads to a (post)transcriptional upregulation of antimicrobial effector mechanisms, including the acidification of the phagolysosomes and the expression of inducible nitric oxide synthase [18,24]. TNF-α is a key cytokine mediating T cell-mediated inflammation. It is involved in leukocyte recruitment by increasing expression of adhesion molecules on vascular endothelium and increasing angiogenesis. Although TNF-α promotes increased macrophage activation, and contributes to control of *Leishmania* parasites, deleterious consequences of excessive TNF-α production have been reported. The high levels of TNF-α and IFN-γ secreted by mononuclear cells from these patients is positively correlated with lesion size and the use of drugs that down modulate production of TNF-α in combination with antimony increases the rate of healing and allows the cure of refractory cases of mucosal and cutaneous disease [15].

The main biological role of IFN-γ is to activate macrophages, inducing iNOS expression and NO production. This contributes to increase the microbicidal activity of these cells and therefore helps in the elimination of parasites and in the resolution of *Leishmania* infection [5,24]. IFN-γ biological effects can be associated with the activation of STAT1 transcription factors. STAT1/IFN-γ signaling pathway stimulates the expression T-bet, a transcription factor associated with the Th1 profile. STAT1 and T-bet are considered crucial to host protection against *Leishmania* infection in mice, since they are necessary to mount an efficient Th1 immune response [24].

Type I interferons *α* and *β* (IFN-α/β) are proinflammatory cytokines that are able to activate and phosphorylate STAT1 and STAT2. Their functions in innate and acquired immunity to bacterial and parasitic infections are shown in some studies. IFN-*α/β* can act as early regulators of the innate response to infection and are essential for initiating the expression of nitric oxide synthase type 2 (NOS2) and the production of NO. IFN*α/β* play a critical role in the innate immune response to CL infection by mediating events involved in parasite repression, IFN-γ expression, and cytotoxic NK cells activity- all through NOS2. IFN- *α/β* rather than IFN-γ was shown to account for the initial induction of iNOS in the skin and lymph node at day 1 of infection with *L. major*. The task of STAT2 in VL is essentially unknown [5,24]. Known as a proinflammatory cytokine, IL-12 is a heterodimer composed of two subunits, p35 and p40 and is produced primarily by macrophages and dendritic cells (DCs) in response to microbial pathogens. IL-12 functions as the main physiological inducer of gamma interferon (IFN- γ) by activated T cells and promotes Th1-type CD4+ T cell

differentiation, and therefore is a key cytokine for the generation of protective immunity in response to *Leishmania* infection. The specific cellular effects of IL-12 are due to the activation of Janus kinase (JAK)-STAT pathways, primarily to the activation of the specific transcription factor, STAT4. In activated T cells and NK cells, STAT4 functions to induce IFN-γ production in response to IL-12 signaling [5,24].

IL-10, an anti-inflammatory cytokine, is produced by a variety of cells, such as T cells, monocytes, macrophages, DCs, and B cells. Many other cells can produce IL-10, but its main role seems to be on macrophages and DCs, having a part as an anti-immune and anti-inflammatory cytokine. IL-10 inhibits the production of the proinflammatory cytokines IL-1, IL-6, IL-12, and tumor necrosis factor (TNF), preventing the development of a Th1 profile associated with a protective immunity during *Leishmania* infection. IL-10 also promotes the development of a humoral immune response, with the production of antibodies, which aids parasite entry into host cells. Studies demonstrated that IL-10 is a master cytokine in cutaneous and visceral leishmaniasis that is critical for the initial survival and long-term persistence of *Leishmania* parasites in both human and experimental models. Because IL-10 can act as an inhibitor of IFN-γ induced NO synthesis, it is likely that the antagonistic effects of IL-10 are related to its ability to suppress NO production, a critical component for parasite elimination [18,24]. IL-4 is an important cytokine that has been shown to deactivate macrophages and to regulate the induction of Type-2 [20,21]. Furthermore, IL-4 inhibits the responsiveness of CD4+ T cells to IL-12, due to its down regulatory effects on the expression of the IL-12 receptor b2-subunit and also inhibits the deviation of CD4+ T cells towards Th1 cells by modulation of the regulatory function of the transcription factor T-bet [20,21]. Moreover, macrophage activation by IL-4 induces a pathway of arginine metabolism toward arginase with production of polyamines that enhance *Leishmania* growth [21]. Since IL-4 has been shown to suppress macrophages and Th1 cells and enhances *Leishmania* growth, it is conceivable that the host ability in production of this cytokine may determine the susceptibility to CL. This hypothesis is supported by recent report on the association of IL-4 gene polymorphisms with susceptibility to visceral leishmaniasis [21].

3.2. Regulatory T Cells

To achieve cure in Leishmaniasis, the infected host must develop an immune response capable of eliminating the parasite, but harmless to itself. This balance is given by regulatory T cells, which exhibit two well-defined subpopulations: naturally occurring CD4+CD25+ Tregs, which originate in the thymus during ontogeny, and inducible Tregs, which develop in the periphery from conventional CD4+ T cells [46]. The first subpopulation of Tregs was initially described as a population of CD4+ T cells that prevent the expansion of self-reactive lymphocytes and, therefore, autoimmune disease in mice [47]. This population can be defined by their constitutive expression of the IL-2 receptor α chain (CD25), the cytotoxic T lymphocyte antigen (CTLA4), the TNF receptor family member GITR (glucocorticoid-induced TNF-receptor-related protein), and the a chain of the αεβ7 integrin (CD103) [48]. However, expression of these molecules is not specific to Tregs. In contrast, the forkhead/winged helix transcription factor Foxp3 is thought to program the development and function of Tregs and is specifically expressed in natural Tregs in mice, as well as in $CD25^-$ T cells with regulatory activity [49,50,51].

Cells with regulatory functions have been frequently described in *Leishmania* infections, and the existence of concomitant immunity is discussed [52,53,54]. This phenomenon consists in the long-term persistence of pathogens in a host that is also able to maintain strong resistance to reinfection. In the murine model of infection with *L. major*, $CD4^+CD25^+$ T cells accumulate in the dermis, where they suppress – by both interleukin-10-dependent and interleukin-10-independent mechanisms – the ability of $CD4^+CD25^-$ effector T cells to eliminate the parasite from the site. The sterilizing immunity achieved in mice with impaired IL-10 activity is followed by the loss of immunity to reinfection, indicating that the equilibrium established between effector and regulatory T cells in sites of chronic infection might reflect both parasite and host survival strategies [53].

Regarding the experimental infection with *Leishmania (Viannia) braziliensis*, a Treg activity has also been related. $CD4^+CD25^+$ cells expressing GITR, CD103 and Foxp3 were detected throughout the duration of clinical disease both at the ear and in draining lymph nodes of infected mice. In both sites, they were capable of suppress $CD4^+CD25^-$ proliferation. Interestingly, in the outcome of a reinfection, parasites were mainly detected in the LN draining the primary infection site where a high frequency of $CD4^+IFN\text{-}\gamma^+$ T cells was also present. Thus, in this model, Tregs are present in healed mice but this population does not compromise an effective immune response upon reinfection with *L. braziliensis* [54].

Suppression of T cell response is thought to be involved in the pathogenesis of human leishmaniasis. In patients with CL caused by *L. braziliensis*, a frequency of CD4+CD25+ cells was observed in the skin lesions, along with expression of CTLA-4 and GITR markers and secretion of IL-10 and TGF-β. Moreover, CD4+CD25+ T cells in peripheral blood (PB) from the same patients exhibited higher levels of CTLA-4 than healthy individuals[55]. Because CTLA-4 is highly expressed on Treg cells [56,57], and because it is supposed that this molecule plays an important role in their suppressor function[57], it is possible that the suppressor activity of CD4+CD25+ T cells was increased in the patients with CL.

A similar immune regulation in human visceral leishmaniasis is observed. The presence of CD4+CD25+ in the bone marrow, one of the disease sites, and the production of IL- 10 by these lymphocytes may inhibit T cell activation in IL-10 dependent manner [58]. In contrast, CD4+CD25+ lymphocytes did not accumulate in and were not a major source of IL-10 in the spleen, and their removal did not rescue antigen-specific interferon-γ responses. Thus, in different sites the regulation of immune response may be performed by different T cell subpopulations, once IL-10 is secreted in the spleen by CD25-Foxp3- T cells [59].

It is also interesting to investigate whether there is an influence of mechanisms of immune regulation on the response to chemotherapy. The analysis of the frequency of CD25+ cells in PB from patients with active and cured CL showed a higher presence of cells expressing this marker after treatment. Thus, CD25+ T cell expansion, presented by patients, may be due the role of these cells in the modulation of an exacerbated response by effector T cells, and maintenance of a small number of parasites in the localized lesion as an antigenic stimulus to prevent reinfection [60]. Among all the data obtained so far, immune regulation seems to happen as a way to maintain a homeostatic environment to allow the achievement of clinical cure by the host and the parasite persistence. Nevertheless, conclusive role of Treg cells in suppression of immunity in patients and its consequences is yet to be well defined.

3.3. Th17 Responses

Similar to the Th1 and Th2 subsets, the Th17 subset is orchestrated by specific cytokines and transcription factors [61]. The Th17 response has been studied since 1995, when it was found that T helper cells can produce IL-17 under stimulation with specific antigens [62]. Nowadays, it is known that the production of Th17 specific cytokines is present in allergy and inflammatory diseases [63,64]. However, these inflammatory mediators can orchestrate protective responses to several agents, as it is shown in *M. tuberculosis* and *T. cruzi* infections[65,66].

The Th17 response is activated by a combination of the cytokines IL-6 and TGF-β, and the transcription factors RORγt, RORα and Stat3 are essential for Th17 commitment [60,67]. IL-6 plays an important role in the differentiation of the Th17 subset, since TGF- β can also induce Foxp3, a transcription factor required for the generation of regulatory T (Treg) cells, and the presence of IL-6 suppresses the induction of Foxp3 [67].

Th17 cells produce cytokines such as IL-17A, IL-17F, IL-22, IL-21 and IL-23, which promote Th17 responses functionality. The cytokine IL-27, on the other hand, is the main negative regulator of the Th17 response, despite its structure's similarity to IL-6 [60]. Research over the role of these cytokines in many infections is under constant development. The research of the influence of Th17 cells in leishmaniasis is primordial to understand the mechanisms related to protective or damaging immune responses in this disease. In the next section, some features of these cytokines are described.

3.3.1. IL-17

The IL-17 cytokines include a family of six members (IL-17A-F), of which at least two of them exhibit potent proinflammatory properties: IL-17A (also known as CTLA-8) and IL17-F, which seem to have similar functions. IL-17B and IL-17C are members of the family whose cellular sources are unknown at this point, and whose biology seems unrelated to IL-17A. IL-17D and IL-17E (alternative names: IL-27 and IL-25), in turn, are the two members of the IL-17 family with lowest homology to IL-17A. None of the last is produced by Th17 cells, and they exert a negative control on the Th17 subset development[60]. In this chapter, we will refer to IL-17A as IL-17.

By signaling through the receptor IL-17RA, IL-17 can induce the production of different kinds of proteins, many of them related to inflammation, including chemokines (CXCL-1, CXCL-2, CXCL-8-10, CCL-2, CCL-20), cytokines (IL-6, TNFα, G-CSF, GM-CSF), proteins of the acute phase response, tissue remodeling factors (MMP1, MMP3, MMP9, MMP13, TIMP2), and anti-microbial products (β-defensins, mucins, calgranulins) [60]. The role of IL-17 in immune responses is being widely studied. It is known that IL-17 is a potent activator of neutrophils. Increased levels of this cytokine are responsible for neutrophil immigration, most likely via CXCL2, whereas IFN-γ is responsible for activating macrophages to kill intracellular pathogens [68]. IL-17 seems to have a role in the protective immunity against many bacterial and fungi infections, as in the case of *Klebsiella pneumoniae*, *Mycobacterium tuberculosis*, *Candida albicans* and *Aspergillus fumigate* infections [69,70]. IL-17 could also be defensive against some parasites, as in the infection with the protozoan *Toxoplasma gondii* [71]. Also, IL-17 production appears to be downstream of IL-1αβ in several pathological conditions. DC derived IL-1 is important for efficient Th1 induction in leishmaniasis [68].

Whilst in some models IL-17 and IL-23 seem to have a protective role on the outcome of the infection, as in the case of extracellular pathogens (e.g., *Klebsiella pneumonia* bacteria, *Toxoplasma gondii* parasites and *Cryptococcus neoformans* fungi) [72], in *Schistosoma mansoni* infections, increased levels of IL-23 and IL-17 are associated with disease exacerbation [73].

As to leishmaniasis, Kostka et al (2009) reported that BALB/c mice produced increased levels of IL-17 after infection with *L. major* and that IL-17-deficient (IL-17$^{-/-}$) BALB/c mice exhibited dramatically attenuated disease despite typical Th2 development. They also demonstrate that elevated levels of IL-17A in BALB/c mice were associated with increased production of IL-23, but not IL-6 and TGF-β1, by infected DC.

In humans, studies have shown that IL-17 is present at the initial phase of the immune response in the cutaneous forms of leishmaniasis [68,74,75], leading to the conclusion that this cytokine could be injurious for the disease resolution. On the other hand, Novoa et al (2011) observed an increase in IL-17 levels in individuals with subclinical ACL, in comparison to patients with active lesions, concluding that this cytokine presents a protective part in the immune response. Pitta et al (2009) have also shown that *L. donovani*, a visceral leishmaniasis agent, strongly induces IL-17 and IL-22 production in PBMCs of healthy individuals, suggesting that these cytokines can present a protective role in *Leishmania* infections.

3.3.2. IL-21

Although IL-21 does not look like an essential factor for Th17 lineage commitment, it is able to induce IL-17 expression in collaboration with TGF-β even in the absence of IL-6. Furthermore, generation of Th17 cells is attenuated by blocking IL-21, and loss of its expression, or its receptor, results in defective Th17 differentiation. Similar to IL-6, IL-21 inhibits Foxp3 expression induced by TGF-β. IL-21 is produced by Th17 cells under IL-6 induction and autocrinally induces its own synthesis and the expression of IL-23R to allow IL-23 responsiveness [68].

Furthermore, IL-21 has been recently proven to induce IL-10 production under stimulation with *L. donovani* antigens. It is also known to critically regulate Ig production, and could be a contributing factor to the high titers of anti-leishmanial Abs in VL patients [76].

3.3.3. IL-22

IL-22 is also produced by Th17 cells, and to a lesser extent by Th1 and NK cells, and is involved in immunity at the epithelium and mucosal surfaces [77,78]. The functional IL-22 receptor is expressed on hepatocytes, keratinocytes, and fibroblasts. IL-22 increases the production of proinflammatory molecules, such as the S-100A proteins and CXCL5. IL-17 and IL-22 synergistically increase the production of antimicrobial peptides, such as β-defensins, by epithelial cells [68,78].

Both IL-17 and IL-22 have been shown to increase protection against certain bacterial and fungal pathogens in experimental models [78]. As to protozoans, Pitta et al (2009) stated that IL-17 and IL-22 are the cytokines most strongly associated with protection in the visceral forms of leishmaniasis.

These cytokines may contribute to protective immunity to *L. donovani* in several ways. Studies using animal models suggest that neutrophils could be involved in controlling the

Leishmania infection through the generation of skin and liver granulomas that form around *Leishmania* at early stages of infection. Furthermore, IL-22 is involved in epithelial repair and liver protection in chronic infections. Both the increases in epithelial protective barrier function and the recruitment of inflammatory cells, including neutrophils, to the skin and liver, could contribute to protection against *L. donovani* [78].

3.3.4. IL-23

The function of IL-23 in promoting Th17 cell expansion or survival has been proposed. A recent report suggests that IL-23 maintains the Th17 phenotype without affecting proliferation or survival. On the other hand, IL-23 has been demonstrated to maintain the pathogenic Th17 functions compared with culture under TGF-β and IL-6, depending on IL-10 production by Th17 cells [60].

Recent studies have also implicated IL-23 and IL-17 in immunity against extracellular pathogens, as bacteria (*Klebsiella pneumoniae*), *Toxoplasma gondii* parasites and fungi (*Cryptococcus neoformans*). In *Schistosoma mansoni* infections, increased levels of IL-23 and IL-17 are associated with disease exacerbation[72]. Kosksta et al (2009) suggests that DC-derived IL-23, in addition to IL-1β and IL-12p80, can contribute to disease susceptibility in BALB/c mice infected with *Leishmania* parasites.

3.3.5. IL-27

IL-27 is one of the main negative regulators of Th17 development. This cytokine is structurally related to IL-6, but has many different actions. Research studies show a damaging role of IL-27 on IL-17 producer cells. These studies conclude that the absence of IL-27 signaling exacerbates chronic inflammation in correlation with increased number of Th17 cells.

Moreover, IL-27 is able to promote IL-10 production, another negative player in the network of Th17 activity regulation [60].

Novoa (2009) reported a higher expression of mRNA for IL-27 *ex vivo* or in cultures stimulated with soluble *Leishmania* antigen in patients with active lesions compared to individuals with subclinical disease. Ansari et al (2011) also associated active visceral leishmaniasis with elevated levels of IL-27 in plasma and IL-27 mRNA in spleen.

IL-27 produced by macrophages, along with IL-21 from T cell sources, are suggested to be disease-promoting cytokines in visceral leishmaniasis by virtue of their roles in promoting the differentiation and expansion of Ag-specific, IL-10–producing T cells.

The studies support the notion that IL-27 is a key instructional cytokine involved in regulating the balance between immunity and pathology in human visceral leishmaniasis [76].

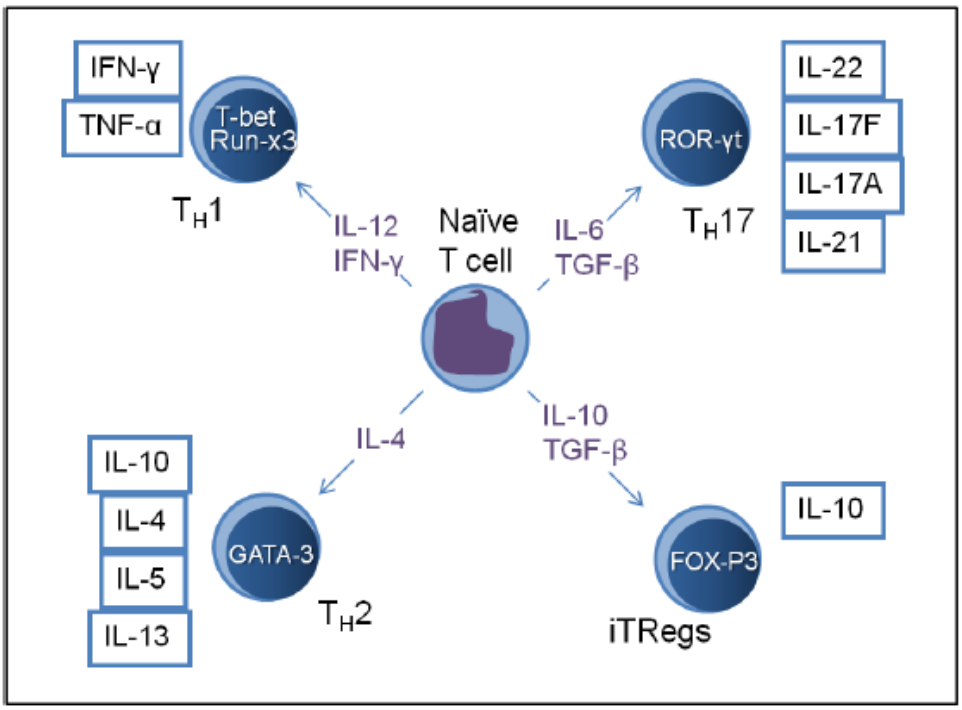

Figure 2. To all of the clinical forms of Leishmaniasis, the adaptive immune response is mainly executed by $CD4^+$ T-cells, through cytokine production. The development of a T cell profile may vary according the cytokine microenvironment. The presence of IFN-γ and IL-12 will contribute to set a Th1 profile, initially beneficial to the host, but harmful if exacerbated expressed. Th2 cells will dominate under influence of IL-4, which contributes to parasite growth and development of the disease. Cytokines such as IL-17A, IL17F and IL22 will be mainly secreted by Th17 cells, which develop in the presence of IL-6 and TGF-β. IL-27 works as a down-regulator of the profile. TGF-β is also required to the development of a regulatory response, mediated by IL-10 secretion by $Foxp3^+$ induced regulatory T cells.

3.4. Humoral Response

The infection by *Leishmania* in humans is characterized by the appearance of anti-leishmanial antibodies in the patients' serum. In respect of the humoral immune response, a successively high titer of specific antibodies can be observed in Localized ATL, Mucocutaneous Leishmaniasis (MCL) and Diffuse ATL. An exceptionally high titer of antibodies against *Leishmania* antigens can be detected in the most severe form of the disease, Visceral Leishmaniasis (VL), as a consequence of polyclonal activation of B cells, resultant of the presence of large numbers of parasites in the bone marrow and spleen [79].

To evaluate the humoral immune response on Leishmaniasis, works have shown the role of the immunoglobulins on immunopathological mechanisms which are involved in the resistance and/or pathogenesis of the infection [80,81,82]. In some studies the presence of antibodies against *Leishmania braziliensis* in the sera of infected patients is still unclear but these antibodies have been monitored and they are utilized for diagnosis and prognosis of ATL [84,85]. Contrastingly, strong anti-leishmanial antibody titers are as well documented in VL [86,87].

However, it has been shown that the class IgG not only offers protection against this intracellular parasite, but indeed, it contributes to the progression of the infection. Previous analysis of *Leishmania* antigen-specific immunoglobulin isotypes and IgG subclasses in VL patients' sera has shown that elevated levels of IgG, IgM, IgE and IgG subclasses were lasting [79]. This is due to differential patterns of immunoglobulin isotypes observed during the disease progression. Drug resistance and cure were specific for antigens of *Leishmania donovani*. IgG subclass analysis has revealed expression of all the subclasses, with a prevalence of IgG1 during the disease [87], nevertheless, some studies have shown the advantage of using specific subclass antibodies for the diagnosis of VL [79,88,89].

Although studies have been evaluating the humoral immune response on ATL, the role of specific antibodies on the immunity against *Leishmania* is still not completely clear. On Cutaneous Leishmaniasis (CL) and Mucocutaneous Leishmaniasis (MCL), the cellular immunity and the prevalence of the isotypes IgG1, IgG2 and IgG3 have been associated with the Th1 response; on the other hand, the Th2 profile has been related to Diffuse Cutaneous Leishmaniasis (DCL), with the presence of IgG4. Studies lead the attention to the correlation of the subclasses of IgG with the clinical manifestations of ATL. Therefore, high levels of the isotypes IgG1, IgG2 and IgG3 and low levels or absence of the IgG4 isotype can be detected in the sera of patients with CL. In patients with MCL, there are high levels of IgG1 while the levels of IgG2, IgG3 and IgG4 are similar to the findings on the sera of patients with CL. The levels of IgG4 in patients with DL are highly elevated, as the level of IgG1 and IgG2 are similar to the patients with CL and MCL. Studies show that all specific isotypes anti-*Leishmania,* except for IgD, are detected in the sera of patients with ATL. There are high levels of IgE in patients with more time of disease evolution and high levels of IgA in patients with MCL [82].

The intensity of the antibody response appears to reflect both the parasite load and the chronicity of the infection and it also can be observed high titers of antibodies in all clinical manifestations of ATL [90]. Studies with immunological and serological methods which are available to the research of antibodies in ATL, showed controversial results due to its low sensibility and specificity [91,92]. However, studies have shown the advantages of using specific antibodies in the diagnosis of VL [79,88,89].

4. Final Considerations

Classically, *Leishmania* infections can induce the host to mount an immune response, which is characterized by the enrichment of T CD4+ cells, with Th1 or Th2 cytokines profile. Although this definition exists, the complexity in host-parasite interaction has promoted the investigation of other response profiles, in which cytokines, molecules and mediators take part. These may contribute favorably or not in the evolution of the different clinical forms in leishmaniasis. The scientific community has evaluated distinct cell subtypes, such as regulatory T cells, that accumulate in the lesion site and also acts mediating immune response through effector cells. Recently the Th17 profile was evidenced, and it was firstly related to the pathogenesis of chronic inflammatory disease or autoimmunity. Furthermore, the involvement of antibodies in diagnostic evaluations and as a criterion of cure must be considered.

In the balance between cure and progress of the disease, studies have shown that regardless of the cell and or molecules of a given profile, none is sufficient to act independently in the immune response. Thus, the balance between the innate and adaptive immune system and the parasite evasion mechanisms is critical for the decision if disease is observed and if (lifelong) immunity develops [13].

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