UNIVERSIDADE FEDERAL DE PERNAMBUCO

Programa de Pós-Graduação em Inovação Terapêutica

RAFAEL DE FREITAS E SILVA

IDENTIFICAÇÃO E AVALIAÇÃO IMUNOLÓGICA DE POTENCIAIS EPÍTOPOS DE LINFÓCITOS T CD4^{+} E T CD8^{+} NO PROTEOMA DE *Leishmania (Viannia) braziliensis*

Recife

2016

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Inovação Terapêutica da Universidade Federal de Pernambuco, como parte dos requisitos para obtenção do Título de Doutor em Inovação Terapêutica

Orientadores: Drª. Valéria Pereira Hernandes
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Recife
2016

Catalogação na fonte
Elaine Barroso
CRB 1728

Silva, Rafael de Freitas e
Identificação e avaliação imunológica de potenciais epítopos de linfócitos T CD4+ e T CD8+ no proteoma de *Leishmania* (Viannia) *braziliensis* / David Ítallo Barbosa– Recife: O Autor, 2016.

200 folhas: Il., fig., tab.
Orientadores: Valéria Pereira Hernandes e Antonio Mauro Rezende
Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Biociências. Inovação Terapêutica, 2016.
Inclui referências, apêndices e anexos

1. Leishmaniose 2. Vacinas 3. Linfócitos I. Hernandes, Valéria Pereira (orientadora) II. Rezende, Antonio Mauro (orientador) III. Título

616.9364 CDD (22.ed.) UFPE/CCB-2016-327

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Nome: DE FREITAS E SILVA, Rafael

Título: IDENTIFICAÇÃO E AVALIAÇÃO IMUNOLÓGICA DE POTENCIAIS EPÍTOPOS DE LINFÓCITOS T CD4+ E T CD8+ NO PROTEOMA DE *Leishmania (Viannia) braziliensis*

Tese apresentada à Universidade Federal de Pernambuco para obtenção do título de Doutor em Inovação Terapêutica

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DEDICO ESTE TRABALHO AOS INDIVÍDUOS PORTADORES DE LEISHMANIOSES E À TODOS OS PORTADORES DE OUTRAS DOENÇAS NEGLIGENCIADAS.

AGRADECIMENTOS

À Drª Valéria Pereira (FIOCRUZ), por ter me acolhido em seu grupo de pesquisa, pela orientação, apoio, compreensão, amizade e paciência durante todos os momentos da realização desse trabalho. Muito obrigado!

Ao Dr. Antônio Rezende (FIOCRUZ), pela orientação, apoio e suporte na realização do trabalho, por nossas reuniões sempre muito produtivas e com muita ciência (!).

Ao Prof. Marcelo Zaldini (UFPE), pelo suporte e por ter feito possível acontecer as simulações moleculares dessa pesquisa. Também pela orientação científica, pelos conselhos e pelas reuniões em que eram feitos sempre muitos questionamentos.

Ao Sr. Dr. Osvaldo Pompílio (FIOCRUZ), pelas leituras sempre muito críticas e enriquecedoras, pelos questionamentos feitos e pelo apoio científico dado para realização da pesquisa.

À Drª Edileuza Brito (FIOCRUZ), pelo apoio dado e suporte na coleta das amostras de pacientes com leishmanioses.

Aos colegas e aos amigos que fiz no Laboratório de Imunogenética, pelo apoio, suporte, paciência e companherismo durante a elaboração desse trabalho. Em especial Ailton Álvaro, Beatriz Coutinho (Beatrix), Carla, Diego, Marina, Andreza e Renata.

À minha amiga Carolina Brelaz, pela amizade, paciência e apoio durante a realização desse trabalho.

À banca de Professores e Doutores que contribuiu para a qualificação desse trabalho e contribuiu para a defesa da tese.

Aos meus pais, pelo amor, educação, carinho e apoio dados durante a minha vida.

Aos meus irmãos, Ana Freitas e Luciano Freitas, e em especial à Valéria Freitas por acompanhar de perto minha formação acadêmica e por todo apoio e força dados, obrigado! Ao meu cunhado Onilson Emerson pela paciência.

Aos meus colegas dos colegiados de Medicina e Biologia da Universidade de Pernambuco, pelo apoio e compreensão nas muitas horas que precisei estar ausente.

Aos meus colegas e amigos da Universidade de Pernambuco, Jaciara, Cristina, Zélia, e em especial Marina Araújo e Vladimir Silveira, sobretudo pela paciência e apoio nas horas que precisei. Obrigado!

Aos meus estimados alunos da Universidade de Pernambuco que sempre estiveram acompanhando o desenrolar desse trabalho.

Aos meus amigos Jana Sandes e Marcos Ely, por todo carinho, paciência e momentos de descontração.

Aos meus amigos biomédicos, por nossos tantos momentos de alegria juntos!

À minha grande amiga Isabelle Freire, pois mesmo distante fisicamente sempre me deu muito apoio e carinho durante a realização desse trabalho e na minha vida!

Às minhas amigas Marcela Pinto e Neyliane Santos por sempre torcerem e me darem apoio quando precisei.

Ao meu amigo Guillaume Costa e sua família por terem me acolhido tão bem e recebido durante me estada em Paris. Merci!

À minha amiga Rafaella Pitzurra, por sua amizade e momentos de descontração, mesmo que tão distantes! Vielen dank!

Ao programa de pós-graduação em inovação terapêutica (PPGIT) por ter me dado oportunidade para realizar essa pesquisa. Ao secretário do PPGIT, Paulo Germano, por sua grande eficência e calma.

Às agências de fomento, Conselho Nacional de Pesquisa Científica (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e Fundação de Amparo à Ciência e Tecnologia de Pernambuco (FACEPE), pelo apoio financeiro aos projetos para realização desse trabalho.

“SABER NÃO É SUFICIENTE; DEVEMOS APLICAR. QUERER NÃO É SUFICIENTE; DEVEMOS FAZER”

- Es ist nicht genug zu wissen – man muss auch anwenden. Es ist nicht genug zu wollen – man muss auch tun.

Johann Wolfgang von Goethe

“DEVEM EXIGIR QUE EU PROCURE A VERDADE, NÃO QUE A ENCONTRE”

- On doit exiger de moi que je cherche la verité, mais non que je la trouve

Denis Diderot

RESUMO

FREITAS-SILVA, R. **Identificação e Avaliação Imunológica de Potenciais Epítopos de Linfócitos T CD4$^+$ e T CD8$^+$ no Proteoma de *Leishmania (Viannia) braziliensis***. 2016. Tese (Doutorado). Universidade Federal de Pernambuco, Recife, Pernambuco, Brasil.

As leishmanioses são doenças causadas por protozoários do gênero *Leishmania* e estão presentes em 98 países e territórios e possuem incidência anual de 2 milhões de casos. A *Leishmania (Viannia) braziliensis* (*L.V.* braziliensis) é uma das principais espécies causadoras da leishmaniose cutânea (LC) no Brasil. Apesar disso, ainda não há uma vacina segura e eficaz para ser utilizada em seres humanos. Nesse sentido, o objetivo deste trabalho foi identificar no proteoma predito de *L.V. braziliensis*, potenciais epítopos de linfócitos T e avaliá-los por meio de ensaios imunológicos. No primeiro capítulo, o proteoma predito de *L. braziliensis* foi comparado ao de outras espécies e analisado quanto a presença de epítopos. Nessa etapa foram encontrados epítopos derivados de mais de 8 mil proteínas conservadas entre diferentes espécies de *Leishmania*. Os epítopos foram clusterizados e então utilizados para etapa de docagem molecular com estruturas de MHC I e MHC II depositadas no *Protein Data Bank*. A docagem molecular resultou em epítopos peptídicos de 15 aminoácidos com alta afinidade de ligação às moléculas de MHC I e MHC II. Os 10 melhores resultados foram então sintetizados e avaliados, *in vitro*, quanto à capacidade de estimular a proliferação de células mononucleares do sangue periférico (PBMC) de indivíduos com LC após o tratamento (PT). Os resultados indicaram que 50% das moléculas testadas apresentaram capacidade de estimular, significativamente (p<0,05), a proliferação celular quando comparado às células de indivíduos saudáveis que não vivem em região endêmica para LC. No segundo capítulo, os peptídeos foram avaliados quanto à capacidade de estimular a proliferação de PBMC de indivíduos com LC em sua fase ativa (AD) e indivíduos moradores de área endêmica para LC resistentes à infecção (RT). Em paralelo, quantificou-se a expressão do fator de transcrição T-bet em PBMC de indivíduos PT, e citocinas dos perfis Th1, Th2 e Th17 foram mensuradas no sobrenadante de cultura das células de indivíduos PT e AD. Os resultados demonstraram altos níveis de proliferação nas células do grupo RT para todos os peptídeos testados. Além disso, níveis significativos de T-bet foram observados em linfócitos T CD4$^+$ e CD8$^+$ após estímulo com seis peptídeos. Níveis significativos de IFN-γ, TNF e IL-6 foram observados no sobrenadante das células do grupo PT com quatro dos peptídeos testados. Altos níveis dessas citocinas também foram encontrados no sobrenadante do grupo AD. No terceiro capítulo, avaliou-se o efeito dos peptídeos sobre células dendríticas de medula (BMDC) murinas, produção de citocinas de sobrenadante, e células dendríticas esplênicas murinas após estímulo com os peptídeos. Verificou-se altos níveis de MHC II e CD40 em uma subpopulação de BMDC estimuladas com as moléculas e altos níves de TNF e IL-6 após 48h de estímulo. Para as células esplênicas, foram observados altos níveis de subpopulações celulares expressando CD11b$^+$, IL-12p70$^+$, CD205$^+$ e CD11b$^+$ após estímulo com o peptídeo que teve o melhor resultado *in silico*. Por fim, os resultados indicam o grande potencial imunogênico que os epítopos identificados apresentam, o que dá suporte ao desenvolvimento futuro de abordagens vacinais.

Palavras-chave: Doenças tropicais negligenciadas, Leishmanioses, *Leishmania braziliensis*, Epítopos de linfócitos T CD4$^+$ e T CD8$^+$, Vacinas.

ABSTRACT

FREITAS-SILVA, R. **Identification and Immunological Evaluation of Potential CD4$^+$ and CD8$^+$ T cell Epitopes in the Proteome of *Leishmania (Viannia) braziliensis*.** 2016. Thesis (Doctorate). Universidade Federal de Pernambuco, Recife, Pernambuco, Brasil.

The leishmaniasis are diseases caused by protozoans from the genus *Leishmania* which are present in 98 countries and territories, with an annual incidence of 2 million cases. Among the other species, *Leishmania (Viannia) braziliensis* is the main specie implicated with cutaneous leishmaniasis (CL) in Brazil. Besides that, there is no safe and effective vaccine against leishmaniasis to be applied in humans. In this context, the aim of this work was to identify in the predicted proteome of *L. braziliensis* potential CD4$^+$ and CD8$^+$ T cell epitopes and evaluate them by immunological assays. In the first chapter, the predicted proteome of *L. braziliensis* was compared with other species and analyzed for the presence of epitopes. In this step, epitopes from more than 8,000 conserved proteins were found among other species of *Leishmania*. The epitopes were clustered and then used for the molecular docking with MHC I and MHC II structures deposited in the Protein Data Bank. This approach resulted in 15 aminoacids peptide epitopes with high binding affinity for MHC I and MHC II. The 10 best results were synthesized and evaluated *in vitro* for their capacity to stimulate the proliferation of peripheral blood mononuclear cells (PBMC) of individuals with CL post treatment (PT). The results have shown that 50% of the tested molecules had the capacity to stimulate, significantly (p<0.05), cell proliferation when compared with cells of healthy individuals living in non-endemic regions. For the second chapter, the peptides were evaluated for their capacity to stimulate the proliferation of PBMC from CL individuals with active disease (AD) and of individuals resistant to infection (RT) living in endemic region. In parallel, the T-bet transcription factor expression was quantified in PBMC of PT individuals, and cytokines from the Th1, Th2 and Th17 profiles were measured in culture supernatant of PT and AD groups. High levels of cell proliferation in the RT group were demonstrated for all peptides tested. Moreover, significant levels of T-bet in CD4$^+$ and CD8$^+$ T cells were verified after stimulation with six peptides. For IFN-γ, TNF and IL-6, significant levels were detected in the supernatant of cultures from the PT group with four peptides tested. High levels of these same cytokines were also present in the supernatant of AD group. In the third chapter, the peptide effects over murine bone marrow dendritic cells (BMDC), the production of cytokines in the supernatant and murine spleen dendritic cell subsets were evaluated after peptide stimuli. High levels of MHC II and CD40 were verified for stimulated BMDC and high levels of TNF and IL-6 after 48h of stimuli. For spleen cells, high levels of cells expressing CD11b$^+$, IL-12p70$^+$, CD205$^+$ e CD11b$^+$ were observed after stimulation with the peptide which showed the best *in silico* result. In conclusion, the results indicate the great immunogenic potential of the identified peptides and support the further development of vaccine approaches using those molecules.

Keywords: Neglected tropical diseases, Leishmaniasis, *Leishmania braziliensis*, CD4$^+$ and CD8$^+$ T cell epitopes, Vaccines.

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Revisão de Literatura e Metodologia

Capítulo I

LISTA DE ABREVIAÇÕES E SIGLAS

ABNT	Associação Brasileira de Normas Técnicas
AD	*Active Disease* (Doença Ativa)
ANN	*Artificial Neural Networks* (Redes Neurais Artificiais)
ANVISA	Agência Nacional de Vigilância Sanitária
APC	*Antigen Presenting Cell* (Célula Apresentadora de Antígeno)
BMDC	*Bone Marrow Dendritic Cell* (Célula Dendrítica de Medula Óssea)
CD	*Cluster differentiation* (Grupamento de diferenciação)
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CPqAM	Centro de Pesquisas Aggeu Magalhães
DC	*Dendritic Cells* (Células Dendríticas)
DNA	*Desoxiribonucleic acid* (Ácido desoxirribonucléico)
DPDx-CDC	*Laboratory Identification of Parasitic Diseases of Public Health Concern – Center for Disease Control and Prevention*
ELISA	*Enzyme-linked immunosorbent assay* (Ensaio imunoenzimático)
EMA	*European Medicine Agency* (Agência Européia de Medicamentos)
FIOCRUZ	Fundação Oswaldo Cruz
HLA	*Human Leukocyte Antigen* (Antígeno Leucocitário Humano)
IFN-γ	Interferon-gama
IL	Interleucina
LMC	Leishmaniose Mucocutânea
LPG	Lipofosfoglicano
LT	Leishmaniose Tegumentar
LV	Leishmaniose Visceral
LZ	Leishmanização
mDC	*Myeloid Dendritic Cells* (Células Dendríticas mielóides)
MenB	Meningococo B
MHC	*Major Histocompatibility Complex* (Complexo Principal de Histocompatibilidade)
MST	*Montenegro Skin Test* (Intradermorreação de Montenegro
NK	*Natural Killer cells* (Células natural killer)

NNN	McNeal, Novy & Nicole medium
NTDs	*Neglected Tropical Diseases* (Doenças Tropicais Negligenciadas)
OMS	Organização Mundial da Saúde
PBMC	*Peripheral Blood Mononuclear Cells* (Células mononucleares do sangue periférico)
PCR	*Polymerase Chain Reaction* (Reação em cadeia da polimerase)
PFR	*Peptide Flanking Regions* (Regiões flanqueadoras de epítopos centrais)
PHA	*Phytohemagglutinin* (Fitohemaglutinina)
PPGIT	Programa de Pós-Graduação em Inovação Terapêutica
PT	*Post Treatment* (Pós-tratamento)
RNA	*Ribonucleic Acid* (Ácido ribonucléico)
RPMI	Roswell Park Memorial Institute medium
RT	*Resistant* (Resistente)
TCLE	Termo de Consentimento Livre e Esclarecido
TCM	*T Central Memory* (Linfócitos T de memória central)
TEM	*T Effector Memory* (Linfócitos T de memória efetores)
TGF	*Transforming Growth Factor* (Fator de transformação do crescimento)
Th	*T helper* (Linfócito T auxiliar)
TLR	*Toll Like Receptor* (Receptor ligado a Toll)
TNF	Tumor necrosis factor (Fator de necrose tumoral)
TR	Termo de Referência
Treg	*Regulatory T Cell* (Célula T regulatória)
UFPE	Universidade Federal de Pernambuco
WHO	*World Health Organization* (Organização Mundial da Saúde)
TAP	*Transporter Associated with Antigen Processing* (Proteína transportadora de antígenos associada com processamento)
DBMS	*Data Base Management System* (Sistema de gerenciamento de banco de dados)

SUMÁRIO

1 INTRODUÇÃO

As leishmanioses são doenças parasitárias causadas por protozoários do gênero *Leishmania*, constituindo um importante grupo de doenças tropicais negligenciadas (DTNs). Essas doenças atingem principalmente as populações mais pobres; contudo, o cenário atual indica que a doença vem ganhando espaço em áreas mais urbanas e que antes eram consideras livres. As leishmanioses estão presentes em 98 países e territórios e apresentam uma incidência anual de 2 milhões de casos. Além disso, estimativas indicam que cerca de um quarto da população mundial, que corresponde a 1,7 bilhão de pessoas, vivem em áreas de risco.

A transmissão das leishmanioses acontece por meio da picada de flebotomíneos infectados com protozoários do gênero *Leishmania*. Esses ao realizarem o respasto sanguíneo, regurgitam material contendo os protozoários em sua forma infectiva. Após a entrada no hospedeiro, os parasitas acabam por infectar, principalmente, os macrófagos e estabelecer a infecção que poderá levar ao desenvolvimento da doença. As leishmanioses apresentam um grande espectro de formas clínicas, desde formas tegumentares que acometem a pele e podem vir a curar espontâneamente, até formas viscerais potencialmente fatais, se não tratadas.

No caso da leishmaniose tegumentar (LT), a doença manifesta-se clinicamente pelo surgimento de uma ou múltiplas úlceras, em geral na região da face, que podem persistir por anos ou mesmo curar espontaneamente. A LT apresenta uma incidência anual de 0,7 a 1,2 milhão de casos reportados. A *Leishmania (Viannia) braziliensis* (*L.V. braziliensis*) é uma das principais espécies envolvidas com casos da doença em países da América Latina onde a doença é endêmica.

Apesar dos números, as opções disponíveis para controlar as leishmanioses são o controle de vetores e hospedeiros e a quimioterapia de indivíduos doentes. Porém, tais estratégias são de alto custo e estão relacionadas com o desenvolvimento de parasitas e vetores resistentes. Além disso, os agentes quimioterápicos apresentam alta toxicidade para o hospedeiro, podendo levar ao óbito em decorrência de danos causados durante o tratamento. Nesse sentido, o desenvolvimento de uma vacina contra

as leishmanioses para ser aplicada em seres humanos, mostra-se com uma das melhores opções para controlar a doença, limitar o uso de quimioterápicos ou mesmo melhorar a resposta imunológica dos indivíduos acometidos.

Análises de custo e efetividade indicam que a aplicação de uma vacina contra LT com proteção de até 10 anos e 70% de eficácia em países da América Latina poderia prevenir entre 41 e 144 mil casos de LT. Dessa maneira, os dados indicam que o custo-benefício de se aplicar uma vacina é maior do que os gastos com medicamentos. Contudo, a falta de uma vacina ideal decorre de uma série de fatores, como a complexidade do parasita e da resposta do hospedeiro.

Indivíduos que se recuperam da infecção por *L. braziliensis* podem ficar resistentes a uma nova infecção, indicando que é possível desenvolver uma vacina contra a LT. Entretanto, as vacinas com parasitas completos (ou de primeira geração) apresentam uma série de limitações em termos de produção e segurança. Assim, uma das etapas limitantes ao desenvolvimento é a identificação de antígenos molecularmente definidos que possam estimular uma resposta protetora.

Abordagens clássicas já foram utilizadas e levaram a identificação de uma série de moléculas. Algumas chegaram até fases de testes clínicos, mas se mostraram ineficazes em induzir uma resposta protetora. Esses métodos identificaram antígenos com base em sua capacidade de estimular uma resposta potente de anticorpos; no entanto, falharam em estimular uma resposta imune celular.

Nesse contexto complexo, surgem métodos alternativos para o encontro de antígenos e essa é a proposta deste trabalho de pesquisa. No primeiro capítulo apresentado, buscou-se identificar no proteoma predito de *L. braziliensis,* potenciais epítopos de linfócitos T CD4$^+$ e T CD8$^+$ por meio de uma combinação de métodos *in silico*. Uma vez identificados, as moléculas foram sintetizadas e avaliadas quanto a capacidade de estimular a proliferação de células de indivíduos com leishmaniose após cura clínica. No segundo capítulo, os epítopos foram testados com células de outros grupos de indivíduos portadores de leishmaniose ativa e indivíduos residentes em área endêmica. Além disso, foi investigado a presença do fator de transcrição T-bet e das citocinas produzidas no sobrenadante de cultura das células. No terceiro e

último trabalho apresentado, buscou-se verificar se os peptídeos induziram efeito imunológico em células dendríticas murinas. Os resultados do presente trabalho, indicam que os epítopos apresentam grande potencial de induzir uma resposta protetora contra a leishmaniose tegumentar.

2 OBJETIVOS

2.1 Geral

- Identificar no proteoma de *Leishmania (Viannia) braziliensis* potenciais epítopos de linfócitos T CD4$^+$ e T CD8$^+$ e avaliá-los imunologicamente.

2.2 Específicos

- Pesquisar epítopos imunogênicos no proteoma predito de *L.V. braziliensis* utilizando ferramentas de bioinformática;
- Testar a estabilidade dos epítopos frente à moléculas de MHC de classe I e II utilizando o docagem molecular;
- Avaliar a capacidade dos peptídeos sintéticos em estimular a proliferação de células mononucleares humanas (PBMCs) de indivíduos com leishmaniose cutânea em sua forma ativa, após o tratamento e indivíduos resistentes à infecção;
- Detectar e quantificar citocinas dos perfis Th1, Th2, e Th17 no sobrenadante de cultura de PBMC dos indivíduos envolvidos na pesquisa;
- Avaliar os níveis de expressão do fator de transcrição T-bet em linfócitos T CD4$^+$ e T CD8$^+$ de pacientes com leishmaniose tegumentar após o tratamento;
- Avaliar a expressão dos marcadores de superfície MHC I, MHC II, CD11c e CD40 em células dendríticas mielóides murinas (BMDC) estimuladas, *in vitro*, com os peptídeos e a produção de citocinas dos perfis Th1, Th2 e Th17 nos sobrenadantes de cultura;
- Vacinar camundongos BALB/c com os peptídeos e após a vacinação avaliar a expressão de CD11b, CD8α, CD103, IL-12p70, CD317, CD205 e MHC II em células esplênicas.

3 REVISÃO DE LITERATURA

3.1 Leishmanioses

As leishmanioses são consideradas um grupo de doenças infecciosas parasitárias causadas por protozoários do gênero *Leishmania*, transmitidos por fêmeas de flebotomíneos infectados (MURRAY *et al.*, 2005; “WHO | Leishmaniasis”, 2015). Essas doenças podem ser transmitidas ao homem e a outras espécies de mamíferos e, portanto, possuem um complexo ciclo de vida envolvendo os vetores e espécies animais que funcionam como reservatórios naturais (ASHFORD, 1996; READY, 2013). Devido a essas características, as leishmanioses são consideradas hoje um dos grupos de doenças tropicais negligenciadas (DTNs) mais importantes em termos de distribuição mundial e grande impacto econômico e social nas populações afetadas.

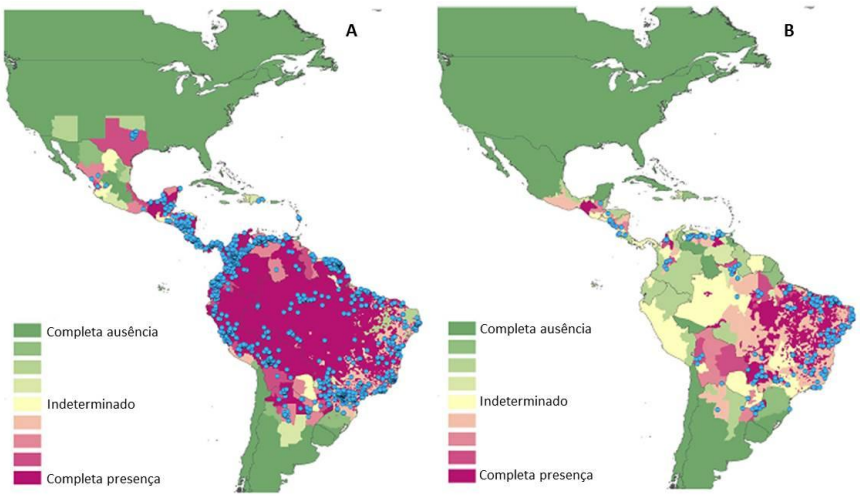
3.1.1 Distribuição, impacto econômico e social das leishmanioses

As leishmanioses estão presentes em diferentes partes do globo (Figuras 1 e 2) e de acordo com a Organização Mundial da Saúde (OMS), estima-se que há transmissão endêmica das leishmanioses em 98 países, em 3 territórios localizados em 5 continentes (ALVAR *et al.*, 2012). Recentemente, Pigott *et al.* (PIGOTT *et al.*, 2014) estimaram que o número de pessoas vivendo em áreas de risco de transmissão é de cerca de 1,7 bilhões de pessoas, ou seja, um quarto da população mundial. Nos últimos anos, o número de regiões endêmicas para as leishmanioses tem aumentado e, consequentemente, o número de casos subnotificados da doença é cada vez maior (ALVAR *et al.*, 2012; BOETTCHER *et al.*, 2015; OKWOR; UZONNA, 2016a).

Em termos de distribuição, há casos de leishmaniose tegumentar (LT) notificados nas Américas, no Mediterrâneo, no Oriente Médio e na região central da Ásia. Nos últimos anos, têm-se observado um aumento significativo no número de indivíduos acometidos em regiões de guerra civil, especialmente na Síria e no Afeganistão (HOTEZ *et al.*, 2012). Já a leishmaniose visceral (LV), que é fatal se não tratada corretamente, está presente em 6 países que respondem por 90% dos casos: Bangladesh, Brasil, Etiópia, Índia, Sudão do

Sul e Sudão (HOTEZ; FUJIWARA, 2014). Outra forma clínica das leishmanioses é a mucocutânea (LMC), na qual os casos são mais concentrados na América do Sul. A LMC acomete principalmente as regiões de mucosa do nariz, boca e garganta (ALVAR *et al.*, 2012).

Figura 1 : Distribuição dos casos de leishmaniose cutânea (A) e visceral (B) no Novo Mundo.



Fonte: PIGOTT *et al.* 2014

Nota: O gradiente de cor indica áreas onde a doença está ausente, indeterminada ou presente. A cor verde escuro indica a completa ausência da doença, a cor amarela indica que para aquela área ainda é indeterminada a presença de casos da doença e a cor rosa indica completa presença da doença naquela área. As áreas em azul indicam pontos de ocorrência da doença.

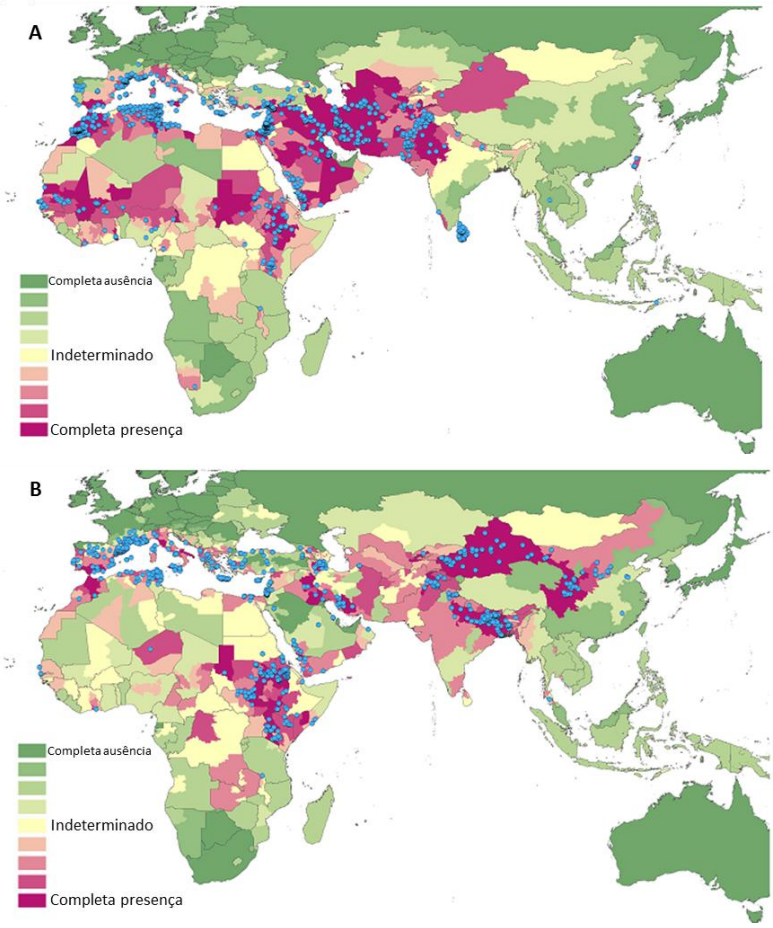
As leishmanioses apresentam alta taxa de morbidade e mortalidade e no ano de 2010 cerca de 50 mil mortes ocorreram em decorrência das leishmanioses (LOZANO *et al.*, 2012). Outra medida estima que as leishmanioses são a terceira causa mais importante em anos de vida perdidos e vividos com incapacidades (do inglês, DALY, *Disability Adjusted Life Years*) (SAVOIA, 2015).

Em termos econômicos, o impacto das leishmanioses se traduz por uma perda expressiva da produtividade, sobretudo de produtores rurais (SARNOFF *et al.*, 2010; THAKUR, J. *et al.*, 2011). Isto ocorre em decorrência da incapacidade de trabalhar que é muitas vezes imposta pela doença, redução

na eficácia do trabalho ou mesmo devido a presença de numerosas lesões que desfiguram e descaracterizam o indivíduo (HOTEZ *et al.*, 2009).

Vários fatores de risco estão presentes em populações com alta prevalência das leishmanioses, tais como: pobreza, má nutrição, mudanças climáticas e ambientais. Além disso, os processos de globalização e urbanização, acelerados nas últimas décadas, aumentou cada vez mais o contato das populações com florestas endêmicas para flebotomíneos (DESJEUX, 2001; “WHO | WER 2002 Index”, 2012).

Figura 2: Distribuição dos casos de leishmaniose cutânea (A) e visceral (B) no Velho Mundo.



Fonte: PIGOTT *et al.* 2014

Nota: O gradiente de cor indica áreas onde a doença está ausente, indeterminada ou presente. A cor verde escuro indica a completa ausência da doença, a cor amarela indica que para aquela área ainda é indeterminada a presença de casos da doença e a cor rosa indica completa presença da doença naquela área. As áreas em azul indicam pontos de ocorrência da doença.

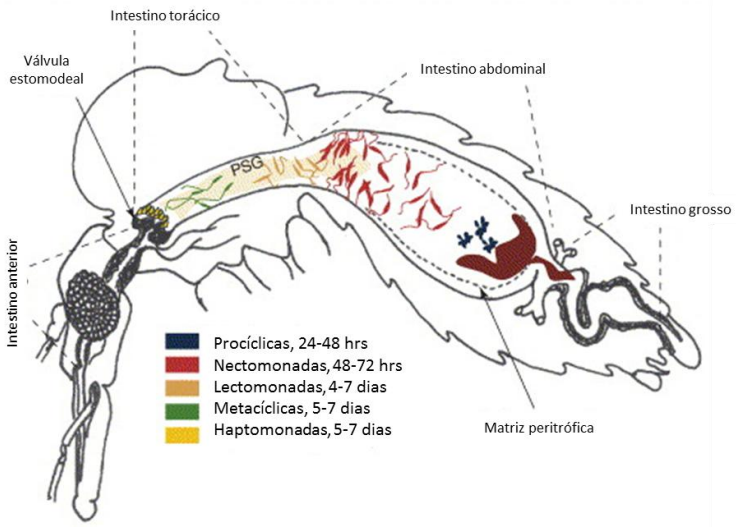
3.1.2 Ciclo de vida de *Leishmania* spp.

O ciclo de vida das leishmanias envolvem sempre uma fase nos insetos flebotomíneos e outra fase num hospedeiro mamífero. A transmissão das leishmanioses pode acontecer de forma antroponótica ou zoonótica. Os flebotomíneos do gênero *Phlebotomus* spp. e *Lutzomyia* spp. são os principais vetores transmissores do parasita no Velho e Novo Mundo, respectivamente (KILLICK-KENDRICK, 1999). Outras formas mais raras de transmissão dos parasitas é por meio de acidentes de laboratório ou seringas contaminadas (FELINTO DE BRITO *et al.*, 2012).

Os dados mais atuais mostram que mais de 90 espécies de flebotomíneos são conhecidos como transmissores dos parasitas para mais de 70 espécies animais que podem atuar como reservatórios naturais, incluindo a espécie humana. Até o momento cerca de 20 espécies do protozoário atuam como agentes etiológicos da leishmaniose em humanos (BEAUMIER *et al.*, 2013). O parasita passa por duas formas principais ao longo de seu ciclo de vida: promastigota e amastigota. As promastigotas, são a forma flagelada e infectiva do parasita, encontrada primeiramente nos insetos. Esta forma sofre alterações morfológicas, até se transformar nas promastigotas metacíclicas, que são a forma infectiva do parasita transmitidas durante a picada do flebotomíneo (Figura 3) (DOSTÁLOVÁ; VOLF, 2012).

O ciclo tem início no intestino dos flebotomíneos quando há proliferação das promastigotas prociclícas. Estas por sua vez se diferenciam em promastigotas metacíclicas, infectivas, que não são capazes de se dividir e se alojam na válvula estomodeal. Durante o repasto sanguíneo, os flebotomíneos regurgitam as promastigotas metacíclicas e também uma gama de moléculas presentes na saliva que possuem efeitos imunomodulatórios, como os proteofosfoglicanos (KAYE; SCOTT, 2011b).

Figura 3: Diferentes formas evolutivas do parasita no intestino do flebotomíneo vetor.

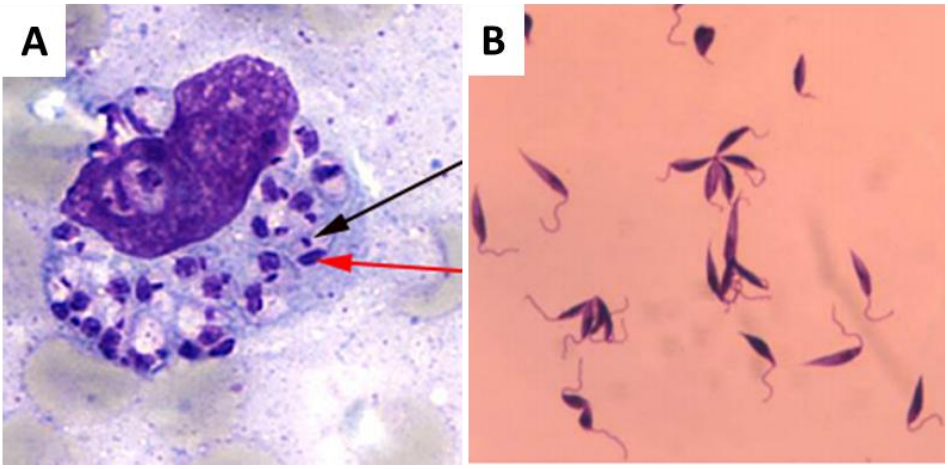


Adaptado de (KAMHAWI, 2006)

Nota: As formas procíclicas (azul) são encontradas na porção posterior do intestino do flebotomíneo 24h a 48h após a entrada das leishmanias. Entre 48 a 72h os parasitas se encontram na forma nectomonadas (vermelho), em seguida com 4 a 7 dias nas formas lectomonadas (laranja). As lectomonadas posteriormente evoluem para formas metacíclicas (verde) 5 a 7 dias após a entrada inicial das leishmanias.

As promastigotas (Figura 4-B) metacíclicas são rapidamente fagocitadas por diferentes tipos celulares presentes no microambiente tecidual. No meio intracelular, dentro de vesículas fagocíticas, as promastigotas se transformam em amastigotas aflageladas. As amastigotas (Figura 4-A) são capazes de se proliferar no interior do vacúolo fagocítico até que haja rompimento da célula contendo grande número de parasitas. Assim, ocorre a infecção de outras células circunvizinhas. O ciclo somente estará completo quando um flebotomíneo realizar o repasto sanguíneo e capturar células infectadas com amastigotas. Essas últimas se diferenciam no intestino do inseto, reiniciando o ciclo (Figura 5) (WHEELER; GLUENZ; GULL, 2011).

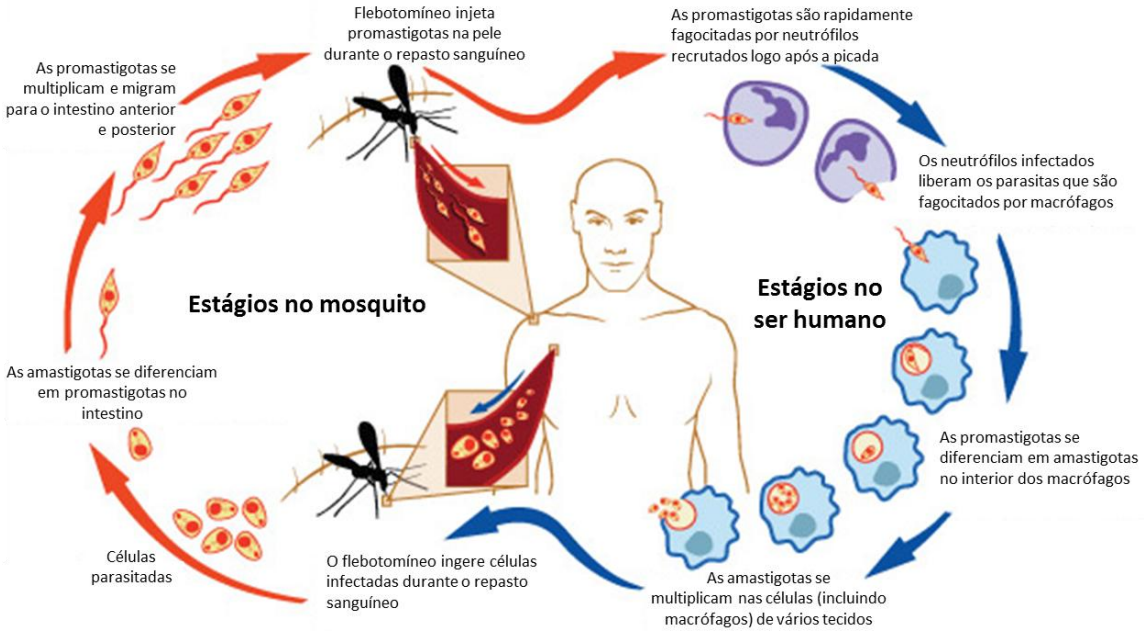
Figura 4: Formas amastigota (A) e promastigota (B) de *Leishmania* spp.



Fonte: (“CDC - DPDx - Leishmaniasis”, 2013)

Nota: A seta em vermelho indica a presença do núcleo da amastigota e a seta em preto indica a presença do cinetoplasto, estrutura importante de ser encontrada para confirmar o diagnóstico.

Figura 5: Ciclo de vida das *Leishmanias*.



Adaptado de: (“Leishmaniasis Life Cycle”)

3.1.3 Infecção e mecanismos de evasão por *Leishmania* spp.

Logo após a picada do flebotomíneo e injeção das promastigotas, os neutrófilos desempenham um papel importante para o estabelecimento da infecção. Essas células rapidamente fagocitam as promastigotas metacíclicas

e, assim, permitem que elas ganhem acesso ao interior celular. De outra maneira, a fagocitose das promastigotas por macrófagos teciduais induziria ativação celular e consequente destruição dos parasitas (LASKAY; VAN ZANDBERGEN; SOLBACH, 2003). Em seguida, as promastigotas induzem apoptose de neutrófilos com formação de corpos apoptóticos que são fagocitados por macrófagos teciduais, sem que aconteça ativação destes últimos (RAVICHANDRAN; LORENZ, 2007).

Dessa maneira, os neutrófilos têm sido implicados como células cruciais para o estabelecimento da infecção inicial pelas leishmanias. Contudo, apesar dos neutrófilos conseguirem fagocitá-las, eles não possuem o ambiente favorável para sua proliferação e sobrevivência por mais tempo (PETERS, N. C. *et al.*, 2009b). As células dendríticas (DCs) dérmicas fagocitam os parasitas nas primeiras horas após a infecção inicial, enquanto que os macrófagos dérmicos também aparecem infectados, sendo a população dominante cerca de 24 horas após o início da infecção (NG *et al.*, 2008). Um dos possíveis mecanismos de evasão da resposta imune acontece logo após a fagocitose de neutrófilos infectados e ou apoptóticos por DCs no sítio inicial de entrada dos parasitas, uma vez que foi verificado o bloqueio da atividade de co-apresentação de antígenos pelas DCs (RIBEIRO-GOMES *et al.*, 2015).

Outros tipos celulares também têm sido implicados no estabelecimento da infecção, uma vez que as DCs e os macrófagos dérmicos estão presentes em baixo número que justificasse o desenvolvimento de uma lesão. Acredita-se que estas células recrutem monócitos que por sua vez se diferenciam em células dendríticas derivadas de monócitos (moDCs). Este tipo celular apresenta um papel dual que pode tanto favorecer a infecção produtiva ou a resposta imunológica individual (LEÓN; LÓPEZ-BRAVO; ARDAVÍN, 2007; CHARMOY *et al.*, 2010).

As moDCs são células com atividade fagocítica intensa e podem favorecer o crescimento e sobrevivência das leishmanias em seu interior, justificando o elevado número de células infectadas necessárias para o surgimento da doença. Em contrapartida, as moDCs são capazes de expressar grandes quantidades de moléculas do complexo principal de histocompatibilidade classe II (MHC de Classe II) em sua superfície e de produzir e secretar IL-12, uma citocina crucial para uma resposta protetora do

tipo Th1 (LEÓN; LÓPEZ-BRAVO; ARDAVÍN, 2007; DE TREZ *et al.*, 2009). As diferentes espécies de *Leishmania* spp. podem induzir diferentes efeitos em DCs, modificando a capacidade de apresentação de antígenos e favorecendo a evasão do parasita. Nesse sentido, foi observado que *L. infantum* após infectar DCs humanas é capaz de prevenir a apoptose nesse tipo celular, favorecendo a migração dos parasitas para outros sítios orgânicos. Esse mesmo efeito não foi verificado após infecção de DCs com *L. braziliensis*, que não foi capaz de prevenir a apoptose das DCs (FALCÃO *et al.*, 2016).

Além disso, outros tipos celulares também têm sido encontrados infectados por diferentes espécies de *Leishmania* spp. Inicialmente, isto foi observado com *L. major* infectando fibroblastos da pele e de linfonodos (BOGDAN *et al.*, 2000). Sugere-se que a infecção destes outros subtipos celulares está relacionado com mecanismos de evasão do sistema imunológico. Tal fenômeno também foi observado em outras formas clínicas de leishmaniose causada por espécies distintas (SVENSSON *et al.*, 2004). Outros mecanismos de evasão utilizados estão relacionados na tabela seguinte.

Tabela 1: Alvos e efeitos biológicos dos mecanismos de evasão utilizados por *Leishmania* spp.

Alvo	Efeito biológico induzido
Sistema complemento	Os parasitas em sua forma metacíclica previnem a inserção do complexo C5-C9 de ataque na membrana (PUENTES *et al.*, 1990). A proteína GP63 na superfície dos parasitas é essencial para esse mecanismo (BRITTINGHAM *et al.*, 1995).
Fagocitose	A molécula de lipofosfoglicano (LPG) favorece a ligação do parasita a proteínas da membrana do macrófago (BLACKWELL *et al.*, 1985), tais como os receptores de manose/fucose, a proteína reativa ao complemento (CULLEY *et al.*, 1996) e a CR4 (TALAMÁS-ROHANA *et al.*, 1990).
Fagossomo	Parasitas retardam ou previnem a maturação dos endossomos e expressão tardia de Rab-7 ou LAMP-1 (OLIVIER; GREGORY; FORGET, 2005). O LPG previne a maturação dos endossomos induzindo acúmulo de actina-F. As amastigotas do parasita induzem ativação de transportadores de ferro LIT1 e LIT2 para sobrevivência (KAYE; SCOTT, 2011a).
Receptores semelhantes a Toll (TLRs)	*L. major* induz a diminuição das citocinas induzidas pela ativação do TLR2 (DE VEER *et al.*, 2003). As promastigotas de *L. donovani* bloqueiam a liberação de IL-12 e TNF via TLR2 (SRIVASTAV *et al.*, 2012).
Apresentação de antígenos	Sequestro de antígenos e interferência no processo de carregamento de antígenos nas moléculas de MHC de Classe II (KIMA *et al.*, 1996; PRINA *et al.*, 1996). *Leishmania donovani* aumenta a fluidez de rafts de lipídeos da membrana dos macrófagos, reduzindo a eficiência da apresentação de antígenos (CHAKRABORTY *et al.*, 2005). As moléculas de MHC de Classe II são importantes para resistência aos parasitas, mas não as moléculas de MHC de Classe I (HUBER *et al.*, 1998; LOCKSLEY *et al.*, 1993).

Citocinas e quimiocinas	Supressão de IL-12 e indução de IL-10 em monócitos e macrófagos (BELKAID *et al.*, 2000). *Leishmania* inibe os receptores de quimiocina CCR4 e CCR5 (PINHEIRO *et al.*, 2006); inibe as moléculas de adesão E-selectina, ICAM-1, VCAM-1 (LO *et al.*, 1998); inibe a expressão de CCL2 (RITTER *et al.*, 1996).
Resposta de linfócitos T	Indução de resposta do tipo Th2 (SILVA, V. M. G. *et al.*, 2011; TABATABAEE *et al.*, 2011), indução de células T regulatórias (Treg) produtoras de IL-10 e TGF-β em modelos animais e também em humanos (PETERS, N.; SACKS, 2006; RAI *et al.*, 2012).

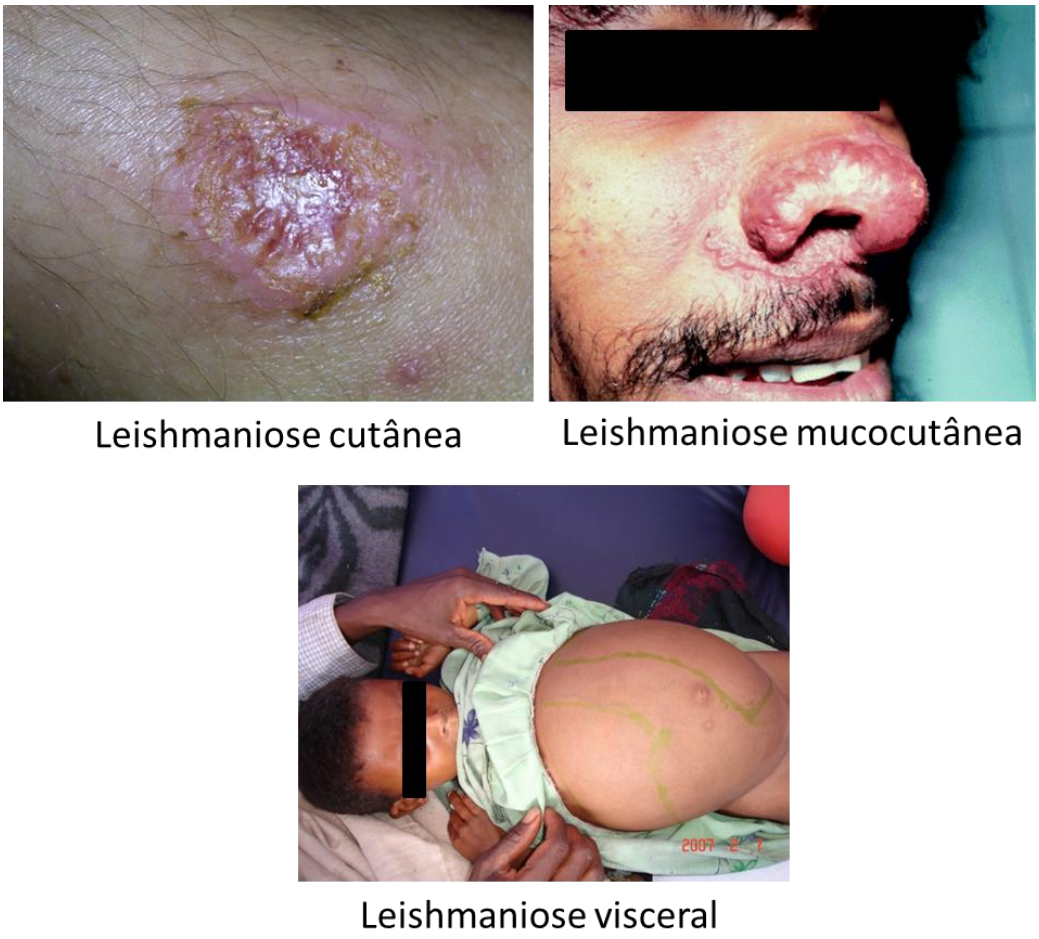
3.1.4 Formas clínicas das leishmanioses

As leishmanioses são doenças que podem ter diferentes apresentações clínicas, desde formas tegumentares que podem acometer pele e mucosas, até formas viscerais potencialmente fatais se não tratadas (CARVALHO *et al.*, 2012). Anualmente, estima-se que ocorram 0,2 – 0,4 e 0,7 – 1,2 milhões de casos das formas visceral (LV) e tegumentar (LT), respectivamente (ALVAR *et al.*, 2012), e de 20 a 30 mil mortes ("WHO | Leishmaniasis", 2015). A Figura 6 abaixo ilustra algumas dessas formas principais de apresentação clínica das leishmanioses.

A leishmaniose cutânea ou tegumentar (LT) é considerada a forma mais branda da doença e apresenta ampla distribuição em países e territórios do Velho e Novo Mundo (GOTO, H.; LAULETTA LINDOSO, 2012). A lesão da LT pode se manifestar de diferentes maneiras, como uma úlcera ou nódulo único próximo ou na região de picada do flebotomíneo, infecção subclínica, ou mesmo disseminar para áreas diferentes do corpo (GOLLOB, KENNETH J. *et al.*, 2008). A LT geralmente acomete áreas do corpo expostas ao vetor transmissor, tais como rosto, antebraço e pernas. A lesão na LT, se não tratada adequadamente, pode perdurar por semanas a meses e curar espontaneamente em alguns casos.

Contudo, a depender de uma série de fatores, tais como genética individual, status imunológico do indivíduo e da espécie do parasita, a doença pode evoluir para a leishmaniose cutâneo difusa (ROGERS *et al.*, 2002). A *Leishmania amazonensis* tem sido implicada na maior parte destes casos, nos quais o indivíduo apresenta lesões nodulares de variados tamanhos em diferentes locais do corpo. Em contrapartida, casos de LT causados por *L. panamensis* e *L. braziliensis* podem progredir para uma forma mais agressiva e mutilante da doença, acometendo as mucosas, a leishmaniose mucocutânea (LMC) (DAVID; CRAFT, 2009).

Figura 6: Diferentes formas clínicas das leishmanioses, leishmaniose cutânea, mucocutânea e visceral.



Fonte de: ("WHO | Cutaneous leishmaniasis", 2010, "WHO | Photos on leishmaniasis for download", 2016)

A LMC é uma manifestação clínica que ocorre meses ou anos após a lesão cutânea em decorrência de um aumento ou expansão da área acometida pela lesão cutânea inicial, atingindo as mucosas (AMATO *et al.*, 2008). A LMC é considerada a forma clínica mais desfigurante, resultado de destruição crônica e progressiva de tecidos do nariz, boca, oro e naso-faringe e pálpebras. A doença pode progredir afetando a função respiratória e dificultar a nutrição do indivíduo acometido (MCGWIRE; SATOSKAR, 2014). A LMC é principamente causada pela *L. braziliensis* e os fatores que resultam na patogênese dessa forma clínica ainda não são bem compreendidos, mas provavelmente envolvem fatores do próprio parasita e fatores individuais (DE OLIVEIRA, C. I.; BRODSKYN, 2012).

A grande parte dos casos de LMC acontece em países da América do Sul, principalmente no Brasil, mas também no Peru e Bolívia. Os casos da doença também têm sido notificados na Colômbia, Equador, Paraguai e Venezuela (CALVOPINA; ARMIJOS; HASHIGUCHI, 2004). Em geral, o prognóstico da doença não é bom, uma vez que a maior parte dos casos é refratária ao tratamento medicamentoso. Assim, muitos indivíduos acometidos vêm a óbito em decorrência de infecções secundárias ou mesmo má nutrição. No Brasil, a incidência desta forma clínica em regiões endêmicas não tem ultrapassado os 5% (BRASIL, 2007; SAVOIA, 2015).

A leishmaniose visceral (LV) ou Kala-azar é considerada a forma clínica mais letal das leishmanioses, pois diferentemente das outras formas que acometem o tegumento, essa é caracterizada por marcante visceralização. Diferentes espécies do parasita estão implicadas na doença, que se não tratada a tempo, pode levar o indivíduo a óbito. A LV é caracterizada pela metastização dos parasitas do local de infecção na pele para infecção dos fagócitos do sistema retículo endotelial, acometendo, principalmente, baço, fígado e medula óssea. Em decorrência da proliferação dos parasitas nestes sítios orgânicos, os indivíduos acometidos frequentemente apresentam hepatoesplenomegalia e supressão das funções da medula óssea (EVANS; KEDZIERSKI, 2012; JAIN; JAIN, 2014).

3.1.5 Estratégias de controle das leishmanioses

Uma vez que não há uma vacina contra as leishmanioses universalmente aceita, as principais estratégias de controle dessas doenças são o diagnóstico, a quimioterapia com antimoniais de indivíduos doentes e, por fim, o controle de vetores. No caso do diagnóstico, ele é realizado por meio de uma associação de técnicas laboratoriais, para então se chegar ao diagnóstico da doença. Dentre as técnicas mais utilizadas, estão a pesquisa de anticorpos anti-leishmania, a cultura celular de amostras clínicas do indivíduo ou a detecção de material genético do parasita (KOBETS; GREKOV; LIPOLDOVA, 2012).

Tabela 2: Distribuição global das diferentes formas clínicas das leishmanioses e as espécies do parasita envolvidas com a doença.

Forma clínica	Espécie
Velho mundo, subgênero Leishmania	
Leishmaniose visceral	*Leishmania donovani* e *Leishmania infantum*
Leishmaniose tegumentar	*Leishmania major, Leishmania tropica* e *Leishmania aethiopica*
Leishmaniose tegumentar difusa	*L. aethiopica*
Novo mundo, subgênero Leishmania	
Leishmaniose visceral	*L. infantum*
Leishmaniose tegumentar	*L. infantum, Leishmania mexicana, Leishmania pifanol* e *Leishmania amazonensis*
Leishmaniose tegumentar difusa	*L. mexicana* e *L. amazonensis*
Novo mundo, subgênero *Viannia*	
Leishmaniose tegumentar	*Leishmania braziliensis, Leishmania guyanensis, Leishmania panamensis* e *Leishmania peruviana*
Leishmaniose mucocutânea	*L. braziliensis* e *L. panamensis*

Adaptado de: (KAYE; SCOTT, 2011b; LEISHMANIASES; GENEVA, 2010)

O teste cutâneo de Montenegro (MST) ou reação de hipersensibilidade tardia ao antígeno de leishmania é muito utilizado para detecção de formas cutâneas subclínicas. Esse teste é realizado em áreas endêmicas e permite identificar indivíduos que não apresentam sinais clínicos da doença, mas que são positivos ao teste. O teste consiste na inoculação subcutânea de antígeno total inativado de leishmania (ALVES *et al.*, 2013). Em paralelo, alguns outros métodos de diagnóstico imunológico também têm sido aperfeiçoados, tais como a imunofluorescência, a citometria de fluxo, o teste rápido e a reação de aglutinação direta (DE PAIVA-CAVALCANTI *et al.*, 2015). Cada uma dessas metodologias apresentam suas vantagens e limitações ao uso, além das condições de custo que podem influenciar na escolha da técnica (DE OLIVEIRA, A. P. *et al.*, 2013; PEREIRA *et al.*, 2012).

Os testes que exploram a pesquisa do material genético do parasita por meio da reação em cadeia da polimerase (PCR) ou de suas variantes têm sido também cada vez mais utilizados como auxiliar no diagnóstico das leishmanioses (POURABBAS *et al.*, 2013; SILVA, L. A. *et al.*, [S.d.]). Além disso, o exame clínico bem realizado é de grande importância no diagnóstico das leishmanioses, uma vez que as formas tegumentares da doença podem ser confundidas com outras doenças, como hanseníase, câncer de pele, tuberculose, esporotricose, entre outras (BRASIL, 2007).

No que se refere ao tratamento, é realizado com drogas não específicas para a doença, nas quais os mecanismos de ação ainda não foram elucidados completamente (SINGH; KUMAR; SINGH, 2012). A principal classe de drogas utilizadas há mais de 70 anos são os antimoniais pentavalentes. Tais drogas são utilizadas há tanto tempo que não são raros os casos que vem surgindo nos últimos anos de parasitas resistentes (MEHEUS *et al.*, 2010; OLIVEIRA, L. F. *et al.*, 2011). Além disso, os antimoniais apresentam custo elevado, requerem repetidas doses e estão frequentemente associados com efeitos adversos importantes (SUNDAR; CHAKRAVARTY, 2010).

O controle de vetores e dos reservatórios é útil em algumas regiões sob determinadas condições, não sendo aplicável em todos os cenários epidemiológicos, pois necessitam de infraestrutura e vigilância que muitas vezes estão além da capacidade de muitos países endêmicos. Outra alternativa utilizada no controle das leishmanioses é o diagnóstico, tratamento ou eutanásia de cães com leishmaniose visceral canina (LVC) (RIBEIRO *et al.*, 2013). O tema ainda é fonte de debate por diferentes grupos que defendem o tratamento correto dos cães infectados e outros que defendem a eliminação dos animais doentes sobretudo nas áreas onde há taxas acentuadas de transmissão.

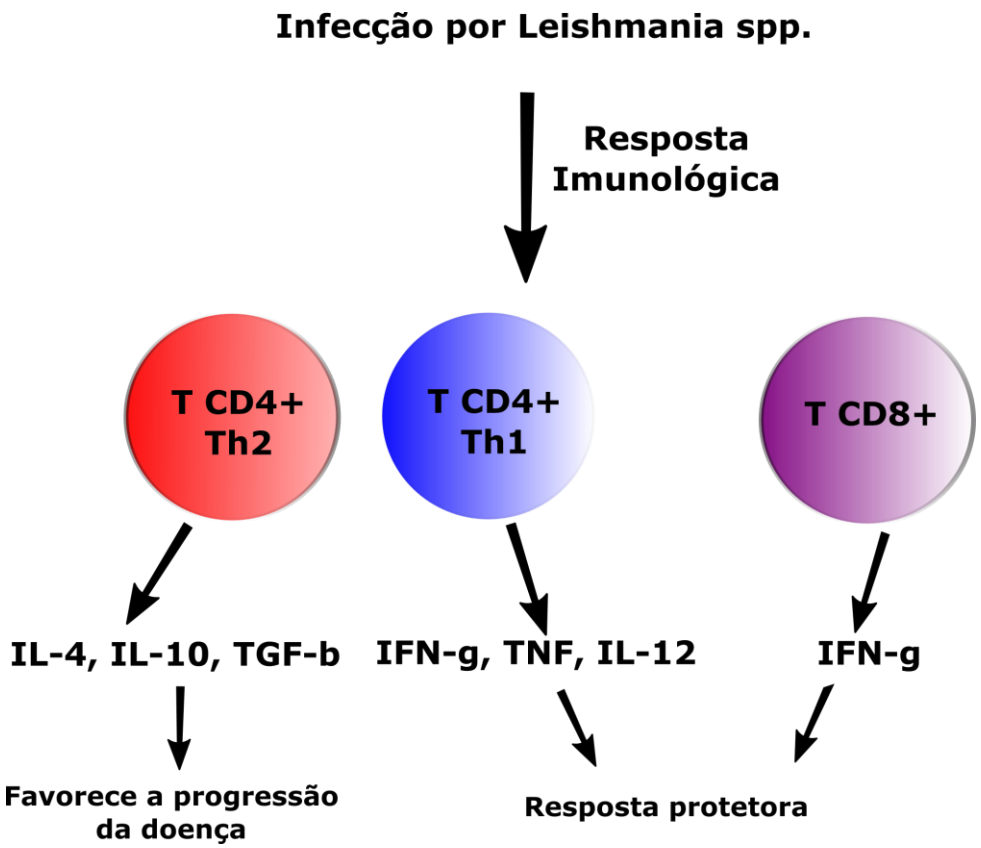
Diante disso, a vacinação é a principal alternativa para controlar todas as formas da doença (COSTA, C. H. N. *et al.*, 2011a; KEDZIERSKI, 2010). Dessa maneira, para que o desenvolvimento de uma vacina ocorra é necessário entender como ocorre a resposta imunológica nas leishmanioses e os correlatos de proteção esperados de uma vacina contra essas doenças.

3.1.6 Resposta imunológica contra *Leishmania* spp.

A resposta imunológica contra *Leishmania* spp. é mediada por interações complexas entre células e moléculas do sistema imunológico (SI) (DUTHIE *et al.*, 2012). Grande parte dos estudos relacionando os mecanismos imunológicos nas leishmanioses aconteceram em modelos murinos. Com esses modelos foi possível compreender melhor vários aspectos da resposta imunológica que governam a resistência ou susceptibilidade à infecção por *Leishmania* spp. Nesse sentido, um dos principais achados é o papel chave das citocinas pró-inflamatórias da via Th1 na resistência. e das citocinas da via Th2 associadas à susceptibilidade (DE LUCA; MACEDO, 2016a). Entretanto, os achados imunológicos em modelos murinos nem sempre foram corroborados em ensaios clínicos com seres humanos (OLIVEIRA, W. N. *et al.*, 2014).

Nesse contexto, modelos murinos vêm sendo utilizados no intuito de compreender os mecanismos imunológicos envolvidos na infecção por *Leishmania* e também auxiliando na pesquisa de drogas e vacinas. Neste sentido, o principal modelo em uso é o de infecção de camundongos com *L. major*, no qual os camundongos C57BL/6 e C3H são resistentes, já a linhagem BALB/c é susceptível. A infecção dessas linhagens com *L. amazonensis* também resulta em resistência e susceptibilidade, como ocorre com *L. major*; entretanto, a infecção de BALB/c com *L. braziliensis* é auto-limitada (MCMAHON-PRATT; ALEXANDER, 2004).

Figura 7: Perfis de resposta imunológica à infecção por *Leishmania* spp. em modelo murinho.



Fonte: o autor.

Nota: Em modelos murinos, observa-se que logo após a infecção por *Leishmania* spp., a resposta imunológica induzida pode favorecer a progressão da doença por meio da indução de um perfil de resposta com linfócitos T CD4^{+} produzindo citocinas como IL-4, IL-10 e TGF-β. Ou, a resposta imunológica pode induzir a proteção contra a doença por meio de um perfil de linfócitos T CD4^{+} e T CD8^{+} produtores, principalmente, de IFN-γ.

No caso humano, a resposta imune do indivíduo pode ter um papel favorecendo a cura da doença por meio da indução de células e produção de mediadores que favorecem a destruição do parasita ou mesmo contém sua proliferação (imunobalanço). Por outro lado, essa mesma resposta que é capaz de conter a progressão da doença e curar o indivíduo, também pode ser responsável por favorecer o parasita e consequentemente a progressão da doença (DE ASSIS SOUZA *et al.*, 2013). Nesse sentido, a presença de células como macrófagos, células NK (do inglês, *Natural Killer*), linfócitos T, e moléculas efetoras, citocinas e anticorpos, é crítica para que ocorra o controle

da doença (BIRNBAUM; CRAFT, 2011; LIESE; SCHLEICHER; BOGDAN, 2008; OGHUMU *et al.*, 2010).

No caso em particular dessas doenças, os mecanismos protetores têm sido relacionados principalmente com a presença de linfócitos T CD4+ com o perfil Th1 produzindo IFN-γ, TNF e IL-12 (Figura 7). Tais fatores têm sido associados com a ativação de macrófagos e consequente eliminação de parasitas (ENGWERDA *et al.*, 2004; ROBERTS, 2005; SOLBACH; LASKAY, 2000; SQUIRES *et al.*, 1989). Em contrapartida, citocinas do perfil Th2 (IL-4, IL-10, TGF-β) tem sido relacionadas com alta carga parasitária, inibição da produção de óxido nítrico por macrófagos ativados por IFN-γ, assim como inibição da diferenciação de linfócitos Th1 (BARATTA-MASINI *et al.*, 2007; GOMES-SILVA *et al.*, 2007), principalmente por IL-10 (GAUTAM *et al.*, 2011).

Contudo, apesar do papel-chave já bem reconhecido dos linfócitos T CD4$^+$ Th1 como mecanismo protetor nas leishmanioses, cada vez mais tem se visto que os linfócitos T CD8+ também apresentam um papel protetor importante (DA SILVA SANTOS; BRODSKYN, 2014). Além disso, foi visto que em camundongos com depleção transitória de linfócitos T CD4$^+$ suceptíveis a infecção por *L. major*, os linfócitos T CD8$^+$ foram essenciais para manter os animais resistentes à infecção (MÜLLER *et al.*, 1991; STÄGER, SIMONA; RAFATI, 2012). Parte desse papel protetor dos linfócitos T CD8$^+$ produtores de IFN-γ está ligado com a capacidade dessas células de alternar a resposta Th2 para a resposta Th1 (UZONNA; JOYCE; SCOTT, 2004). Outras vias também podem estar envolvidas, como a Th17.

Os linfócitos Th17 são produtores de algumas citocinas, tais como a IL-17, IL-22 e IL-6. O papel dessas citocinas na infecção por *Leishmania* spp. e doença ainda não está completamente claro. Contudo, os resultados de diversos trabalhos indicam que o papel dessas citocinas depende da espécie do parasita e de fatores do hospedeiro. Em humanos foi observado que a IL-17 e a IL-22 apresentam um papel protetor contra a LV causada por *L. donovani* (PITTA *et al.*, 2009). Em paralelo, outro trabalho sugere que a infecção por *L. infantum* induz a produção de IL-17a que, por sua vez, potencializa a resposta do tipo Th1 e previne expensão de células Treg e linfócitos produtores de IL-10 (NASCIMENTO *et al.*, 2015). Em modelos murinos foi observado que a IL-17 é um importante mediador da patologia da doença em camundongos C57BL/6

que não expressam IL-10 infectados com *L. major* (GONZALEZ-LOMBANA *et al.*, 2013). Já em camundongos BALB/c susceptíveis a IL-17 também foi envolvida com a progressão da doença após infecção com *L. major* (LOPEZ KOSTKA *et al.*, 2009).

Durante a investigação de uma estratégia vacinal é necessária uma compreensão dos mecanismos de resposta imunológica protetora, bem como da geração e da manutenção de células de memória imunológica. Sendo esse último um dos aspectos mais importantes e um dos mais negligenciados durante o desenvolvimento da vacina (MUTISO *et al.*, 2012). Durante uma resposta imunológica são gerados linfócitos T de memória com diferentes funções e fenótipos. Duas populações principais podem ser identificadas, os linfócitos T de memória central (TCM) que funcionam como um reservatório de linfócitos T antígeno-específicos e os linfócitos T de memória efetores (TEM), capazes de migrar rapidamente para os tecidos e produzir citocinas efetoras (COLPITTS; SCOTT, 2010). Pesquisas sugerem que a presença de linfócitos T de memória imunológica é crítica para o desenvolvimento de vacinas contra a leishmaniose (PAKPOUR; ZAPH; SCOTT, 2008; SCHROEDER; AEBISCHER, 2011).

Com base no que foi discutido, a Figura 8 abaixo sumariza alguns dos aspectos importantes esperados para uma vacina ideal contra as leishmanioses.

3.2 Vacinas contra as leishmanioses

O maior desafio para uma vacina é o de ativar corretamente o sistema imunológico do hospedeiro para que haja o desenvolvimento de uma resposta imune protetora contra um patógeno em específico. De modo geral, a formulação de uma vacina contém dois elementos essenciais: o antígeno e o adjuvante (KOCOURKOVA *et al.*, 2016). Diferentes moléculas estão sendo utilizadas como adjuvantes vacinais, por terem a capacidade de ativar o sistema imune do indivíduo e permitir uma melhor resposta ao antígeno (AWATE; BABIUK; MUTWIRI, 2013). Um dos exemplos que vem ganhando uso crescente em ensaios experimentais é o DNA CpG. Esse consiste em sequências de oligonucleotídeos com motivos citosina-fosfato-guanina não

metilados e que são capazes de estimular receptores Toll 9 (TLR-9) (GURSEL; GURSEL, 2016).

As vacinas têm seguido uma ordem de classificação com base na composição do antígeno principal. As vacinas de primeira geração são aquelas nas quais o antígeno é o parasita completo, inativado ou morto, por processos físicos e ou químicos. Já as vacinas de segunda geração são compostas por antígenos molecularmente definidos, sendo uma ou mais proteínas, por exemplo. No caso das vacinas de DNA, elas são classificadas como vacinas de terceira geração. Atualmente, somente três vacinas anti-*Leishmania* spp. foram aprovadas e licenciadas para uso. Duas destas vacinas são para humanos, incluindo uma vacina com parasita morto para imunoterapia no Brasil e uma vacina com parasito vivo no Uzbekistão; a outra vacina é composta por moléculas recombinantes e é utilizada para profilaxia em cães no Brasil (MAYRINK *et al.*, 2006; PALATNIK-DE-SOUSA, 2008; PARRA *et al.*, 2007).

Apesar dessas vacinas terem o uso aprovado pelas agências de regulação nestes países, a eficácia dessas vacinas permanece controversa, principalmente quando se compara com vacinas contra infecções bacterianas e virais. A falta de uma vacina segura, eficaz e que possa conferir profilaxia contra as leishmanioses sugere que os fatores que regulam a indução e manutenção da imunidade contra *Leishmania* spp. ainda não são bem compreendidos (SACKS, 2014). Assim, o melhor entendimento destes fatores é um passo essencial para o desenvolvimento de uma vacina efetiva contra as leishmanioses (OKWOR *et al.*, 2012).

3.2.1 Vacinas de primeira geração

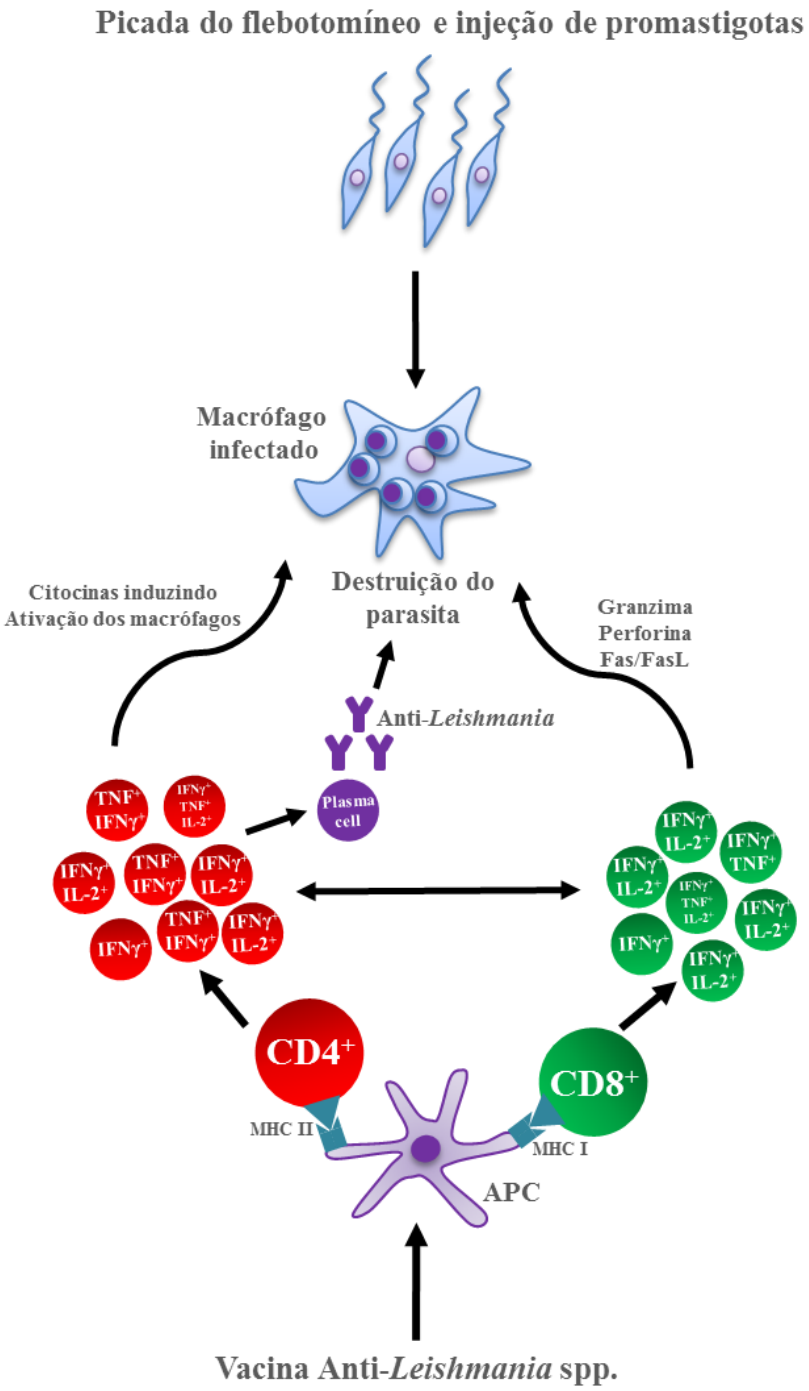
Dentre as vacinas anti-*Leishmania* spp. licenciadas para uso em humanos, uma vacina de primeira geração foi registrada no Brasil para ser utilizada como adjuvante a terapia com antimoniais. Há muito tempo o processo conhecido como leishmanização (LZ) vem sendo utilizado em populações de alto risco. Esse processo de LZ consiste na inoculação de uma cepa viva virulenta de *Leishmania major* para produzir a lesão. A LZ provou ser um método eficiente de induzir uma resposta protetora contra exposição subsequente a *Leishmania* spp (GREENBLATT, 1980; HANDMAN, 2001).

Apesar da LZ induzir uma lesão que, tipicamente, se cura espontaneamente, em alguns casos as lesões podem vir a cronificar e se tornarem de difícil tratamento (KHATAMI *et al.*, 2007; MODABBER *et al.*, 2007). Dois pontos principais são levados em consideração em vacinas utilizando parasita completo para vacina contra *Leishmania*, um destes pontos é a segurança para ser utilizado em populações humanas. A segurança é um dos critérios mais importantes, vacinas com parasitas vivos devem induzir a resposta imune protetora sem persistir por muito tempo no indivíduo vacinado (BRETON *et al.*, 2005). Outro ponto de grande importância nessas vacinas está relacionado com a padronização das condições de cultura para se obter o antígeno que são difícil para estabelecer e a infectividade dos parasitas que diminui progressivamente com os repetidos sub-cultivos (DUTHIE *et al.*, 2012; MODABBER, 2010).

Com o objetivo de testar esta hipótese, ensaios clínicos mais consistentes foram realizados no intuito de estabelecer os critérios de segurança e reprodutibilidade da LZ (KHAMESIPOUR *et al.*, 2005; NADIM *et al.*, 1997). As vacinas de primeira geração foram eficazes em algumas populações, como no Sudão (LV) e Irã (LV), mas a imunidade conferida pela LZ nestes países não foi vista no Brasil (RAFATI *et al.*, 2005). Contudo, mesmo considerando essas limitações, os pesquisadores continuam revisitando o tema e propondo novas abordagens baseadas em LZ com diferentes espécies de *Leishmania* spp (MCCALL *et al.*, 2013).

Dentre as limitações observadas no uso dos parasitas completos em vacinas, as evidências têm mostrado que as vacinas com parasitas mortos apresentam falhas na proteção contra a infecção. Isto se deve a uma possível perda na indução ou manutenção de uma resposta rápida e robusta no local de inoculação do parasita na LZ que é requerido para se previnir doenças após inoculação dos parasitas pela infecção natural por meio do vetor (PETERS, N. C. *et al.*, 2009a). Outro ponto limitante importante para as vacinas que tem por base parasitas mortos são a sua eficácia limitada para manter células de memória (OKWOR; UZONNA, 2008).

Figura 8: Infecção e mecanismo de ação desejado para uma vacina em potencial contra *Leishmania* spp.



Fonte: o autor

Nota: Uma vacina desejada contra *Leishmania* spp. deve ser capaz de ativar células apresentadoras de antígeno (APC), capazes de expressar via MHC I e MHC II antígenos do parasita e, consequentemente, ativar linfócitos T CD4$^+$ e T CD8$^+$. Parte destas células se diferenciam em produtores de IFN-γ, TNF, IL-2, entre outras citocinas. Tais mecanismos combinados da resposta imune seriam capazes de levar a destruição dos parasitas no organismo humano.

Uma vez que as vacinas que utilizam parasitas vivos atenuados ou mortos apresentam uma série de limitações por diferentes fatores, alternativas ao uso dessas foram criadas no sentido de obter melhores resultados. Neste contexto, o *targeting* de genes de virulência em *Leishmania* spp. tem sido visto como uma alternativa promissora para produção de uma vacina segura e eficaz.

Nesta categoria são incluídas as vacinas produzidas com organismos geneticamente modificados, *knock-out* de genes de virulência de *Leishmania* spp. (COSTA, C. H. N. *et al.*, 2011a), tais como dihidrofolato-redutase timidilato sintase (CRUZ; COBURN; BEVERLEY, 1991), cisteíno-proteinase (ALEXANDER, J; COOMBS; MOTTRAM, 1998; SOUZA *et al.*, 1994) ou transportador de biopterina (PAPADOPOULOU *et al.*, 2002). Esses parasitas modificados possuem um curto ciclo de vida, suficiente para induzir uma resposta imune específica suficiente para eliminar a infecção e não induzir doença no homem. Outra alternativa que tem sido vislumbrada é a introdução de cassetes suicidas no genoma de *Leishmania* spp., incluindo genes de sensibilidade a drogas. Alguns exemplos deste último são *L. major* expressando o gene da timidina kinase sensível a ganciclovir do Herpes vírus I (MUYOMBWE *et al.*, 1998) ou o gene da citosina deaminase de *Saccharomyces cerevisae* sensível ao 5-fluorocitosina (DAVOUDI *et al.*, 2005). Apesar desses parasitas não apresentarem alguns genes de virulência ou mesmo apresentarem genes de susceptibilidade a drogas, ainda se considera eticamente inaceitável o desafio de seres humanos com parasitas vivos (BORJA-CABRERA *et al.*, 2008; REQUENA *et al.*, 2004). Uma das grandes vantagens do uso desses parasitas geneticamente atenuados é que eles persistem no hospedeiro por um tempo maior, provendo antígeno em quantidade e tempo suficiente para induzir a geração de células específicas de memória imunológica que podem combater uma reinfecção (SELVAPANDIYAN *et al.*, 2012).

3.2.2 Vacinas com antígenos recombinantes

Nesse cenário, as vacinas que são baseadas no uso de antígenos recombinantes podem fornecer resultados encorajadores e amenizar dúvidas

quanto à segurança para uso em humanos e ou animais. Para isto, antígenos em potencial de espécies de *Leishmania* tem sido investigados e testados quanto a sua imunogenicidade. Os mais promissores foram a proteína 11 de membrana do kinetoplastídeo (AGALLOU; MARGARONI; KARAGOUNI, 2011; CARRILLO *et al.*, 2008), esterol 24-c-metiltransferase (GOTO, Y. *et al.*, 2007), proteína A2 específica do amastigota (GHOSH, A.; ZHANG; MATLASHEWSKI, 2001), cisteína proteinase B (RAFATI; ZAHEDIFARD; NAZGOUEE, 2006), fator de alongamento e iniciação de *L. braziliensis* (SKEIKY, Y. A. *et al.*, 1998), K26/HASPB (STÄGER, S; SMITH; KAYE, 2000), kinase C ativada de *Leishmania* (BENHNINI *et al.*, 2009), antígeno 2 da superfície de promastigota (HANDMAN *et al.*, 1995), nucleosídeo hidrolase (AL-WABEL *et al.*, 2007) e glicoproteína expressa de superfície gp63 (CONNELL *et al.*, 1993).

Algumas vacinas foram formuladas com base na combinação de uma série de moléculas diferentes, tal como a Leish-111F/MPL-SE. Esta vacina é composta pelo homólogo de *L. major* do antioxidante tiol-específico de eucarioto, proteína 1 de estresse de *L. major* e fator de iniciação e alongamento de *L. braziliensis*, combinados com o adjuvante MPL-SE. Em modelos murinos de LC e LV, Leish-111F/MPL-SE induziu proteção (COLER *et al.*, 2007; SKEIKY, Y. A. . *et al.*, 2002), mas não protegeu contra LV canina (GRADONI *et al.*, 2005). Nesta última, o desafio com o antígeno foi realizado pela picada do flebotomíneo.

A vacina Leish-111F/MPL-SE demonstrou ser segura, imunogênica e reatogênica em voluntários nos EUA e em pacientes de LC e LM no Brasil e Peru, respectivamente (GHALIB; MODABBER, 2007). Em contrapartida, a vacinação com os componentes da Leish111f, com a proteína de choque térmico 83 (Lbhsp83) e GM-CSF, combinado à quimioterapia, induziu uma melhora clínica na resposta de pacientes e na cura de seis pacientes com LM (BADARO *et al.*, 2006).

A histona H1 que protegeu camundongos (SOLIOZ *et al.*, 1999) e macacos contra LC (MASINA *et al.*, 2003), a HASPB1 (proteína de superfície acetilada hidrofílica B1) ou ambos em combinação com Montanide (MORENO *et al.*, 2007), e a proteína Q, um antígeno quimérico composto por cinco fragmentos das proteínas ácida ribossomal Lip2a, Lip2b, P0 e a histona H2A

utilizada com BCG induziu proteção parcial contra LV em cães (MOLANO *et al.*, 2003).

3.2.3 Vacinas de DNA

A imunização genética emergiu como uma boa alternativa para induzir imunidade específica em hospedeiros. As diversas vantagens deste método são a expressão seletiva de genes muito próximos a sua conformação nativa natural, indução de resposta imune celular específica, persistência da expressão do antígeno por um período de tempo, e, por fim, indução de células de memória para proteger contra reinfecção (TANG; DEVIT; JOHNSTON, 1992). Uma importante característica da vacinação com DNA é a indução de linfócitos T CD8$^+$ citotóxicos e T CD4$^+$ auxiliares e a produção de anticorpos (DAS, A.; ALI, 2012).

Para tal, diferentes antígenos têm sido utilizados para imunização de camundongos com proteínas ribossomais ou seus genes em formulações para vacina de DNA. Para isso, Masih *et al.* (MASIH; ARORA; VASISHTA, 2011) investigaram a eficácia do gene P1 de *L. donovani* na vacina de DNA pVAX-P1. Esta vacina, administrada com reforço, foi capaz de imunizar hamsters e proteger contra a infecção por *L. donovani* reduzindo a mortalidade e a carga de parasitas no baço e induziu um aumento da expressão de citocinas de resposta Th1. As análises de bibliotecas de cDNA de *Leishmania* permitiram a identificação de diferentes antígenos, tais como as histonas (MELBY *et al.*, 2000). Com base nisso, Carrión (CARRIÓN, 2011) *et al.* (CARRIÓN; FOLGUEIRA; ALONSO, 2008) demonstrou a contribuição de diferentes vacinas de DNA construídas com genes de histonas (H2A, H2B, H3, H4) na proteção de BALB/c contra a infecção por *L. major.*

Os resultados demonstraram que a vacinação com histonas H3 e H4 reduziram o edema na pata infectada com *L. major*, a carga de parasitas nos linfonodos popliteais e baço. Com base nisso, as histonas H3 e H4 podem ser consideradas candidatos vacinais em potencial. A combinação de vacinas DNA-proteínas também tem demonstrado ser uma alternativa promissora, e tal metodologia foi utilizada por Todolí *et al*. (TODOLÍ *et al.*, 2012) utilizando

diferentes estratégias para vacinação com DNA e com as proteínas KMPII, TRYP, LACK, e PAPLE22.

Com isso, este trabalho comparou a vacinação com estas proteínas e DNA individualmente ou em combinação, e os resultados indicaram que uma estratégia DNA-proteína parece ser muito mais eficaz para induzir uma resposta imunológica contra LV quando comparado à proteína ou DNA administrados individualmente. Em outros modelos animais diferentes do murino, a imunização com antígenos de *Leishmania* spp. em uma vacina de DNA não parece proteger contra leishmaniose. Em 2007, Rodríguez-Cortés *et al*. (RODRÍGUEZ-CORTÉS *et al.*, 2007) desenvolveram uma vacina de DNA multiantigênica contendo KMPII, TRYP, LACK e GP63 para proteger cães contra LV causada por *L. infantum*. Os cães foram imunizados e foi realizado o desafio com *L. infantum*, contudo, nem resposta celular nem humoral foram detectadas contra os antígenos.

Recentemente, alguns autores testaram uma vacina de DNA contra o receptor de hemoglobina do parasita (HbR) em modelo murino de LV (GUHA *et al.*, 2013). As leishmanias não são capazes de sintetizar o grupo heme, assim, elas utilizam o HbR para capturar a hemoglobina e também em reações metabólicas. Por essas propriedades, a vacina de DNA com o gene do HbR demonstrou proteção em hamsters e camundongos contra o desafio com *L. donovani* sem o uso de adjuvantes. Contudo, deve-se ficar claro que vacinas de DNA não demonstraram utilidade em humanos e por isso impede o desenvolvimento de outras etapas para produção de uma vacina.

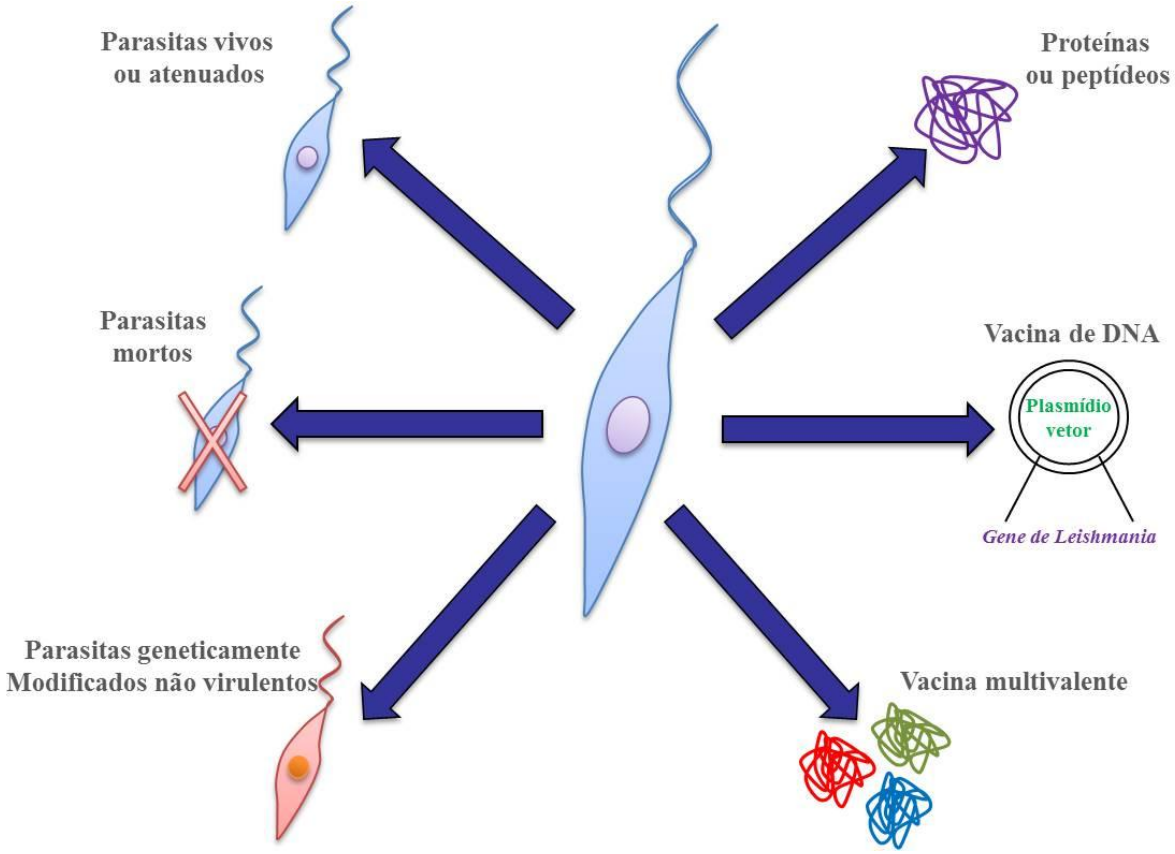
Atualmente, vacinas que são seguras para uso em humanos e possuem em sua composição bactérias ou vírus vivos e atenuados são aceitas para uso em humanos e animais. Com base nisto, estas vacinas tem sido testadas como sistemas vetoriais para entrega de antígenos de outros patógenos, como de parasitas (SAXENA *et al.*, 2013). Neste sentido, Breton *et al.* (BRETON *et al.*, 2005) obtiveram resultados que indicam que *L. tarentolae* pode ser utilizada como um bom candidato para vacina viva que consegue chegar até células dendríticas e órgãos linfóides, dando suporte, assim, para apresentação de antígenos e para geração de resposta celular e de memória.

Acredita-se que a exposição contínua a antígenos seja uma etapa crucial para o desenvolvimento de células T de memória (ZINKERNAGEL *et al.*,

1996). Apesar disso, outros parâmetros parecem influenciar nesta resposta, tais como o tipo de células que são ativadas e o nível e duração da estimulação (ZAPH *et al.*, 2004). As vacinas com parasitas mortos são limitadas devido sua ineficácia em manter células de memória, contudo, Okwor *et al*. (OKWOR; KURIAKOSE; UZONNA, 2010) demonstraram que injeções repetidas de parasitas mortos pode resultar na expansão de populações de células de memória resultando em proteção contra o desafio com vírus.

Neste contexto, as principais estratégias utilizadas para vacinas contra as leishmanioses estão ilustradas na Figura 9. A Tabela 4 relaciona alguns dos principais candidatos vacinais para leishmaniose e o andamento dos ensaios clínicos.

Figura 9: Principais estratégias utilizadas para uma vacina anti-*Leishmania* spp.



Fonte: o autor

3.2.4 Papel das células dendríticas e vacinas que utilizam células dendríticas

A resposta imune contra a leishmaniose é orquestrada pelas DC, pois essas células se destacam como uma importante classe de células apresentadoras de antígeno para o sistema imunológico (GORAK; ENGWERDA; KAYE, 1998; LEÓN; LÓPEZ-BRAVO; ARDAVÍN, 2007; LIU; UZONNA, 2010). A regulação da atividade das DC parece ser espécie específica, visto que algumas espécies de *Leishmania*, como a *L. major* e a *L. donovani* são capazes de ativar as DC para expressarem moléculas co-estimulatórias e produzir IL-12 (GHOSH, M. *et al.*, 2006; WOELBING *et al.*, 2006). Em contrapartida, algumas espécies de *Leishmania* induzem uma ativação deficiente das DC ou mesmo falham neste processo, como a *L. amazonensis* e a *L. mexicana* (BENNETT *et al.*, 2001; XIN; LI; SOONG, 2007). Já a *L.V.braziliensis* está associada com forte indução de resposta imune, iniciada principalmente pela ativação de DC e consequente ativação de linfócitos T efetores (VARGAS-INCHAUSTEGUI, D. A.; XIN; SOONG, 2008).

Neste contexto de ativação celular, diversos trabalhos têm utilizado DC estimuladas com antígenos como estratégia no combate a diferentes tipos de doenças infecciosas e câncer (BROWNING, 2013; GARCÍA *et al.*, 2013). Todavia, ainda é limitado o número de estudos que têm se concentrado no desenvolvimento e investigações de vacinas para a leishmaniose utilizando as DC como modelos celulares carreadores de antígeno (COSTA, C. H. N. *et al.*, 2011a; KEDZIERSKI, 2011). As DC são derivadas de precursores medulares e são encontradas nos tecidos do organismo em um estado imaturo. Ao entrar em contato com antígenos que induzem sua ativação, as DC amadurecem e passam a expressar diversos marcadores de superfície, como o MHC de classe II e moléculas co-estimulatórias (OLEX *et al.*, 2010).

Apesar de serem capazes de iniciar e direcionar a resposta imune de maneira eficiente, os fatores que governam os mecanismos de ação das DC ainda não são bem compreendidos. Evidências experimentais indicam que uma das maneiras pelas quais as DC agem é por meio da indução dos diferentes subtipos efetores de linfócitos T helper (Th) (KLECHEVSKY *et al.*, 2008), sendo os linfócitos Th1, produtores de INF-γ, associados com a resistência à infecção

por *Leishmania* (DARRAH *et al.*, 2010). A sinalização via Toll Like Receptors (TLR) é uma das responsáveis por induzir a secreção de citocinas pelas DC, estimulando, assim, uma resposta do tipo Th1 (SOONG, 2008). Uma das moléculas que se ligam ao TLR-9 é o DNA CpG e trabalhos realizados por outros grupos de pesquisa demonstraram que a utilização de DNA CpG com parasitas de *Leishmania* vivos ativam as DC dérmicas a produzirem citocinas, especialmente a IL-6 (WU; WEIGAND; MENDEZ, 2009). A IL-6 induz diferentes efeitos, agindo como fator de desenvolvimento para linfócitos, células mesangiais e linfócitos Th17.

Uma das grandes limitações na área de vacinologia é o encontro de antígenos ideais para o trabalho. Neste sentido, a área de vacinas se desenvolveu com o uso de métodos clássicos para o encontro de antígenos. Contudo, estes métodos apresentam uma série de limitações que dificultam o processo como um todo. Dessa maneira, métodos modernos de investigação aplicando ferramentas de bioinformática têm sido utilizados para a pesquisa e desenvolvimento de vacinas (FLOWER, 2013).

Tabela 3: Relação de marcadores moleculares de superfície utilizados no estudo de células dendríticas e outros subtipos celulares.

Molécula de superfície	Tipo celular
CD11c	Presente em altos níveis em células dendríticas, mas também em monócitos, macrófagos, neutrófilos e alguns linfócitos B (STEINMAN; PACK; INABA, 1997; STEWART; THIEL; HOGG, 1995).
CD11b	Presente em células dendríticas, macrófagos, células NK, granulócitos e linfócitos ativados (DEMBIC; SCHENCK; BOGEN, 2000; ZHANG, Y. *et al.*, 2002).
CD8α	Expresso em alguns tipos de células dendríticas residentes e não migratórias. As células que expressam esse marcador, não expressam o CD11b (SHORTMAN; HEATH, 2010a).
CD103	Expresso em células dendríticas presentes em muitos

	órgãos linfócitos e alguns órgãos não linfoides. Células dendríticas que expressam CD103 são eficientes em realizar apresentação cruzada de antígenos exógenos para linfócitos T CD8$^+$ (DEL RIO *et al.*, 2010).
CD205	Expresso em altos níveis por células dendríticas CD8α e também por células Gr1+, células de Langerhans, células dendríticas mielóides e células epiteliais tímicas (PACK *et al.*, 2008; PARK; RODRIGUEZ; STEINMAN, 2012; SHRIMPTON *et al.*, 2009).
CD317	Marcador específico de células dendríticas plasmocitóides que são também conhecidas como células produtoras de IFN do tipo I (CAO; BOVER, 2010; LOSCHKO *et al.*, 2011)

Tabela 4: Candidatos a vacina contra leishmaniose e andamento dos ensaios pré-clínico e clínicos de fase I e II.

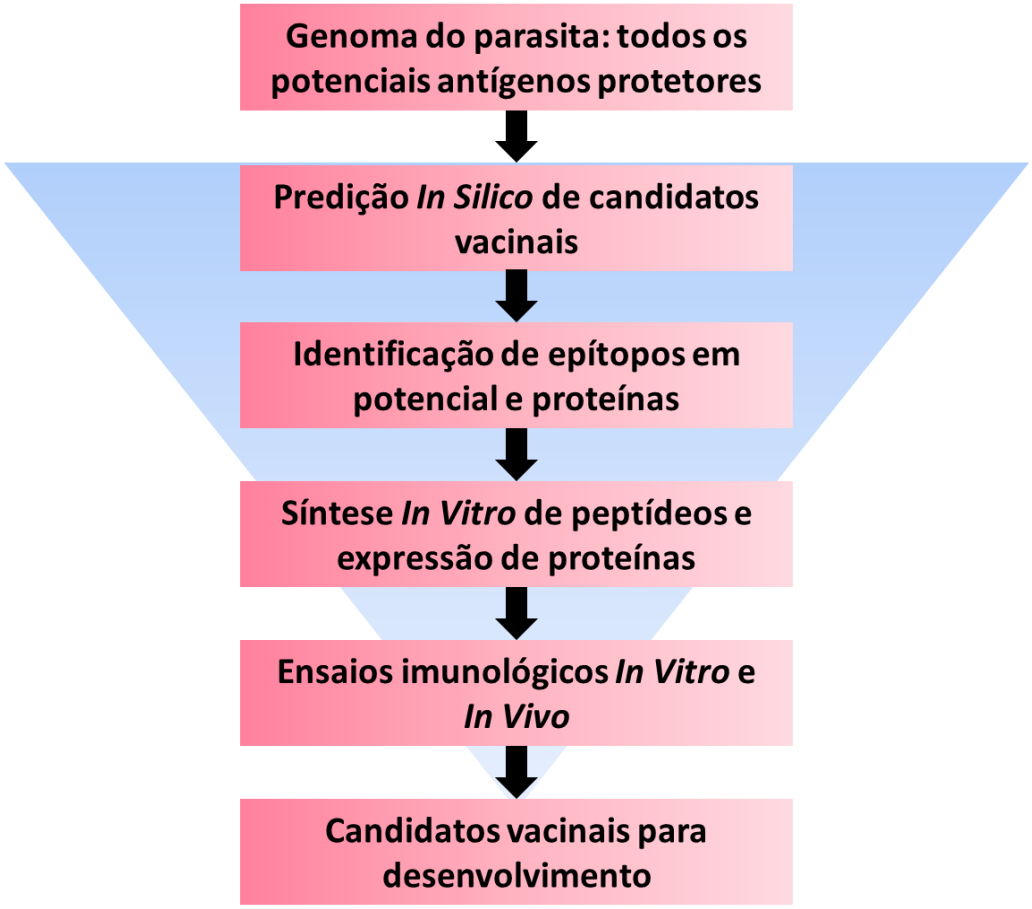
Identificação da vacina candidata	Referência	Ensaio pré-clínico	Ensaio clínico de fase I	Ensaio clínico de fase II
LEISH-F2	("A Study of the Efficacy and Safety of the LEISH-F2 + MPL-SE Vaccine for Treatment of Cutaneous Leishmaniasis - Full Text View - ClinicalTrials.gov", [S.d.])			**X**
LEISH-F3	("LEISH-F3 + GLA-SE and the LEISH-F3 + MPL-SE Vaccine - Full Text View - ClinicalTrials.gov", [S.d.], "Phase 1 LEISH-F3 + SLA-SE Vaccine Trial in Healthy Adult Volunteers - Full Text View - ClinicalTrials.gov", [S.d.])		**X**	
Vários antígenos de flebotomíneos do gênero *Lutzomyia*	("Immune Responses After Human Subject Challenge With Sand Fly Bites - Full Text View - ClinicalTrials.gov", [S.d.])	**X**		
Várias vacinas de segunda geração baseadas em proteínas recombinantes	(REZVAN, HOSSEIN; MOAFI, 2015)	**X**		
Várias vacinas de DNA e abordagens heterólogas de vacinação	(REZVAN, HOSSEIN; MOAFI, 2015)	**X**		

Adaptado de (GILLESPIE *et al.*, 2016)

3.3 Vacinologia reversa na busca de epítopos imunogênicos

Nos últimos anos houve um aumento crescente do número de sequências genômicas de patógenos ao homem e animais. A disponibilidade de muitas dessas sequências permitiu a escolha racional de antígenos utilizando métodos computacionais ou *in silico* (SETTE; RAPPUOLI, 2000).

Figura 10: Fluxograma destacando os passos críticos na metodologia da vacinologia reversa.



Fonte: o autor

A vacinologia reversa foi utilizada primeiramente no desenvolvimento de uma vacina contra o meningococo B (MenB), responsável por cerca de 50% dos casos de meningite bacteriana no mundo. Utilizando a metodologia da vacinologia reversa foi possível a identificação de 600 antígenos em potencial, que foram expressos em *Escherichia coli* e testados com soro de animais imunizados. Destes, foram identificados 90 novos antígenos que nunca tinham

sido descritos na literatura e desses 29 apresentaram capacidade de induzir anticorpos capazes de destruir a bactéria *in vitro*.

Comparada a vacinologia clássica que só tinha chegado a 5 antígenos (PIZZA *et al.*, 2000; TETTELIN *et al.*, 2000) com atividade bactericida, a vacinologia reversa se mostrou muito promissora ao fazer a busca no genoma dos parasitas. A vacina para MenB desevolvida por este método encontra-se aprovada pela agência européia de medicamentos (EMA, do inglês *European Agency of Medicines*) e pela ANVISA (Agência Nacional de Vigilância Sanitária) (GIULIANI *et al.*, 2006).

Além de permitir a identificação de antígenos com potencial protetor, a vacinologia reversa permitiu identificar o papel biológico de novas proteínas nos patógenos (LAUER *et al.*, 2005; MADICO *et al.*, 2006). Os melhores exemplos foram a descoberta de pili em bactérias Gram-positivas e do fator H em meningococos. Seguindo esta linha, estão sendo desenvolvidas vacinas contra estreptococos do grupo B (MAIONE *et al.*, 2005), estreptococos do grupo A, *Staphylococcus aureus*, *Streptococcus pneumoniae*, e Chlamydia (THORPE *et al.*, 2007).

A maior parte dos antígenos vacinais que foram desenvolvidos por meio de vacinologia reversa são capazes de induzir a produção de anticorpos que são capazes de inativar os patógenos diretamente ou cooperar com o complemento e outras células do sistema imunológico. Contudo, para grande parte das doenças parasitárias a presença de anticorpos não é capaz de conferir proteção e, assim, não é o correlato primordial desejado para uma vacina (SACKS, 2014).

Neste sentido, para a maioria das doenças parasitárias a imunidade celular com linfócitos T CD4$^+$ e T CD8$^+$ é um dos componentes principais para prevenção ou controle (DUTHIE *et al.*, 2012; SACKS, 2014). Para tal, a metodologia da vacinologia reversa é capaz de identificar epítopos em potencial presentes em proteínas codificadas por patógenos e que podem ser reconhecidos por linfócitos T CD4$^+$ e T CD8$^+$ (DE GROOT *et al.*, 2009; DOOLAN *et al.*, 2003). Assim, a vacinologia reversa lança mão de ferramentas capazes de realizar a predição da ligação de peptídeos especificamente às moléculas do Complexo Principal de Histocompatibilidade (MHC, do inglês *Major Histocompatibility Complex*).

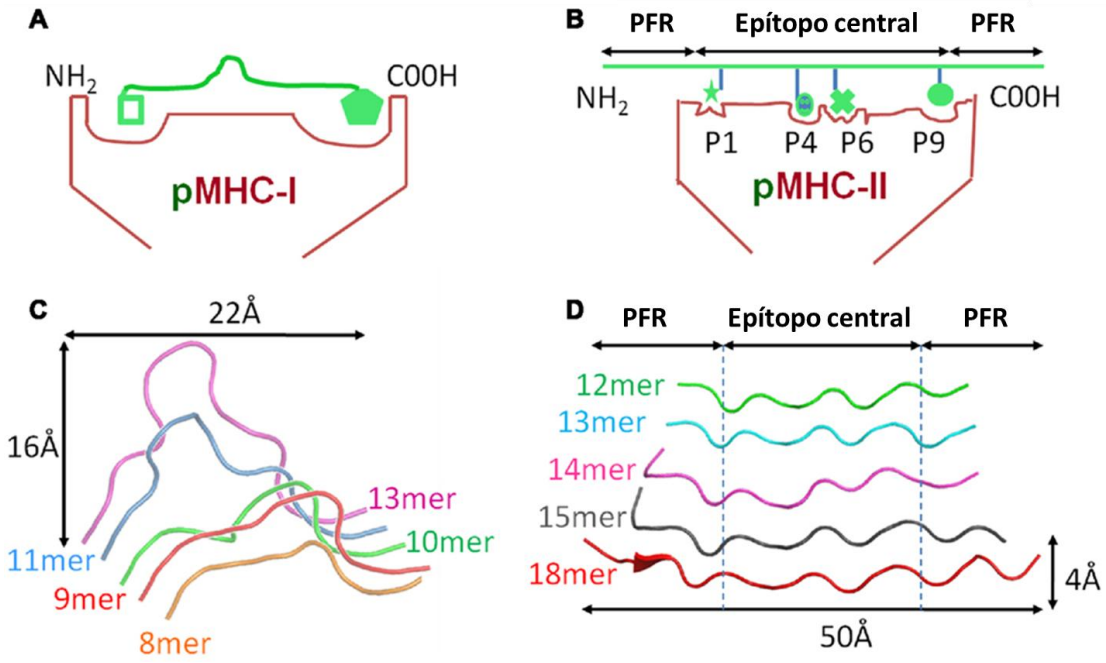
Alguns trabalhos procurando identificar antígenos de *Leishmania* spp. utilizaram a vacinologia reversa. Um desses utilizou somente métodos lineares de busca de epítopos no proteoma de diferentes espécies de *Leishmania* (JOHN; JOHN; KHOLIA, 2012), já outro trabalho concentrou esforços para identificar potenciais epítopos contidos em proteínas candidatas já previamente identificadas (MARIA *et al.*, 2013). Os autores desse último trabalho também apresentaram posteriormente o desenvolvimento de uma vacina com múltiplos epítopos de *Leishmania* (AGALLOU *et al.*, 2014).

3.3.1 Apresentação de antígenos via MHC de Classe I e II

As moléculas de MHC de Classes I e II apresentam diferenças em sua estrutura e também na função, estando a molécula de MHC de Classe I presente na superfície de todas as células nucleadas e capaz de se ligar à peptídeos ou epítopos com 8 a 15 aminoácidos derivados de proteínas estranhas ou próprias. De outra maneira, a molécula de MHC de Classe II somente está presente na superfície de células apresentadoras de antígeno profissionais, tais como as DC, e se ligam a peptídeos com 8 a 20 aminoácidos derivados de proteínas presentes no meio extracelular (FLOWER, 2013).

Os peptídeos apresentados pelas moléculas de MHC Classe I são derivados de proteínas presentes no interior celular, assim, são capazes de interagir com o receptor de linfócitos T CD8$^+$. Neste caso, havendo reconhecimento específico há ativação do linfócito T CD8$^+$. Em contrapartida, os peptídeos apresentados pelas moléculas de MHC de Classe II expressas por células apresentadoras de antígeno são derivados de proteínas presentes no meio extracelular (Figura 12-B). Assim, em caso de reconhecimento peptídeo-MHC de Classe II há ativação de linfócitos T CD4$^+$ que se desenvolvem nos diferentes perfis (KOBAYASHI; VAN DEN ELSEN, 2012). No caso das DC, essas células também são capazes de realizar a apresentação cruzada de epítopos, onde peptídeos exógenos podem ser apresentados via MHC de Classe I (Figura 12-A) (GUTIÉRREZ-MARTÍNEZ *et al.*, 2015; KLECHEVSKY *et al.*, 2010; TEL *et al.*, 2013).

Figura 11: Complexos peptídeo-MHC de Classes I e II.



Adaptado de: (HOLLAND; COLE; GODKIN, 2013b)

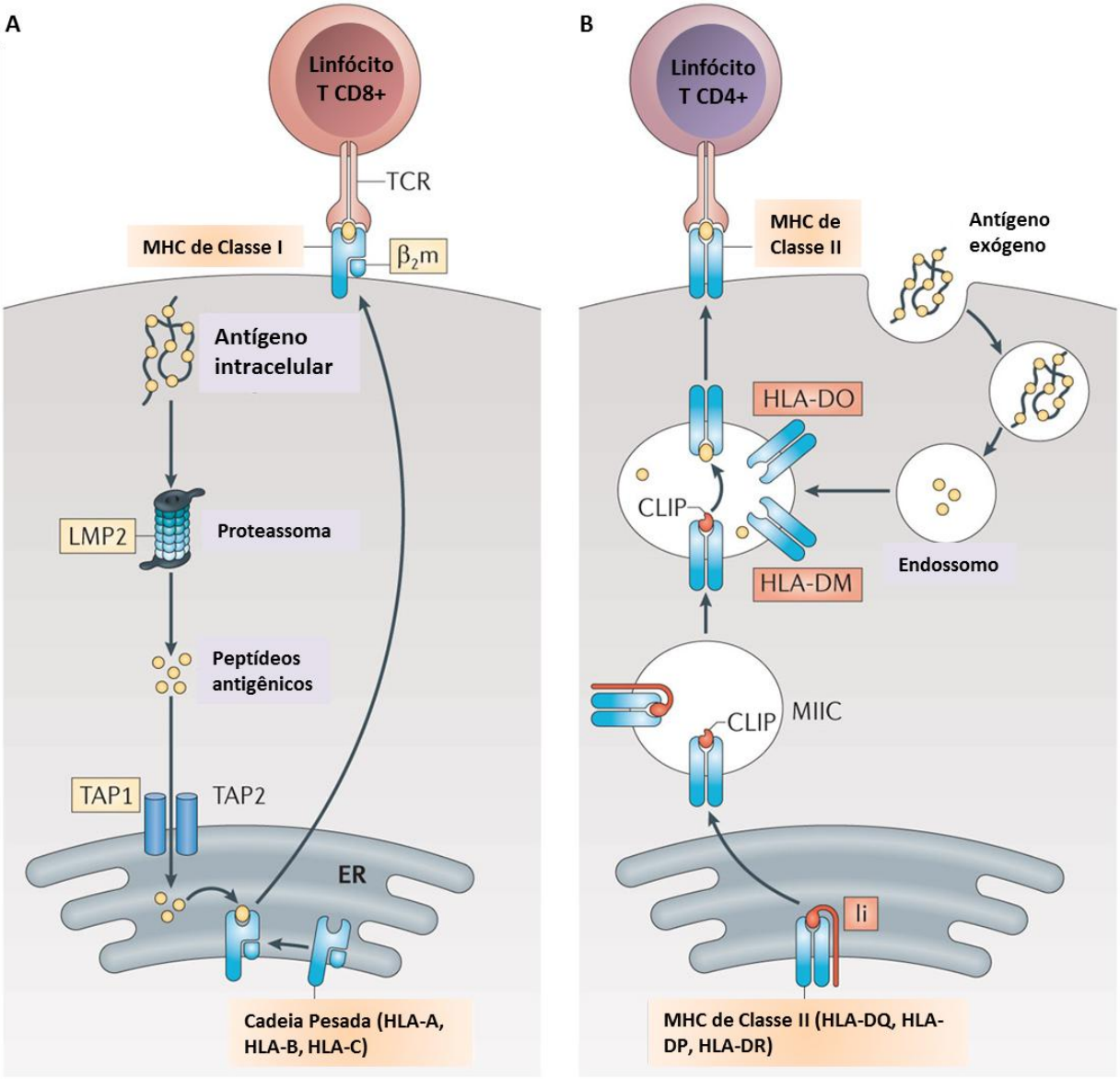
(A e B, respectivamente. Em C e D o tamanho médio e conformação assumida dos peptídeos no bolsão formado por MHC-I e MHC-II, respectivamente. Em B e D estão indicadas as regiões flanqueadoras dos epítopos centrais (PFR).

A região do MHC no genoma humano é a mais polimórfica e, por isso, apresenta um alto grau de variação entre as moléculas, sendo este um fator importante para ser considerado nas predições de epítopos (SETTE; RAPPUOLI, 2000). Dessa maneira, os resultados predizem milhares de epítopos para as diferentes formas alélicas do MHC que apresentam diferentes afinidades de ligação e especificidade. Apesar disso parecer uma limitação importante ao método, estratégias diferentes têm sido utilizadas para contornar tal problema.

Uma dessas estratégias é a de somente utilizar um número limitado de moléculas de MHC que são expressas pela população inteira. Outra estratégia são os trabalhos com metodologias pan-MHC que extrapolam os dados de afinidade de ligação de alelos bem conhecidos e caracterizados para outros alelos mais raros (DOOLAN *et al.*, 2008). Mesmo que cada alelo do MHC possua uma especificidade distinta de peptídeo, eles podem ser agrupados em uma grande categoria de supertipos de MHC que por sua vez estão associados

com sequências peptídicas que se sobrepõem (KAROSIENE *et al.*, 2013; NIELSEN *et al.*, 2010).

Figura 12: Apresentação de antígenos via MHC de Classes I (A) e II (B).



Adaptado de (KOBAYASHI; VAN DEN ELSEN, 2012)

Nota: Em A, os antígenos intracelulares são processados pelo proteassoma, gerando peptídeos antigênicos que são, então, transportados para o interior do retículo endoplasmático (ER) através da TAP. No ER os peptídeos são então conjugados com as moléculas de MHC (ou HLA) de Classe I. Em B, os antígenos exógenos são incorporados no interior da célula dentro de um endossomo que, por sua vez, se acidifica, cliva o antígeno em peptídeos e se fusiona com um endossomo proveniente do ER contendo moléculas do MHC de Classe II e chaperonas (HLA-DM, HLA-DO) que auxiliam no posicionamento correto dos peptídeos ao bolsão do MHC de Classe II.

3.3.2 Predição de epítopos com base na estrutura primária das proteínas

É cada vez maior o número de sequências de genomas completos de organismos disponibilizadas em bancos públicos. De posse dessas sequências é possível inferir o proteoma completo de espécies (proteoma predito). Assim, tais sequências podem fornecer diversas informações sobre os organismos em questão, no caso de patógenos humanos e animais, a presença de potenciais epítopos peptídicos ligantes de moléculas de MHC de Classe I e II. Em decorrência do grande número de moléculas depositadas nesses bancos, faz-se necessário o uso de ferramentas computacionais que permitam a predição de epítopos num intervalo de tempo menor. Tais ferramentas podem utilizar a sequência das proteínas para realizar a predição de epítopos peptídicos lineares necessários para estimular uma resposta contra algum patógeno (LUNDEGAARD *et al.*, 2010). Algumas das ferramentas mais utilizadas para realizar este tipo de análise são: NetCTL (LARSEN, M. V. *et al.*, 2005), NetMHC (NIELSEN *et al.*, 2003) e NetMHCII (NIELSEN *et al.*, 2009). A Tabela 5 relaciona estas ferramentas, suas características principais e fontes.

O NetCTL é uma ferramenta utilizada para predição de epítopos de MHC de Classe I, já tendo sido utilizado com dados de parasitas (RESENDE *et al.*, 2012). Esta ferramenta apresenta um diferencial por integrar no resultado final da predição do epítopo a predição de afinidade ao MHC de Classe I, a predição da eficiência de TAP (do inglês *transporter associated with antigen processing*) e a clivagem proteassômica do C-terminal (BHASIN; RAGHAVA, 2004). O NetCTL realiza a predição por meio de redes neurais (ANN), possuindo uma rede para cada supertipo de molécula de MHC. Cada ANN foi treinada com peptídeos de 9 aminoácidos com afinidade de ligação conhecida para aqueles alelos de MHC de Classe I (NIELSEN *et al.*, 2003).

Os resultados do NetCTL são fornecidos numa tabela com informações sobre o posicionamento do aminoácido, onde o epítopo começa e termina, a proteína fonte daquele epítopo, sequência do epítopo, predição de afinidade ao MHC, predição de clivagem pelo proteassoma, predição de TAP, e por fim, um valor de pontuação combinado.

No caso da predição de epítopos de MHC de Classe II, isto pode ser realizado com algumas ferramentas, tais como o NetMHCII desenvolvido pelo mesmo grupo do NetCTL. Assim como este último, o NetMHCII também realiza a predição utilizando ANN (*NN-align*) capazes de identificar simultaneamente a região central de ligação do epítopo e a afinidade de ligação. Além disso, a ANN no NetMHCII é treinada com um algoritmo que permite a correção de erros e é capaz de aumentar a acurácia dos dados pela incorporação de informações adicionais sobre os resíduos que flanqueiam a região central do epítopo. Assim como acontece no NetCTL, os dados são fornecidos ao NetMHCII no formato fasta e o resultado final é também disposto de maneira muito similar ao NetCTL, na forma de tabela.

Além dessas ferramentas acima citadas, há uma série de outros preditores disponíveis para uso, como: OpitMer (FLOWER, 2013), EpiMer (SIDNEY *et al.*, 2008), SYFPEITHI (LUNDEGAARD *et al.*, 2010), TEPITOPE (MEISTER *et al.*, 1995), CTLPred (RAMMENSEE *et al.*, 1999), WAPP (BHASIN; RAGHAVA, 2004), EpiJen (LARSEN, M. V. *et al.*, 2005), EpiTOP (DÖNNES; KOHLBACHER, 2005) e PREDIVAC (DOYTCHINOVA *et al.*, 2006). Cada um desses varia, por exemplo, em aspectos como alelos do MHC para os quais realizam predição e nos métodos utilizados para realizar e integrar a predição.

Os métodos de predição de epítopos peptídicos baseados na estrutura primária da proteína são bastante precisos e cada vez mais modernos. Contudo, as análises estruturais são também cada vez mais utilizadas no intuito de complementar e assegurar o dado obtido com as ferramentas lineares. Dessa forma, as análises de *docking* molecular, tão utilizadas pela indústria farmacêutica para a descoberta de novos fármacos, podem ser utilizados para descoberta de novos epítopos com potencial vacinal (FLOWER, 2013).

3.3.3 Docagem molecular

A estratégia de docagem molecular consiste numa metodologia computacional utilizada cada vez mais nos estudos para descoberta de novas drogas. Nestes, a metodologia é capaz de realização a predição da afinidade

de ligação entre um alvo biológico (receptor) e uma molécula (ligante). A força de ligação ou estabilidade do complexo formado é normalmente determinado por fatores energéticos e estruturais. Para a grande maior parte dos métodos de docagem, o fator energético mensura a energia de interação entre duas moléculas (ligante e alvo), e é definida como uma função de energia potencial (campo de força). Assim, quanto menor a energia do complexo, mais favorável ou estável é a ligação entre o ligante e o alvo. Desta maneira, os métodos de docagem permitem determinar se há ou não interações que estabilizem a ligação entre duas moléculas investigadas. A docagem molecular permite determinar quais são as forças moleculares relacionadas com a estabilidade do complexo molecular formado (PUJADAS *et al.*, 2008; WANG; LU; WANG, 2003; YURIEV; AGOSTINO; RAMSLAND, 2011; YURIEV; RAMSLAND, 2013).

Em se tratando do fator estrutural, a contribuição desta também é importante para o processo como um todo, uma vez que a ligação entre as duas moléculas ocorre numa região em particular da proteína. Dessa forma, a molécula ligante deve adquirir uma conformação tridimensional que seja compatível com o sítio de ligação na proteína. No contexto deste trabalho, o alvo molecular considerado são as regiões das moléculas de MHC de Classe I e II que se ligam aos epítopos peptídicos (ligantes). A Figura 11 ilustra bem as regiões de ligação do epítopos às moléculas de MHC de Classes I e II.

Com base nesta estrutura, é possível observar que as moléculas de MHC de Classes I e II são compostas por subunidades diferentes, mas que são estruturalmente similares em conjunto (Figura 13). Em ambos os casos, as regiões de ligação aos epítopos peptídicos são formadas por duas alfa-hélices anti-paralelas. Este tipo de conformação permite a formação de um canal ou bolsão ao qual os peptídeos podem se ligar numa conformação estendida ou alongada. Na base deste bolsão formado há oito folhas beta anti-paralelas que formam regiões específicas de interação dos peptídeos, conhecidos como *pockets*. Na molécula de MHC de Classe I observa-se a presença de dois *pockets* importantes, enquanto que na molécula de MHC de Classe II são 4 *pockets* (Figura 11) (RUDOLPH; STANFIELD; WILSON, 2006; VAN DER MERWE; DAVIS, 2003).

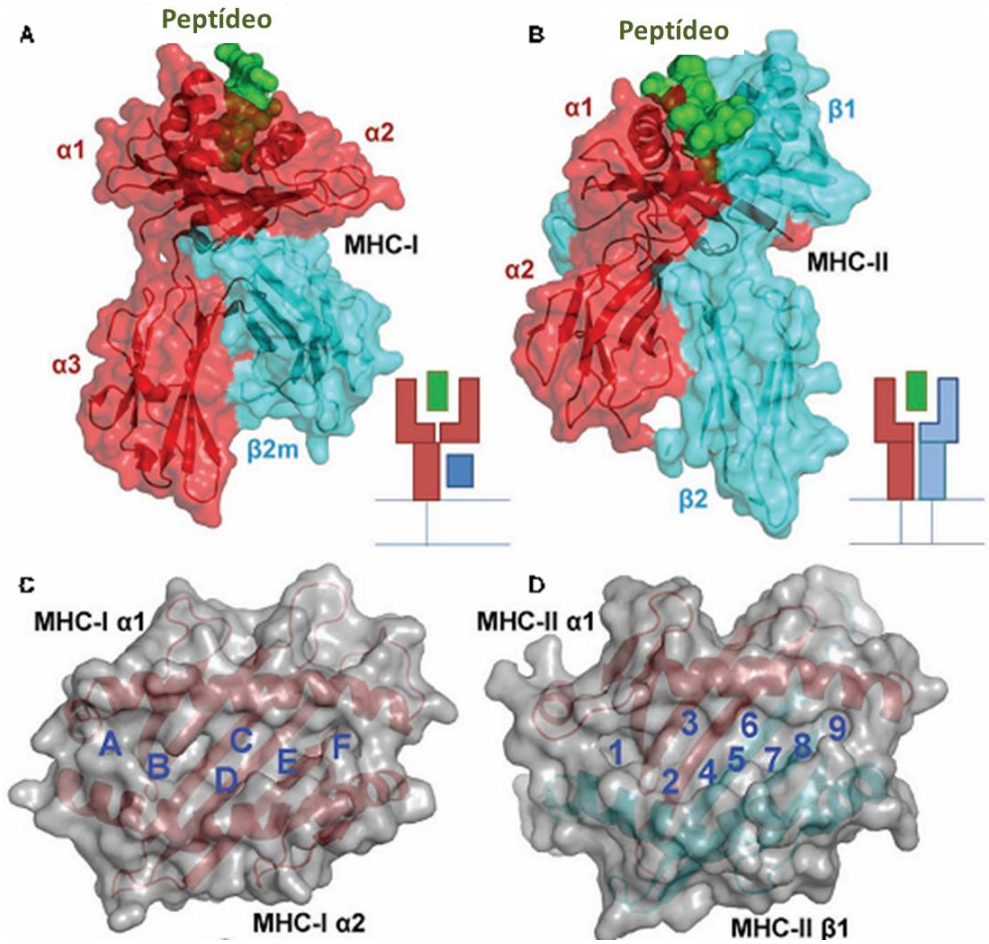
Neste contexto, os métodos de *docking* molecular se apresentam como uma ferramenta útil no sentido de chegar em estruturas moleculares relevantes

que são potencialmente imunogênicas. Assim, diferentes ferramentas computacionais podem ser utilizadas para este fim, tais como o design de cadeias laterais fixas e o design de cadeias laterais flexíveis, ambas metodologias inclusas no pacote do projeto Rosetta (http://www.rosettacommons.org/).

No caso do design de cadeias laterais fixas, o algoritmo utilizado pela metodologia não permite a otimização das cadeias laterais da molécula simulada. Contudo, a utilização desta ferramenta permite a otimização do tempo de simulação e do uso de memória (LEAVER-FAY; KUHLMAN; SNOEYINK, 2005; LEAVER-FAY; SNOEYINK; KUHLMAN, 2008). Em contrapartida, a metodologia flexível (Rosetta FlexPepDock) consiste num protocolo de refinamento que otimiza as cadeias laterais da molécula peptídica simulada, permitindo um refinamento da simulação (DAS, R.; BAKER, 2008; RAVEH; LONDON; SCHUELER-FURMAN, 2010). Utilizando uma base de minimização Monte Carlo, o protocolo flexível permite um refinamento de todos os graus de liberdade do peptídeo e das cadeias laterais do receptor.

Neste contexto, este trabalho buscou identificar no proteoma de *L.V. braziliensis* potenciais epítopos de linfócitos T CD4$^+$ e CD8$^+$ com objetivo de avaliá-los imunologicamente.

Figura 13: Estrutura molecular comparada das moléculas de MHC de Classe I e de Classe II.



Adaptado de (HOLLAND; COLE; GODKIN, 2013a)

Nota: Pode-se observar que as subunidades moleculares do MHC de Classe I (A, PDB:1ZHL) e do MHC de Classe II (B, PDB: 1KG0) são estruturalmente diferentes. Contudo, a conformação que estas moléculas assumem é muito similar para o papel principal destas moléculas na apresentação de epítopos peptídicos (em verde, em A e B). O MHC I (A) é composto por uma cadeia com 3 domínios α (vermelho) e β2m (azul, beta-2-microglobulina), enquanto que o MHC II (B) é composto por duas cadeias com dois domínios α (vermelho) e dois domínios β (azul). Em C e D são representadas as moléculas de MHC I e II, respectivamente, do topo da estrutura. Observa-se que ambas estruturas possuem bolsões de ligação ao antígeno similares formados por duas α-hélices anti-paralelas que forma um canal no qual os peptídeos podem se ligar numa conformação estendida, e oito folhas-β anti-paralelas que formam os sítios específicos de ligação ao peptídeos (*pockets*) na base do bolsão. Os *pockets* são formados por resíduos polimórficos que definem o tamanho e as características químicas de cada *pocket*, dessa maneira a especificidade de ligação ao peptídeo.

Tabela 5: Ferramentas computacionais utilizadas na predição linear de epítopos peptídicos de MHC de Classes I e II.

Ferramenta	Página Web	Características	Referência
NETCTL	http://www.cbs.dtu.dk/services/NetCTL/	Realiza predição de epítopos de linfócitos T citotóxicos em sequências de proteínas	Larsen *et al*. 2007
NETMHC	http://www.cbs.dtu.dk/services/NetMHC/	Realiza predição de ligação de peptídeos a um número diferente de alelos do MHC de Classe I utilizando redes neurais artificiais (ANNs)	Nielsen *et al*. 2003
NETMHCII	http://www.cbs.dtu.dk/services/NetMHCII/	Realiza predição de ligação de peptídeos a HLA-DR, HLA-DQ, HLA-DP do MHC de Classe II e alelos do MHC de Classe II de camundongos utilizando redes neurais artificiais (ANNs)	Nielsen, Lund, 2009

4 METODOLOGIA

4.1 Análises *In Silico*

A Figura 14 abaixo ilustra de forma esquemática o fluxograma metodológico utilizado para realização das análises *in silico* desta pesquisa.

O TriTrypDD foi utilizado para realizar o download do proteoma predito de *Leishmania* spp. As sequências do proteoma foram analisadas por diferentes métodos (B). O banco de dados relacional EpitopeDB foi criado e gerenciado utilizando o MySQL como sistema de gerenciamento. Parsers e algoritmos em linguagem PERL e SQL foram criados e desenvolvidos no intuito de acessar e integrar os resultados (C). Os dados foram então agrupados (D). Os dados agrupados foram utilizados para a docagem molecular (E).

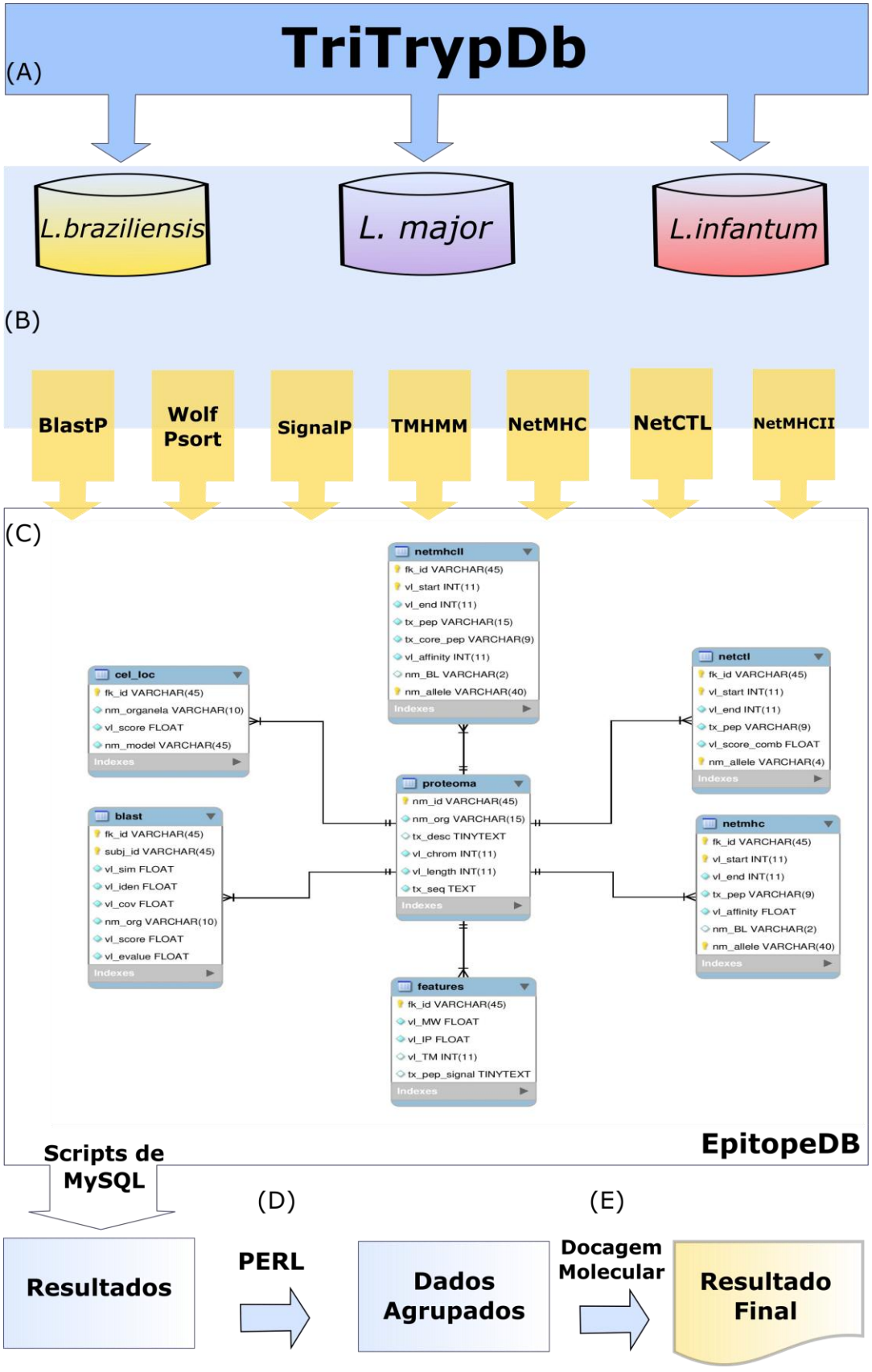
4.1.1 Predição de epítopos lineares

4.1.1.1 Recuperação dos proteomas em bancos de dados e análise da conservação

Inicialmente, foi realizado o download dos proteomas de três espécies de *Leishmania* (*L. braziliensis, L. major* e *L. infantum*) do banco de dados TriTrypDB (ASLETT *et al.*, 2010). A partir dessas sequências foram realizadas análises de bioinformática. As proteínas de *L. braziliensis* foram selecionadas com base no nível de conservação maior que 60% que apresentavam com as outras espécies de *Leishmania*. Essa seleção foi feita utilizando a ferramenta BLAST para alinhamento de sequências de proteínas.

Esse parâmetro foi levado em consideração uma vez que uma vacina ideal deve ser capaz de induzir proteção também contra outras espécies dentro daquele gênero. Além disso, as proteínas conservadas dentro de uma espécie apresentam maiores chances de ter um papel essencial para o funcionamento daquele organismo.

Figura 14: Fluxograma metodológico das análises *in silico* realizadas.



4.1.1.2 Predição de afinidade aos MHC de Classes I e II

A predição dos epítopos ligantes de MHC de Classe I foi realizada com as ferramentas NetMHC e NetCTL, enquanto que o NetMHCII foi utilizado para predição dos epítopos ligantes de MHC de Classe II. Todas as ferramentas utilizadas realizam a predição dos epítopos para os supertipos dos alelos mais prevalentes em populações humanas (LARSEN, M. V *et al.*, 2007; NIELSEN *et al.*, 2009, 2010). Além disso, tanto o NetMHC quanto o NetCTL são preditores de epítopos; contudo, o NetCTL além de realizar a predição de epítopos, também considera outras predições. Essas predições são a eficiência de transporte mediada pela proteína transportadora associada com processamento de antígenos (TAP) e a predição da clivagem no proteassoma. O valor de cut-off definido para selecionar os peptídeos com alta afinidade para as ferramentas foi ≥ 1 no intuito de aumentar as chances de encontrar potenciais epítopos.

4.1.1.3 Similaridade e características biológicas das proteínas candidatas

No intuito de excluir proteínas candidatas que tivessem um alto nível de similaridade com proteínas humanas ou de camundongos, pois estas poderiam inviabilizar testes devido ao risco de indução de autoimunidade, foi realizado um BLAST. Assim, as proteínas com nível de similaridade igual ou maior que 40% com as proteínas de humanos ou de camundongos foram excluídas das análises subsequentes. Além dessas predições, foram realizadas predições do número de hélices transmembranas e da sub-localização celular das proteínas, utilizando as ferramentas TMHMM (KROGH *et al.*, 2001) e WoLF PSORT (HORTON *et al.*, 2007), respectivamente. Essas últimas decisões foram tomadas no sentido de excluir das análises as proteínas que apresentam mais que uma hélice transmembrana, uma vez que o número maior de hélices poderia dificultar a expressão das moléculas (SETTE; RAPPUOLI, 2000). Além disso, buscou-se analisar somente as proteínas secretadas ou que estivessem presentes na membrana do parasita, já que estas estão apresentadas de maneira mais eficiente aos linfócitos T CD4$^+$ e T CD8$^+$ (AEBISCHER, 2014).

Por fim, todos os dados que foram gerados durante o processamento dos métodos acima descritos foram depositados num banco de dados relacional, utilizando o MySQL como sistema de gerenciamento de banco de dados (DBMS). Foram desenvolvidas Parsers e algoritmos nas linguagens PERL e SQL para integrar e acessar os resultados depositados no banco de dados.

4.1.1.4 Clusterização dos epítopos selecionados

Para esta etapa foi construído um algoritmo, *in house*, baseado nos resultados do alinhamento realizado pelo BLAST para agrupar os dados com alta similaridade. Nesse sentido, um limiar de 60% de identidade e 100% de cobertura entre dois epítopos foi utilizado para que eles fossem unidos no mesmo *cluster*. Além disso, a seleção dos grupos envolveu outros dois critérios importantes: os epítopos peptídicos deveriam ter sido preditos com alta afinidade para pelo menos três diferentes alelos do MHC de Classe I ou MHC de Classe II, ou epítopos peptídicos provenientes de pelo menos três proteínas diferentes.

4.1.2 Docagem molecular

4.1.2.1 Recuperação das estruturas do MHC de Classes I e II do *Protein Data Bank*

Esta etapa teve inicío com o download no RSCB Protein Data Bank de 33 estruturas diferentes de alelos para o MHC de Classe I (Identificadores: 2HJL, 3C9N, 3HCV, 3KPP, 3L3D, 3RL1, 3VCL, 3VFS, 3X11, 4F7M, 4G8G, 4HWZ, 4JQX, 4MJ5, 4MJI, 4NQV, 4º2C, 4QRR, 4QRU, 4WU5, 4XXC) e 12 estruturas diferentes de alelos para o MHC de Classe II (Identificadores: 1ª6A, 1BX2, 1H15, 1S9V, 1UVQ, 1YMM, 2NNA, 2Q6W, 3C5J, 3LQZ, 3PL6, 3WEX).

Em seguida, as estruturas foram então preparadas por meio da remoção das moléculas de água, ligantes e resíduos duplicados ou alelos. Adicionalmente, o software PyMol foi utilizado para modificar a cadeia do peptídeo co-cristalizado para que este contivesse o mesmo tamanho dos

peptídeos preditos nas etapas anteriores (9 resíduos para o MHC de Classe I e 15 resíduos para o MHC de Classe II). Além disso, cada aminoácido não canônico encontrado nas estruturas foi substituído manualmente pela alanina.

Por fim, foi utilizado um software desenvolvido, *in house*, chamado GriDoMol para preparar e submeter todos os procedimentos *in silico* em um ambiente de grid computacional. Essa etapa foi realizada para combinar as estruturas do MHC aos epítopos preditos e compilar os resultados de cada etapa em planilhas. O ambiente de grid computacional foi criado com oito computadores, cada um portando placas 2x Intel Xeon quadcore (num total de 8 cores por computador) e 16GB de memória RAM.

4.1.2.2 Produção dos complexos MHC-epítopos

Nesta etapa foi utilizado uma sequência de protocolos utilizando a plataforma Rosetta. Para a substituição dos peptídeos co-cristalizados por cada um dos peptídeos preditos, foi utilizado o protocolo FixBB, disponível no Rosetta. Contudo, o protocolo FixBB não tem capacidade de remover os átomos da cadeia principal. Isso faz com que a substituição do peptídeo co-cristalizado não seja tão bem sucedida e possa gerar estruturas conformacionais instáveis.

Portanto, após a aplicação do protocolo FixBB, foi realizado o protocolo Rosetta Relax para estabilizar energeticamente cada um dos novos epítopos. Além disso, todos os resíduos do MHC foram imobilizados para prevenir mudanças conformacionais no “receptor”, enquanto que os resíduos dos epítopos ficaram livres para realizar os movimentos em busca da conformação mais estável no contexto químico nos quais eles estavam inseridos.

Nesta etapa do processo, a função Score de Interface (Isc) do protocolo Rosetta FlexPepDock foi utilizada no intuito de quantificar a afinidade de ligação entre os epitopos peptídicos e as moléculas de MHC. O Isc corresponde a soma das contribuições energéticas da interface dos resíduos em ambos receptor (MHC) e epítopos. Dessa forma, todas as 32.658 estruturas obtidas por meio da aplicação do protocolo Rosetta Relax passaram por essa estapa utilizando o protocolo Rosetta FlexPepDock, no intuito de obter os valores de Isc. Contudo, as estruturas obtidas com o protocolo Relax foram

mantidas sem alterações nas coordenadas dos átomos. Desse modo, o procedimento realizado teve a função de recomputar as energias com uma melhor função de score mantendo a geometria e sem causar perturbação ao sistema químico.

4.1.2.3 Predição de afinidade dos complexos

No intuito de reduzir o tempo computacional, adotou-se uma estratégia para filtrar e somente selecionar os epítopos mais promissores, com base no score de Isc com receptores e a frequência da afinidade observada ao longo das diferentes estruturas do MHC.

O protocolo do Rosetta FlexPepDock foi aplicado para realizar a docagem molecular, permitindo completa flexibilidade para os resíduos na interface. Esse procedimento buscou encontrar os peptídeos preditos com maior afinidade pelos alelos do MHC. Para cada epítopo predito foram selecionadas as melhores soluções de docagem de acordo com o Isc. Ao final, os epítopos preditos foram ranqueados pela média do valor de Isc das soluções encontradas ao longo dos alelos de MHC. Das centenas de pares de epítopos-MHC descrita, os dez pares de epítopos preditos com os melhores valores de Isc entre os alelos foram selecionados para aplicação de um protocolo avançado do protocolo Rosetta FlexPepDock, aumentando o número de estruturas geradas de 100 para 500. O custo computacional desse tipo de cálculo (500 soluções de docagem) foi cinco vezes maior que o padrão de 100 soluções de docagem.

4.2 Análises *in vitro* com células humanas

Ao término dos ensaios *in silico,* partiu-se para etapas de avaliação biológica dos epítopos peptídicos, identificados pelos métodos de bioinformática.

4.2.1 Grupos investigados e aspectos éticos

As análises envolveram indivíduos de ambos os sexos com idade mediana e provenientes de Moreno, Pernambuco-Brasil. Três grupos foram estudados: pacientes com doença ativa (DA, *n* = 8), pós-tratamento (PT – *n* = 10) e resistentes (RT – *n* = 5). O último grupo foi composto por indivíduos provenientes de áreas endêmicas, positivos para o teste de Intradermorreação de Montenegro (IDRM), mas que nunca manifestaram sinais clínicos da doença. Como grupo controle, foram utilizadas amostras de indivíduos saudáveis (*n* = 5) que não vivem em áreas endêmicas para leishmaniose.

O protocolo utilizado nesta pesquisa foi avaliado pelo Comitê de Ética em Pesquisa com Seres Humanos do Centro de Pesquisas Aggeu Magalhães (CpqAM/FIOCRUZ) (número do protocolo: 522.964). Todos os indivíduos assinaram o Termo de Consentimento Livre e Esclarecido (TCLE).

4.2.2 Síntese, ressuspensão e armazenamento dos peptídeos

As moléculas correspondentes aos 10 melhores resultados do estudo *in silico*, peptídeos com 15 aminoácidos, foram sintetizados comercialmente (Genome Biotechnology, Brasil). Na etapa de síntese, os peptídeos lineares foram purificados por Cromatografia Líquida de Alta Eficiência (HPLC) com uma pureza final superior a 95%. Uma vez produzidos, os peptídeos sintéticos foram ressuspensos individualmente em sulfóxido de dimetilo (DMSO) e estocados a -80°C até o uso. Cada um dos peptídeos foi organizado em ordem numérica, do melhor resultado *in silico*, o número 1, até o 10.

4.2.3 Coleta e obtenção das células mononucleares do sangue periférico (PBMC)

Para cada indivíduo incluído neste estudo, 20 mL de sangue foi coletado de forma asséptica. O sangue foi diluído na proporção 1:1 (v/v) com tampão fosfato-salino (PBS, pH 7,2), depositado na solução de gradiente Ficoll-paque PLUS (GE) e centrifugado por 35 min a 400 x g. Posteriormente, a camada com as células mononucleares do sangue periférico (PBMC) foi removida e

lavada duas vezes com PBS (pH 7,2). As células foram ressuspensas em meio Roswell Park Memorial Institute (RPMI) 1640 contendo 2 mM de L-glutamina, 50 mg/L de sulfato de gentamicina e suplementado com 10% de soro fetal bovino (ambos reagentes da Cultilab, Brasil). Em seguida, as células foram contadas em hemocitômetro e a concentração ajustada para 10^6 células/mL.

4.2.4 Processamento e marcação com 5-(-6)-diacetato de carboxifluoresceína succinimidil ester (CFDA-SE)

Células dos grupos PT, DA, RT, e controle foram marcadas com éster diacetato succinidimil carboxifluoresceína (CFDA-SE, Invitrogen, USA) para avaliar níveis de proliferação celular induzidos pelos peptídeos. Para isso, 4 x 10^6 PBMCs foram ressuspensas em 1 mL de PBS (pH 7,2) contendo 2 µM de CFDA-SE e incubadas a 37°C por 10 min. A concentração do CFDA-SE foi previamente titulada para prevenir inibição da proliferação ou morte celular. Após a incubação, a marcação foi interrompida por meio da adição de 1 mL de meio RPMI 1640 gelado (4°C) contendo 2 mM de L-glutamina, 50 mg/L de sulfato de gentamicina e suplementado com 10% de soro fetal bovino (Cultilab, Brasil). Logo em seguida, as células foram concentradas por centrifugação e lavadas com PBS e ressuspensas em 1 mL de meio RPMI 1640 suplementado na densidade de 2 x 10^6 células/mL. Depois deste período, as células foram retiradas das placas, depositadas em tubos de poliestireno, lavadas com PBS (pH 7,2) e analisadas por citometria de fluxo.

4.2.5 Cultivo celular e estímulo com os peptídeos

2 x 10^5 PBMCs foram depositadas em placas de 96 poços de fundo em U (BD Falcon, USA) e foram estimuladas individualmente com 20 µg/mL (SEYED *et al.*, 2011) de cada peptídeo. Foram realizadas duplicatas para cada peptídeo testado. As placas foram incubadas a 37°C com 5% de CO_2 por 4 dias. Depois deste período, as placas foram centrifugadas por 10 min a 400 x g e o sobrenadante foi coletado e estocado a -80°C para quantificação de citocinas por citometria de fluxo.

No ensaio de proliferação celular, para cada paciente ou controle testado, células não estimuladas e células estimuladas com fitohemaglutinina (PHA) foram avaliadas como controles intra-experimentais para definir os níveis de proliferação celular durante a análise da citometria de fluxo.

4.2.6 Marcação com anticorpos monoclonais

Foram utilizadas células de quatro pacientes após o tratamento e quatro controles para avaliar os níveis de expressão de T-bet após estímulo com os peptídeos. Para isso, as células foram lavadas com PBS acrescido de 0.5% de soro fetal bovino (Sigma, St. Louis, MO) e 0,1% de azida sódica (Sigma, St. Louis, MO), solução chamada PBS-W; centrifugadas (400g, 10 min, temperatura ambiente) e transferidas para tubos de poliestireno. Foram adicionados anticorpos monoclonais anti-CD4 e anti-CD8 aos tubos, ambos conjugados com aloficocianina (APC) e isotiocianato de fluoresceína (FITC), respectivamente (BD Bioscience, San Jose, CA), e as células foram incubadas por 30 min em temperatura ambiente. Posteriormente, as células foram fixadas com paraformaldeído (PFA) 1% em PBS, lavadas por centrifugação com PBS-W (400g, 5 min) e permeabilizadas com PBS acrescido de 0,5% de saponina. Subsequentemente, as células foram lavadas com PBS-W (400g, 5 min) e incubadas com anti-T-bet-phycoerythrin (PE) (BD Bioscience, San Jose, CA) por 30 min, temperatura ambiente. Após a incubação, as células foram lavadas novamente e ressuspensas com PFA 1% em PBS.

4.2.7 Quantificação das citocinas no sobrenadante de cultura

Sobrenadantes de células dos grupos DA, PT e controle foram descongelados e a quantificação de IFN-γ, IL-2, IL-10, IL-4, TNF, IL-6 e IL-17ª foi realizada usando kits humanos de ensaio citométrico de esferas ordenadas Th1, Th2, Th17 (CBA, BD Bioscience). Todos os métodos foram realizados de acordo com as instruções do fabricante. Posteriormente, as amostras foram analisadas no citômetro de fluxo FACScalibur (Becton Dickinson Company, San Jose, USA). Para cada amostra, foram adquiridos 2.100 eventos, como recomentado pelo fabricante. A curva padrão para o ensaio de CBA foi

determinada utilizando 9 diluições, e os dados analisados pelo software FCAP Array, fornecido pela BD.

4.2.8 Aquisições e análises por citometria de fluxo

As análises foram realizadas em um citômetro de fluxo FACSCalibur (Becton Dickinson Company, San Jose, USA) equipado com um laser de argônio (comprimento de onda 488 nm) utilizando o software CELLQuestProTM (BD Bioscience, San Jose, CA) para a aquisição. A fluorescência de 20.000 eventos de um quadrante de linfócitos baseada em parâmetros de tamanho e granulosidade, foi adquirida. Os dados foram analisados e tratados com o software FlowJo v10.1 (Tree Star Inc., USA). Para o ensaio de CFDA-SE, células não estimuladas foram utilizadas durante a análise para estabelecer parâmetros e definir o nível basal de proliferação linfocítica. Para o ensaio de T-bet, células marcadas de indivíduos controles foram utilizadas para definir os padrões dos quadrantes.

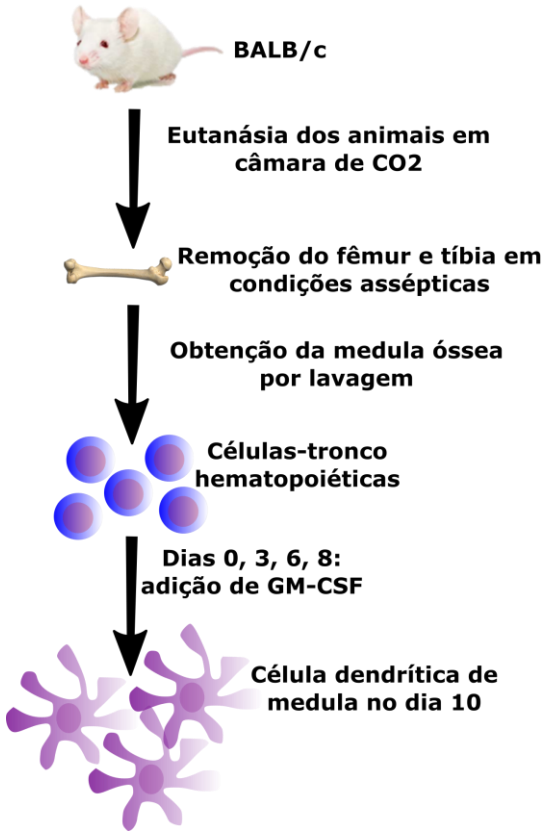
4.3 Análises com células murinas

4.3.1 Aspectos éticos

O modelo animal utilizado nesta pesquisa foram camundongos BALB/c. Os animais foram mantidos e alimentados sob os cuidados do biotério do Centro de Pesquisas Aggeu Magalhães (CpaAM-FIOCRUZ). Os protocolos utilizados no presente trabalho foram todos previamente analisados e aprovados pelo Comitê de Ética no Uso de Animais do CpqAM-FIOCRUZ (Processo CEUA 47/2013).

4.3.2 Avaliações com células dendríticas mielóides (BMDC)

Figura 15: Etapas para obtenção de células dendríticas mielóides murinas diferenciadas com fator de crescimento de granulócitos e macrófagos.



Fonte: o autor.

Nota: Os animais BALB/c foram inicialmente eutaniziados em câmara de CO2, posteriormente foi removido o fêmur e a tíbia dos animais. Em seguida, os ossos foram lavados e foi realizada a obtenção da medula óssea, por meio da remoção das extremidades dos ossos e lavagem com meio de cultivo celular. As células-tronco hematopoiéticas obtidas foram cultivadas por 10 dias na presença do fator de crescimento de colônia de granulócitos e macrófagos (GM-CSF). Esse fator foi adicionado na concentração final de 20 µg/mL no inicío da cultura e após 3, 6 e 8 dias. No dia 10 as células dendríticas estavam diferenciadas

4.3.2.1 Obtenção de células da medula óssea de camundongos BALB/c

O protocolo para geração de BMDC foi adaptado de Lutz *et al*. (1999). Para cada experimento realizado com BMDC, cinco camundongos BALB/c machos foram eutanasiados em câmara de CO_2, em seguida foram removidos o fêmur e a tíbia em condições estéreis. Os ossos foram depositados em recipiente contendo etanol 70% por 1 min e em seguida lavados com meio

RPMI 1640 contendo 2 mM de L-glutamina, 50 mg/L de sulfato de gentamicina, suplementado com 10% de soro fetal bovino (ambos reagentes da Cultilab, Brasil).

Em seguida, as extremidades dos ossos foram removidas e o interior dos ossos foi lavado com meio RPMI 1640 suplementado utilizando uma seringa com agulha de 0,45 mm de diâmetro. Os grumos de células formados foram removidos por meio de pipetagem vigorosa, seguida de passagem por meio de um filtro com poros de 0,45 mm de diâmetro. As células foram lavadas com meio RPMI 1640 (300 x G, por 10 min) e em seguida depositadas em placas de seis poços para descansar por 12 horas.

4.3.2.2 Processamento das células da medula e diferenciação com fator de crescimento de granulócitos e macrófagos (GM-CSF)

Após esse período, as células foram removidas da placa e a viabilidade e concentração de células foi determinada por meio do método de azul de Trypan. A concentração celular foi ajustada e 2 x 10^6 células foram depositadas por poço da placa de cultura de 6 poços. No início da cultura, as células receberam meio RPMI 1640 suplementado e contendo 20 ng/mL de fator de crescimento de colônias de granulócitos e macrófagos (rGM-CSF, Sigma, USA). No terceiro, sexto e oitavo dia da cultura, o meio de cultivo foi renovado e contendo a mesma concentração de rGM-CSF. No décimo dia de cultivo, as células estavam completamente diferenciadas e as células não aderentes foram removidas da placa para serem utilizadas. A morfologia celular foi confirmada por microscopia óptica convencional e microscopia confocal de fluorescência.

4.3.2.3 Cultivo celular e estímulo com os peptídeos

A concentração de BMDC foi ajustada e 2 x 10^5 células foram depositadas em placas de 96 poços com fundo em U. As células foram estimuladas individualmente com 20 µg/mL de cada peptídeo testado. Isto foi realizado em duplicatas, separadamente para cada peptídeo testado. As placas foram incubadas a 37°C com 5% de CO_2 por 24h e 48h.

4.3.2.4 Marcação com anticorpos monoclonais

Após cada período de incubação, as placas contendo as células foram centrifugadas a 400 x g por 10 min. O sobrenadante foi coletado e estocado a -80°C para posterior dosagem de citocinas por citometria de fluxo. Em seguida, as células foram removidas da placa com tampão fosfato-salino (PBS, pH 7,2) e transferidas para tubos de poliestireno nos quais se procedeu com a marcação com anticorpos monoclonais. Para isso, os seguintes anticorpos foram adicionados aos tubos contendo as células: anti-CD11c conjugado ao isotiocianato de fluoresceína (FITC) (Clone N418), anti-MHC I conjugado a ficoeritrina (PE) (Clone AF6-88.5.5.3), anti-MHC II conjugado a aloficocianina (APC) (Clone M5/114.15.2), e anti-CD40 conjugado a APC (Clone 1C10) (todos os anticorpos utilizados foram da Affymetrix eBioscience, USA), sendo as amostras incubadas à temperatura ambiente por 30 min.

4.3.2.5 Quantificação das citocinas no sobrenadante de cultura

Os sobrenadantes de cultura das BMDC foram descongelados para dosagem das citocinas IFN-γ, IL-2, IL-10, IL-4, TNF, IL-6, IL-17a com o kit Th1, Th2, Th17 *Cytometric Bead Array* (CBA) da BD Bioscience (USA). Para realização desta etapa, todos os procedimentos foram seguidos de acordo com indicação do fabricante. Ao final da preparação das amostras, realizou-se a aquisição dos dados no citômetro de fluxo FACSCalibur (Becton Dickinson Company, San Jose, USA). Para cada amostra foram adquiridos 2.100 eventos, como recomendado pelo fabricante. A curva padrão do ensaio de CBA foi determinada com nove diluições dos padrões e os dados foram analisados com o software FCAP Array fornecido pelo mesmo fabricante do kit (BD).

4.3.3 Avaliações com células dendríticas e subpopulações esplênicas

4.3.3.1 Imunização de camundongos BALB/c

Os experimentos de imunização foram realizados com grupos compostos por cinco fêmeas de BALB/c. Cinco peptídeos que apresentaram resultado significativo nos experimentos com células humanas foram escolhidos para se realizar as imunizações. Os animais foram imunizados uma vez por semana, durante três semanas nos folhetos teciduais da orelha esquerda com 20 µg de cada peptídeo e 10 µg de ODN CpG 1688 5'-tccatgacgttcctgatgct-3' (InvivoGen, USA), perfazendo um volume total de 20 µL. Uma semana após a última imunização, cinco animais por grupo foram eutanasiados em câmara de CO_2 para remoção do baço. Os baços foram removidos em condições assépticas, macerados e homogeneizados. Grumos celulares foram removidos por meio de pipetagem, seguido por passagem por um filtro com poros de 0,45 mm de diâmetro. Em seguida, a concentração celular foi ajustada para 10^6 células/mL e 10^6 células foram depositadas por poço em placas de cultura de 6 poços. Cada poço da placa foi estimulado com 20 µg/mL de cada um dos respectivos peptídeos. Esse procedimento foi realizado em duplicata, separadamente para cada peptídeo avaliado. As placas foram incubadas a 37°C com 5% de CO_2 por 48h. Após o tempo decorrido, as células foram removidas das placas para marcação com anticorpos monoclonais.

4.3.3.2 Cultivo celular e estímulo com os peptídeos

Para este procedimento, inicialmente, as células foram lavadas com uma solução de PBS (pH 7,2)-W contendo 0,5% de albumina sérica bovina e 0,1% de azida sódica (ambos reagentes da Sigma, St. Louis, MO). As células foram centrifugadas a 400 x g por 10 min, em seguida transferidas para tubos de poliestireno onde foi iniciada a marcação com anticorpos. Aos tubos, contendo células, foram adicionados os seguintes anticorpos: anti-CD11b-FITC, anti-CD8a-PE, anti-MHC II-APC, anti-CD103-FITC, anti-CD317-PE, anti-CD205-Alexa Fluor (AF)-488 (todos os anticorpos da Affymetrix eBioscience, USA). Após isso, as células foram incubadas por 30 min a 25°C. Em seguida, as células foram fixadas com solução de 1% de paraformaldeído (PFA) em PBS (pH 7,2) por 10 min e centrifugadas com PBS-W a 400 x g por 5 min.

Às células marcadas para IL-12p70, procedeu-se com a permeabilização das membranas com solução de PBS contendo 0,5% de saponina. Em sequência, as células foram lavadas com PBS-W (400 x g, 5 min), e em seguida incubadas com anti-IL-12p70 conjugado a PE (Affymetrix eBioscience, USA) por 30 min a 25°C. Após completado o período, as células foram lavadas com PBS-W (400 x g, 5 min) e ressuspensas com solução de PFA 1% até a realização das aquisições por citometria de fluxo.

4.3.3.3 Aquisições e análises por citometria de fluxo

Todas as aquisições de citometria de fluxo foram realizadas com um citômetro de fluxo modelo FACSCalibur (Becton Dickinson Company, San Jose, USA), equipado com um laser de argônio (comprimento de onda 488 nm) e utilizando o software CELLQuestProTM (BD Bioscience, San Jose, CA) para as aquisições.

Para as amostras de BMDC e de células esplênicas foram adquiridos 20.000 eventos em gate para cada amostra. As gates foram desenhadas com base nos parâmetros de tamanho e granularidade celular. Os dados foram então analisados e tratados com o software FlowJo v10.1 (Tree Star Inc., USA). Para as subpopulações de esplenócitos, gates booleanas foram desenhadas para acessar o nível de expressão de MHC de Class II nas células analisadas.

4.4 Análises estatísticas

A análise estatística foi realizada com o software GraphPad Prism v.7. Os dados foram analisados com os testes não-paramétricos de Kruskal-Wallis e Mann-Whitney. As diferenças foram consideradas estatisticamente significativas quando o valor de $p \leq 0,05$.

5 RESULTADOS E DISCUSSÃO

5.1 Capítulo I

Combination of *in silico* methods in the search for potential CD4$^+$ and CD8$^+$ T cell epitopes in the proteome of *Leishmania braziliensis*

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Keywords: Neglected Diseases, Cutaneous Leishmaniasis, *Leishmania braziliensis*, Vaccine development, CD4$^+$ CD8$^+$ T cell epitopes.

Abstract
The leishmaniasis are neglected tropical diseases widespread throughout the globe which are caused by protozoa from the genus *Leishmania* and are transmitted by infected phlebotomine flies. The development of a safe and effective vaccine against these diseases has been seen as the best alternative to control and reduce the number of cases. To support vaccine development, this work has applied an *in silico* approach to search for high potential peptide epitopes able to bind to different Major Histocompatibility Complex Class I and Class II (MHC I and MHC II) molecules from different human populations. First, the predicted proteome of *Leishmania braziliensis* was compared and analysed by modern linear programs to find epitopes with the capacity to trigger an immune response. This approach resulted in thousands of epitopes derived from 8,000 proteins conserved among different *Leishmania* species. Epitopes from proteins similar to those found in host species were excluded and epitopes from proteins conserved between different *Leishmania* species and belonging to surface proteins were preferentially selected. The resulting epitopes were then clustered, to avoid redundancies, resulting in a total of 230 individual epitopes for MHC I and 2,319 for MHC II. These were used for molecular modeling and docking with MHC structures retrieved from the Protein Data Bank (PDB). Molecular docking then ranked epitopes

based on their predicted binding affinity to both MHC I and II. Peptides corresponding to the top 10-ranked epitopes were synthesized and evaluated *in vitro* for their capacity to stimulate PBMC from post-treated cutaneous leishmaniasis patients, with PBMC from healthy donors used as control. From the ten peptides tested, 50% showed to be immunogenic and capable to stimulate the proliferation of lymphocytes from recovered individuals.

1 Introduction

The leishmaniasis constitute an important group of tropical neglected diseases [1] which affect and impact on "the bottom billion" of people living in poverty, by inducing disfiguration, loss of productivity and a burden of 3.3 million disability-adjusted life years (DALY) [2, 3, 4]. It is estimated that one quarter of the world's population, 1.7 billion people, are living in risk areas for leishmaniasis [5]. Until recently, 98 countries have reported cases of leishmaniasis with 0.7-1.2 and 0.2-0.4 million cases reported annually of cutaneous (CL) and visceral (VL) leishmaniasis, respectively [6]. The leishmaniasis are caused by protozoans from the genus *Leishmania* which are transmitted to humans and other mammals through phlebotomine sandfly bites [7]. These diseases have multiple forms of presentation and the main ones are: CL, widely distributed among targeted populations and which affects the skin and mucous; VL, the more lethal form, if not treated, which affects mainly the reticuloendothelial system from the liver and spleen; and mucocutaenous leishmaniasis (MCL), affecting mucous and with a poor prognosis. In Brazil, most of the CL cases are caused by *Leishmania braziliensis* and the disease is found in different regions [8], but many of the affected individuals experience spontaneous cure that is associated with IFN-γ production [9]. Diverse strategies are in use to control the leishmaniasis, namely the control of infected animals and vectors, and chemotherapy of affected individuals. These approaches, however, have a high cost and can also induce resistance in parasites and vectors [10]. Therefore, a safe and effective vaccine against human leishmaniasis is urgent to address these issues.

Vaccines against *Leishmania* spp. have the major task of correctly activating the immune system to develop a protective response composed mainly by CD4$^+$ and CD8$^+$ T cells producing and secreting IFN-γ. This response has been associated with disease control, macrophage activation, and parasite elimination from the host [11, 12, 13]. Our previous data have shown that cells from *L. braziliensis* infected patients produce high amounts of IFN-γ after stimulation with whole lysed parasite [14, 15]. To initiate cellular response, dendritic cells (DCs), which are specialized antigen presenting cells (APCs), have the unique capacity to prime naïve T cells by presenting peptide antigens bound to major histocompatibility proteins (MHCs), co-stimulating and secreting cytokines and thus mounting a T cell response against *Leishmania* spp. Some *Leishmania* species, e.g. *L. amazonensis* and *L. mexicana*, may fail however to activate DCs, and consequently no T cell effective response is mounted [16, 17]. *L. braziliensis* is capable of activating DCs and inducing a protective immune response [18]. It is estimated that each mature DC expresses 10^6-10^7 MHC Class II (MHC II) and 10^5 MHC Class I (MHC I) molecules [19]. The activation of CD8$^+$ T cells is a result of the specific engagement of 9-mer-peptide to MHC I proteins (9-mer-p-MHC Class I), while CD4$^+$ T cells are activated by 15-mer-peptides bound to MHC II (15-mer-p-MHC Class II).

There is no such thing as an ideal antigen, and the search for antigens that could generate immunogenic epitopes for a potential vaccine against *Leishmania* spp. is thus critical. In this sense, reverse vaccinology has been constantly increasing its value, and now diverse *in silico* approaches are available for identification of potential antigens and epitopes for vaccines. Since experimental methods are difficult and time consuming, reverse vaccinology using *in silico* methods has narrowed the vast amount of molecules to be tested, increasing the odds of finding better candidates [20]. In addition, many pathogen genomes and proteomes are currently available in public data banks and can be assessed regarding their potential antigen diversity and variability. Thus, sequence and structure based methods investigating the binding affinity of peptides to MHC I and MHC II molecules and other parameters may aid in the search for new antigens in order to support vaccine development [21]. John et al. (2012) [22] have used only sequence based methods to search for different epitopes in the predicted proteome of *Leishmania* spp. Agallou et al. (2014) [23] have recently reported the construction of a multi-epitope peptide vaccine against leishmaniasis by analyzing four known proteins from *L. infantum.* In this context, we hypothesized that a combination of modern sequence and protein structure algorithms could help the search, within the whole predicted proteome from *L. braziliensis*, for potential immunogenic epitopes with high affinity for both human MHC I and MHC II. Thus, the aim of this work was to combine robust *in silico* approaches in the search for potential immunogenic T cell epitopes, based on the proteome of *L. braziliensis*, for the development of an anti-*Leishmania* vaccine.

2 Results

2.1 Linear epitope prediction

With the goal of developing a peptide vaccine based on *in silico* approaches, many studies have shown its feasibility and different attempts have been carried out in order to find good epitopes capable of stimulate the immune system and its memory arm [21, 24, 25, 26], but these mainly rely on computational tools which focus on epitope prediction. Here, we have developed an *in silico* pipeline combining the linear prediction of epitopes with a sequence of structural refinements to confirm the potential of some epitopes to bind to MHC molecules and thus stimulate the immune system. Several computational tools were applied with the goal of minimizing the number of candidate epitopes identified as well as maximizing their potential as inducers of protective immunity. The whole strategy used can be seen in the workflow described in Figure 1.

The initial epitope prediction tools used here (NETMHC and NETCTL) were selected based on two criteria, namely: predictors of MHC I and MHC II binding affinity; predictors in which their accuracy and performance applied to trypanosomatid protein sequences have already been assessed by Resende et al. 2012 [27]. NETMHC and NETCTL were then used to predict MHC I binding epitopes and NETMHC Class II was used for a MHC II prediction. For each allele supertype the epitopes predicted were those classified by the tools as strong binders. Simultaneously, different sets of sequence analysis were performed in order to: exclude all epitopes which belonged to proteins conserved in humans and mice, so as to avoid potential autoimmune epitopes; select epitopes belonging to proteins conserved between different *Leishmania* species, potentially able to induce an immune response against multiple species; select epitopes which came from proteins which were predicted as extracellular or secreted and having

a maximum of one *trans*-membrane domain, therefore selecting epitopes from proteins easier to express and which should be generally exposed to the host immune system. The number of predicted epitopes for both MHC I and MHC II derived from these predictions, and the allele supertypes, are summarized in Table 1. If all predictions for different MHC alleles are considered, the total number of epitopes found in this stage are 657, 6710 and 64553, for NETMHC, NETCTL and NETMHCII, respectively. Next, as the total epitope prediction includes some degree of redundancy, a clustering step was performed considering the sequence similarity among the predicted epitopes, and this analysis resulted in 168 groups for MHC I and 2,138 groups for MHC II. Subsequently, each group was dismembered to reveal the exact sequence of each epitope, resulting in 230 individual epitopes for MHC I and 2,319 epitopes for MHC II.

Figure 1: Methodology flowchart used in this work. TriTrypDB was used to retrieve the predicted proteome of *Leishmania* spp. (A). Predicted proteome sequences were analysed by different methods (B). EpitopeDB relational database was created, and managed using MySQL as database management system. Parsers and algorithms in PERL and SQL languages were developed in order to access and integrate the results (C). Data was clustered (D). Clustered data was used for molecular modeling (E).

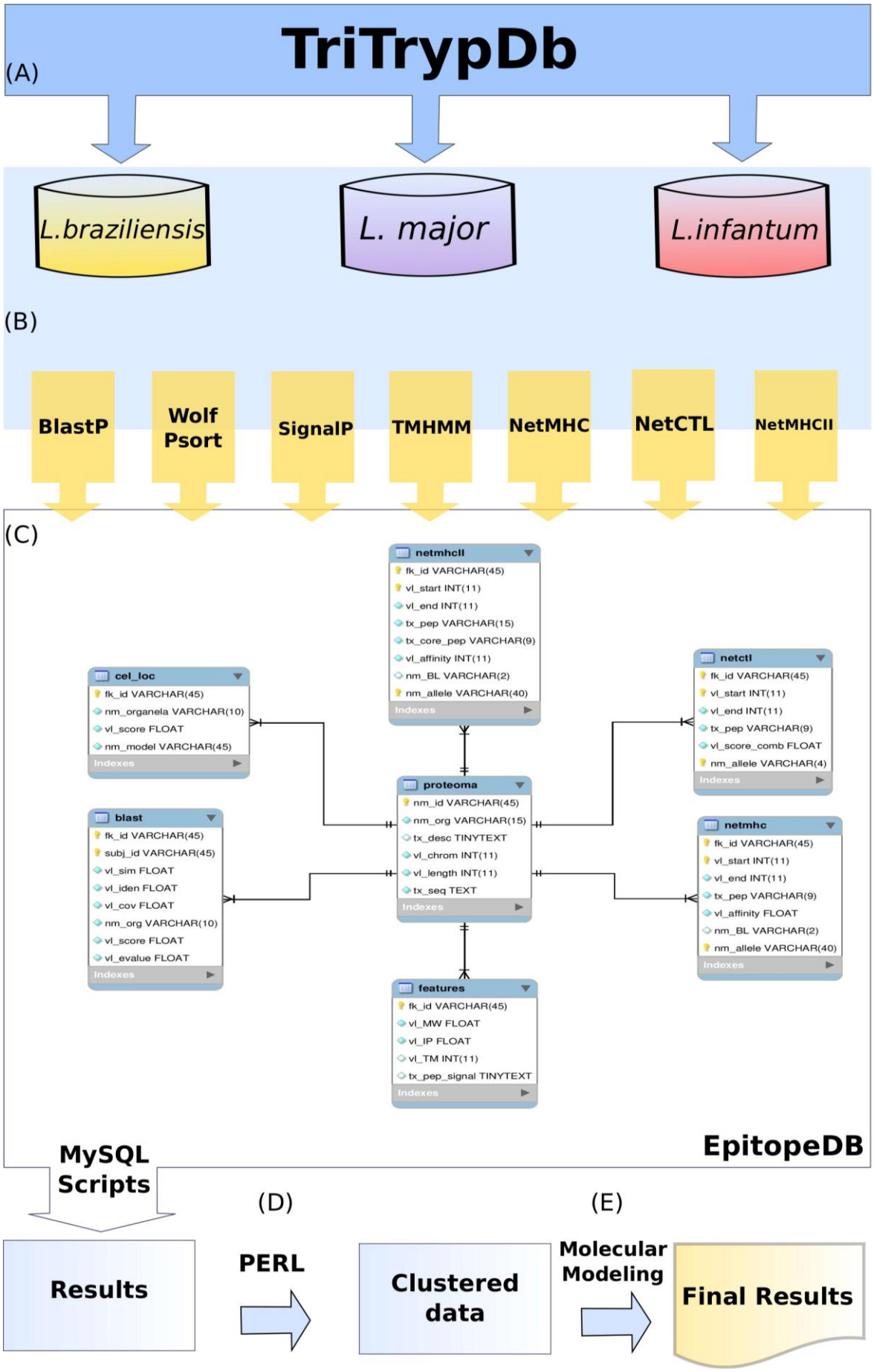

(A)
TriTrypDb
L.braziliensis
L. major
L.infantum
(B)
BlastP
Wolf Psort
SignalP
TMHMM
NetMHC
NetCTL
NetMHCII
(C)
netmhcII
fk_id VARCHAR(45)
vl_start INT(11)
vl_end INT(11)
tx_pep VARCHAR(15)
tx_core_pep VARCHAR(9)
vl_affinity INT(11)
nm_BL VARCHAR(2)
nm_allele VARCHAR(40)
Indexes
cel_loc
fk_id VARCHAR(45)
nm_organela VARCHAR(10)
vl_score FLOAT
nm_model VARCHAR(45)
Indexes
netctl
fk_id VARCHAR(45)
vl_start INT(11)
vl_end INT(11)
tx_pep VARCHAR(9)
vl_score_comb FLOAT
nm_allele VARCHAR(4)
Indexes
proteoma
nm_id VARCHAR(45)
nm_org VARCHAR(15)
tx_desc TINYTEXT
vl_chrom INT(11)
vl_length INT(11)
tx_seq TEXT
Indexes
blast
fk_id VARCHAR(45)
subj_id VARCHAR(45)
vl_sim FLOAT
vl_iden FLOAT
vl_cov FLOAT
nm_org VARCHAR(10)
vl_score FLOAT
vl_evalue FLOAT
Indexes
netmhc
fk_id VARCHAR(45)
vl_start INT(11)
vl_end INT(11)
tx_pep VARCHAR(9)
vl_affinity FLOAT
nm_BL VARCHAR(2)
nm_allele VARCHAR(40)
Indexes
features
fk_id VARCHAR(45)
vl_MW FLOAT
vl_IP FLOAT
vl_TM INT(11)
tx_pep_signal TINYTEXT
Indexes
EpitopeDB
MySQL Scripts
(D)
(E)
Results
PERL
Clustered data
Molecular Modeling
Final Results

Table 1: Number of predicted epitopes by bioinformatics tools

Allele supertype	MHC Class I Prediction	
	NETMHC	NETCTL
	Number of predicted epitopes	
HLA-A1	17	556
HLA-A2	181	685
HLA-A3	86	579
HLA-A24	18	444
HLA-A26	61	515
HLA-B7	20	1620
HLA-B27	229	1092
HLA-B44	31	466
HLA-B58	14	753
Allele supertype	**MHC Class II Prediction**	
	NETMHC Class II	
	Number of predicted epitopes	
HLA-DPA	7021	
HLA-DPB	1558	
HLA-DQA	14762	
HLA-DRB	41212	

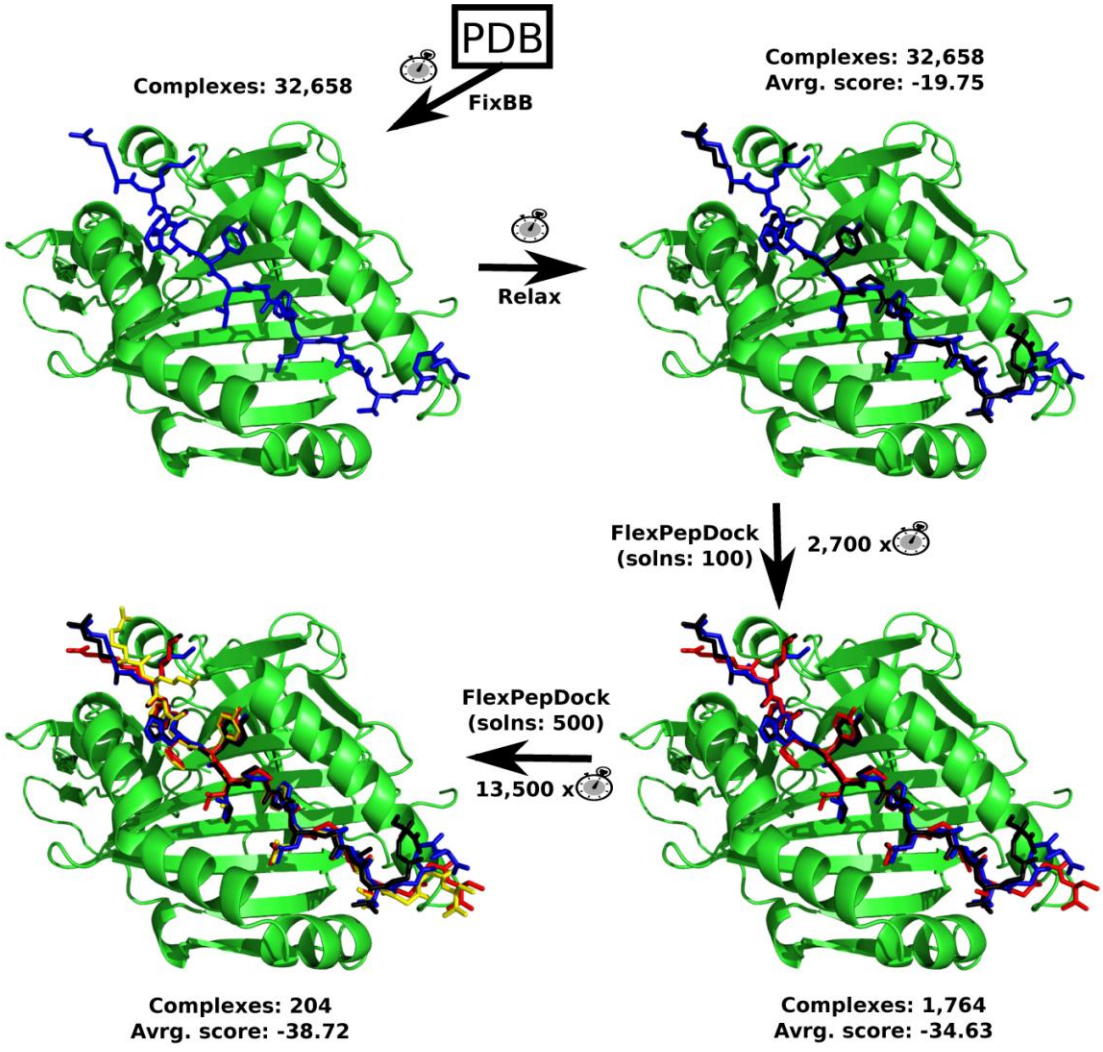
2.2 Fitting linear epitopes into MHC structures

As the main objective of this work was to select predicted epitopes with a good binding affinity to a large number of MHC receptor alleles, a molecular modeling approach was used aiming to find the most *in silico* stable epitope+allele complexes. Structures from 33 different alleles of MHC I (21) and MHC II (12) were downloaded and processed as described in the methods section, prior to modeling their interaction with the individual predicted epitopes. The sequence of steps followed can be found in Figure 2. The first step consists of the replacement of the co-crystalized peptide (present at each downloaded structure) for each one of the predicted epitopes. The total number of complexes is composed of 4,830 complexes for MHC I (21 MHC I alleles times 230 predicted epitopes) and 27,828 complexes for MHC II receptor (12 MHC II alleles times 2,319 predicted epitopes). As the overall combinations of all the MHC structures and predicted epitopes achieved an impressive number of 32,658 complexes, a distributed computing strategy was adopted to process this large scale problem in a feasible time.

With all the 32,658 complexes (receptor + epitope) generated and their respective epitopes energetically relaxed, the molecular docking could be started. This was carried out using the Rosetta's FlexPepDocking protocol. However, during the development of this molecular modeling protocol, several preliminary evaluations were made in order to find a good trade-off between precision and computational demand. First of all, it has been noticed that the use of Rosetta's FixBB protocol to replace the co-crystalized peptide by the predicted epitopes generates typical unstable structures, with high positive Interface scores (Isc). Therefore, three complexes (predicted epitope+allele), that had the best, a regular and the worst Isc values, based solely on the structures obtained by FixBB, were selected and submitted to the Rosetta's Relax protocol. This step was performed to verify if the Relax protocol could stabilize the

selected prediction in more favorable conformations. The Relax protocol results can be found at Figure SI1. It is possible to notice an increase in affinity of the complexes (predicted epitope + MHC receptor) after the relaxing step.

Figure 2: The sequence on which the Rosetta's protocols were used. The total number of complexes generated and the average Interface score (Isc) of it are shown. The computational demand of each protocol compared to the FixBB protocol (clock picture), for a single complex (epitope+allele), can also be found. Every complex image consist of an example containing a MHC II allele (PDB: 3LQZ) and the epitope #1677, after each protocol. The MHC II allele is in green while the predicted epitope color range from blue (FixBB) to black (Relax), red (FlexPepDock with 100 solutions) and yellow (FlexPepDock with 500 solutions).



2.3 Filtering epitope-MHC complexes

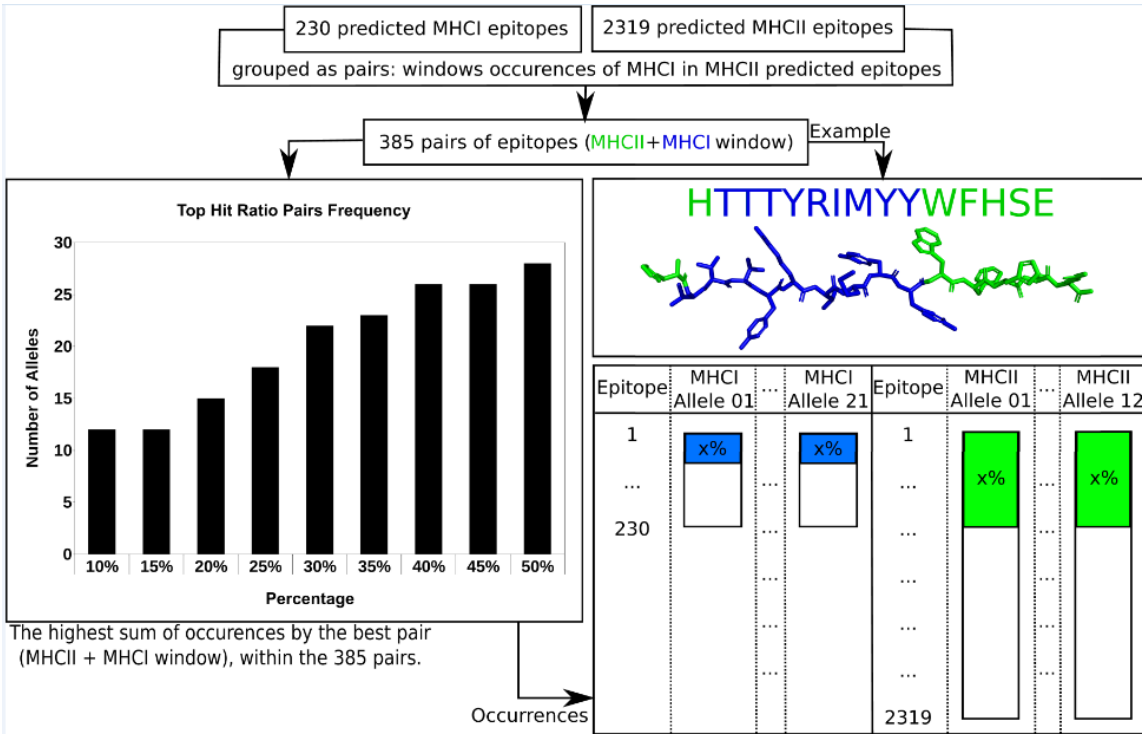
After the analyses performed previously, there was still an impracticable number of complexes (32,658) to be used as input for Rosetta's FlexPepDock protocol. This universe of complexes demands a computational effort that is not feasible even using a computational grid environment. Thus, a filtering strategy had to be adopted in order to select the most promising predicted epitopes to the largest possible number of MHC

receptor alleles. First, knowing that epitopes containing 9 residues (MHC I) can be windows of 15-residue epitopes (MHC II), the epitopes from the Class I prediction were matched with the Class II epitopes in order to find windows of Class I and Class II epitopes. A total of 385 pairs of 15-residue epitopes and their respective 9-residue windows were found.

The filtering strategy required the Isc scores derived from the calculation of the whole set of epitope+allele complexes, computed using the Rosetta's Relax protocol (called re-score procedure, as detailed in the methods section). These Isc scores were used to estimate the frequency that each predicted epitope appears on the list of top 30% ranked candidates for all available MHC structures (Figure 3). If a 15-residue epitope has a good affinity for a MHC II allele, at the top 30% best scored epitopes for each target structure, and its 9-residue window also has a good affinity for a MHC I allele, this "pair" of predicted epitopes (MHC II and MHC I) might be a good candidate for better immunogenic properties. The 385 pairs of 15-residue epitopes and their respective 9-residue windows were then sorted according to the sum of their frequency within the 30% best scored epitopes. After the exclusion of repetitions, the 30% cutoff recovered 81 and 285 predicted epitopes of MHC I and MHC II, respectively, varying from 12 (at 10% and 15%) to 18 (at 25%), within the total 33 alleles (21 for MHC Class I and 12 for MHC Class II). Choosing a cutoff larger than 30% would not increase significantly the number of occurrences. Figure SI2 shows the plot of percentage cutoff for the top ranked candidates, ranging from 10% to 50%, and Figure SI3 presents the filtering algorithm.

Based on such filtering approach, the hundred best ranked pairs (or 1,764 predicted epitope+allele complexes) were used at the next molecular docking step, with Rosetta's FlexPepDock protocol, because this number of calculations required a viable computational demand, about 18-fold lower than the initial set of complexes (32,658).

Figure 3: The filtering approach used in order to reduce the number of complexes calculated at FlexPepDock step. Details can be found in text.

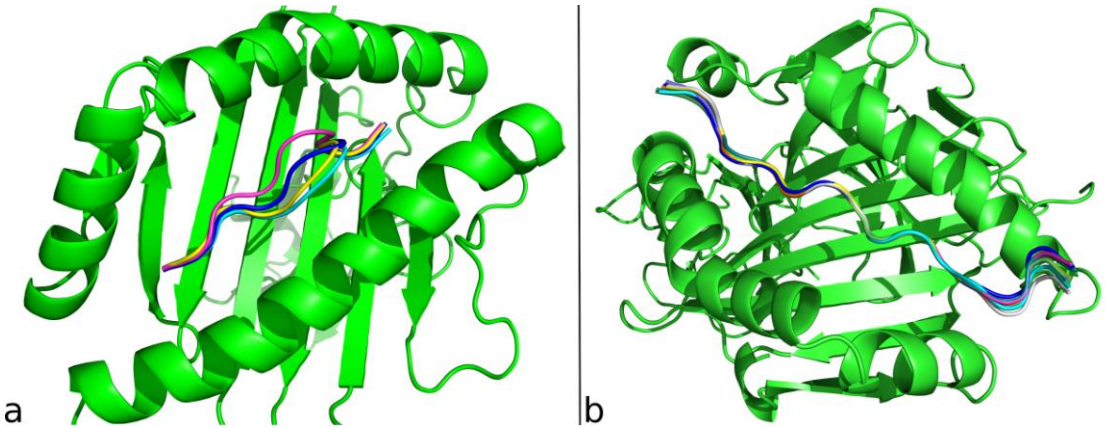


2.4 Molecular docking

The 100 best ranked pairs after the filtering strategy were then used at the molecular docking step with 100 docked solutions each. From this set, the top 10 pairs of predicted epitopes, with the best average Isc among the alleles, were selected for an enhanced run of Rosetta's FlexPepDock protocol with 500 docked solutions, hence increasing the chance of finding new docking solutions with higher affinities for these 10 pairs of predicted epitopes.

The final results obtained through the molecular docking of these ten pairs of predicted epitopes defined a total of four unique 9-residue epitopes predicted for MHC I and ten unique 15-residue epitopes predicted for MHC II. Figure 4 shows the superposition of the best docked solutions derived from the alleles with the highest binding affinity (lowest Isc) to their respective MHC targets (alleles with PDB IDs 4NQV and 3LQZ for MHC I and MHC II, respectively). One can see that the best docking solutions are quite similar in position, displaying a homogeneous result. The way these solutions are positioned are consistent with the known binding mechanism for these MHCs, as each predicted epitope bound to the correct key anchor residues localized within the binding groove of its corresponding MHC target [28]. The MHC I (Figure 4a) predicted epitopes were anchored by residues located at the epitope extremities, while the MHC II predicted epitopes (Figure 4b) were anchored by residues positioned in the middle. The final selected 14 predicted epitopes are those which displayed the best binding affinity among the 2549 candidate epitopes (230 targeting MHC I + 2319 targeting MHC II).

Figure 4: Superimposition of the best solutions for the (a) four predicted MHC I and (b) ten MHC II epitopes.



2.5 Epitope-MHC interaction features

Detailed analyses were then conducted in order to identify important intermolecular aspects responsible for the affinity of these final predicted epitopes to the MHC targets, as follows. First, the correlation between the Interface buried surface area (Ibsa), commonly used to measure the size of the macromolecule interface [29], and the Interface score (Isc) was evaluated. Figure 5 presents the average Ibsa and Isc values for all the complexes (epitope+allele) formed by each one of the 33 MHC structures. A strong correlation between Ibsa and Isc was seen, with bigger Ibsa values being accompanied by lower (more stable) values for Isc. This was to be expected, since when the contact area between the ligand (predicted epitope) and the receptor (allele structure) is larger there are more intermolecular interactions between them stabilizing the complex, with lower (more negative) Isc values. Thus, an increase on Ibsa contributes in a favourable way to the binding affinity of the complexes, by typically lowering the Isc.

In a similar analysis to the one presented in Figure 5, Figure 6 shows the correlation between the average Interface hydrogen bonds (Ihb) and the average Interface score (Isc) values for all the complexes (apitope+allele) formed by each one of the 33 MHC structures. As more hydrogen bonds are formed between the receptor and the predicted epitope, the Isc value is lower, i.e. the complex has a higher binding affinity, emphasizing the fact that the peptides bind to the MHC alleles (particularly MHC II) via an "extensive hydrogen bond network" [28]. To further investigate this correlation, three complexes included in the final results (FPD500) were selected and analyzed (Figure 6). It is important to notice that the larger Class II epitopes (with 15 residues) have a greater natural probability to form hydrogen bonds, because of the higher number of residues (two-thirds more). It is also important to emphasize that the average Interface score (Isc), highlighted in Figure 2 as "Avrg. score", can be observed as progressively more negative with each successive step of the methodology applied, meaning more stable epitope+allele complexes indentified during the *in silico* procedures.

Figure 5: Correlation between the average Interface buried surface area (Ibsa, in square angstroms) and the average Interface score (Isc). This was performed for all the complexes generated with the 33 MHC receptors, after the FlexPepDock protocol (with 500 solutions).

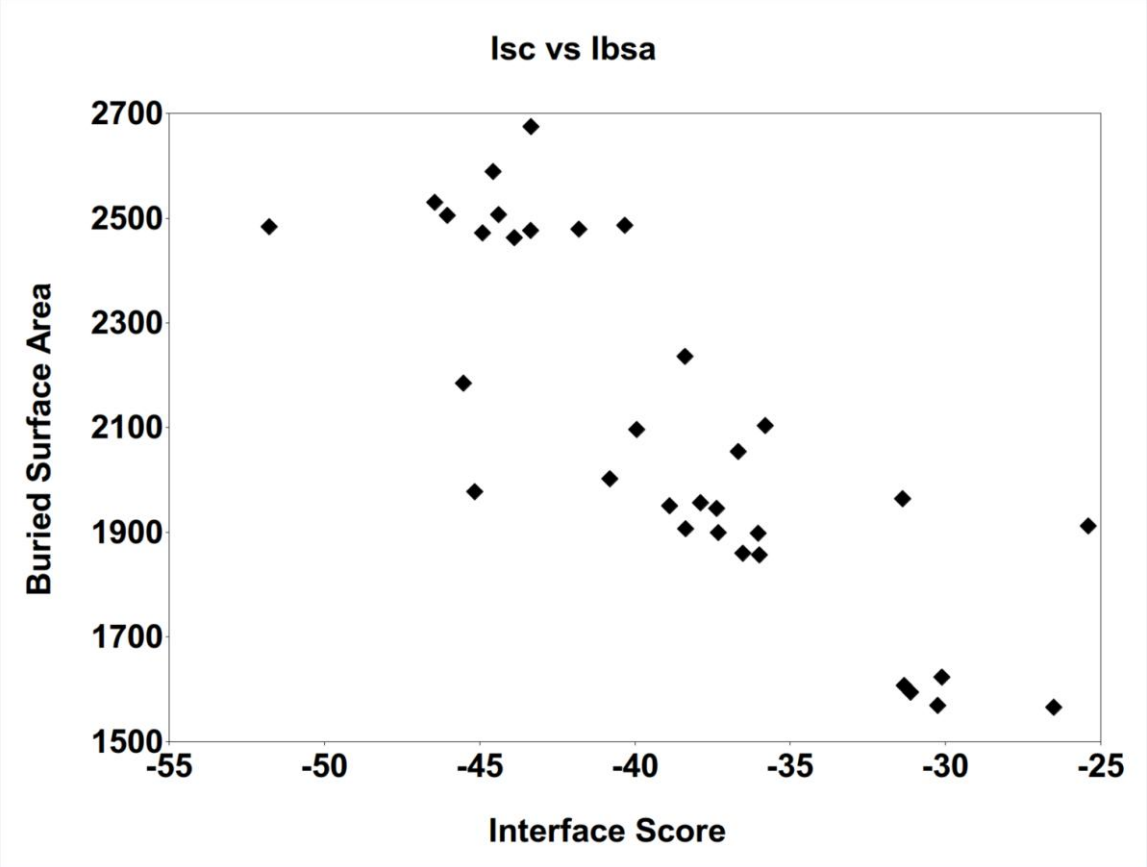
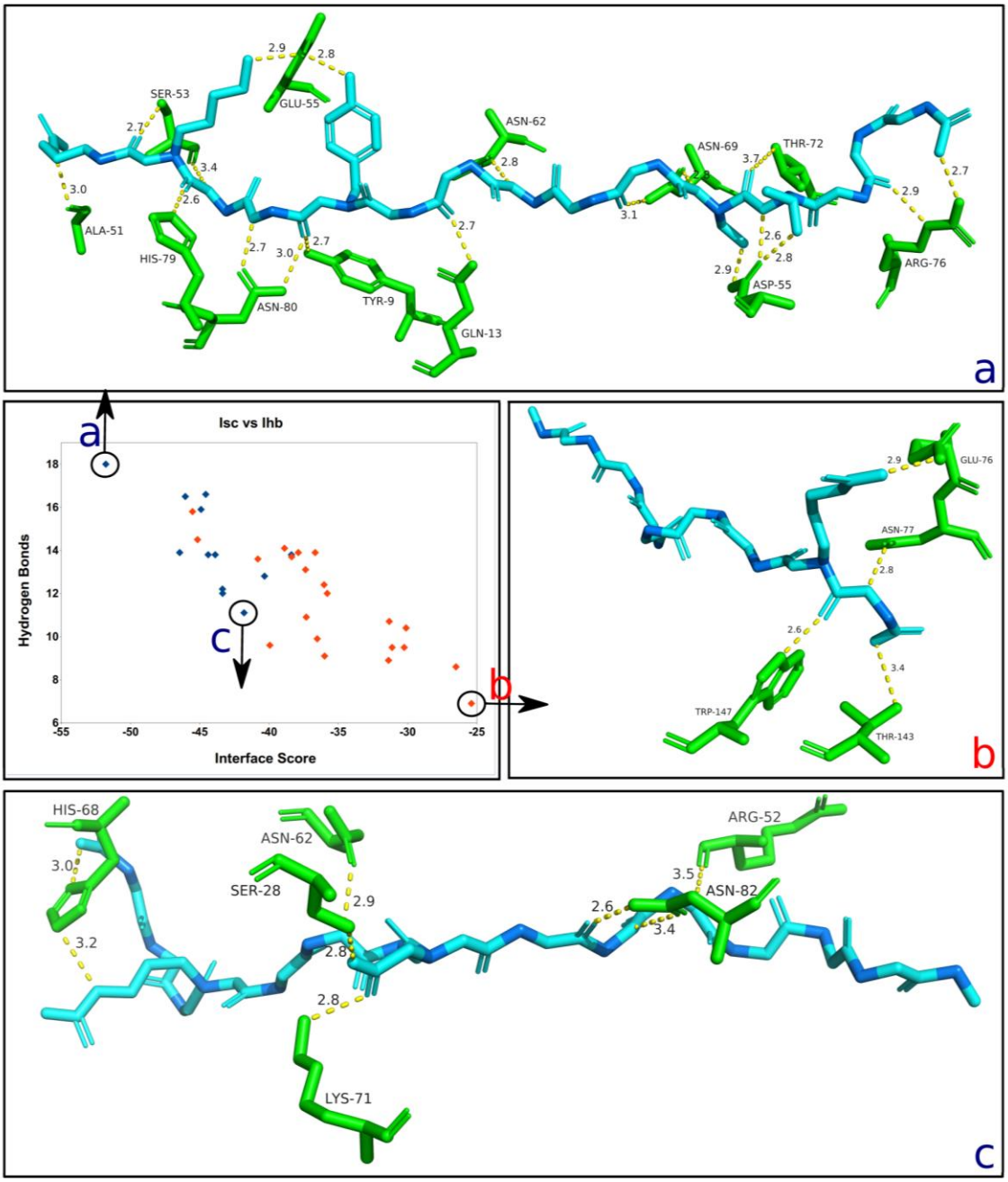


Figure 6: Correlation between the average Interface score (Isc) and the average Interface hydrogen bonds (Ihb), for the complexes obtained by the FlexPepDock protocol with 500 solutions (FPD500). The blue dots correspond to the MHC II alleles (total of 12), while the red dots correspond to the MHC I alleles (total of 21). a) The best docking solution of the final set of results, showing the predicted epitope that formed the highest number of hydrogen bonds and generated the lowest (most stable) Isc. b) The worst overall solution within the final results, having a low number of hydrogen bonds and the highest (less stable) Isc value. c) One of the worst solutions for a MHC II complex, having a reduced affinity between the allele and the predicted epitope. Only the side-chain groups with hydrogen bonds are shown. The epitope´s alpha-carbons are highlighted in dark blue.



2.6 Epitope tracing

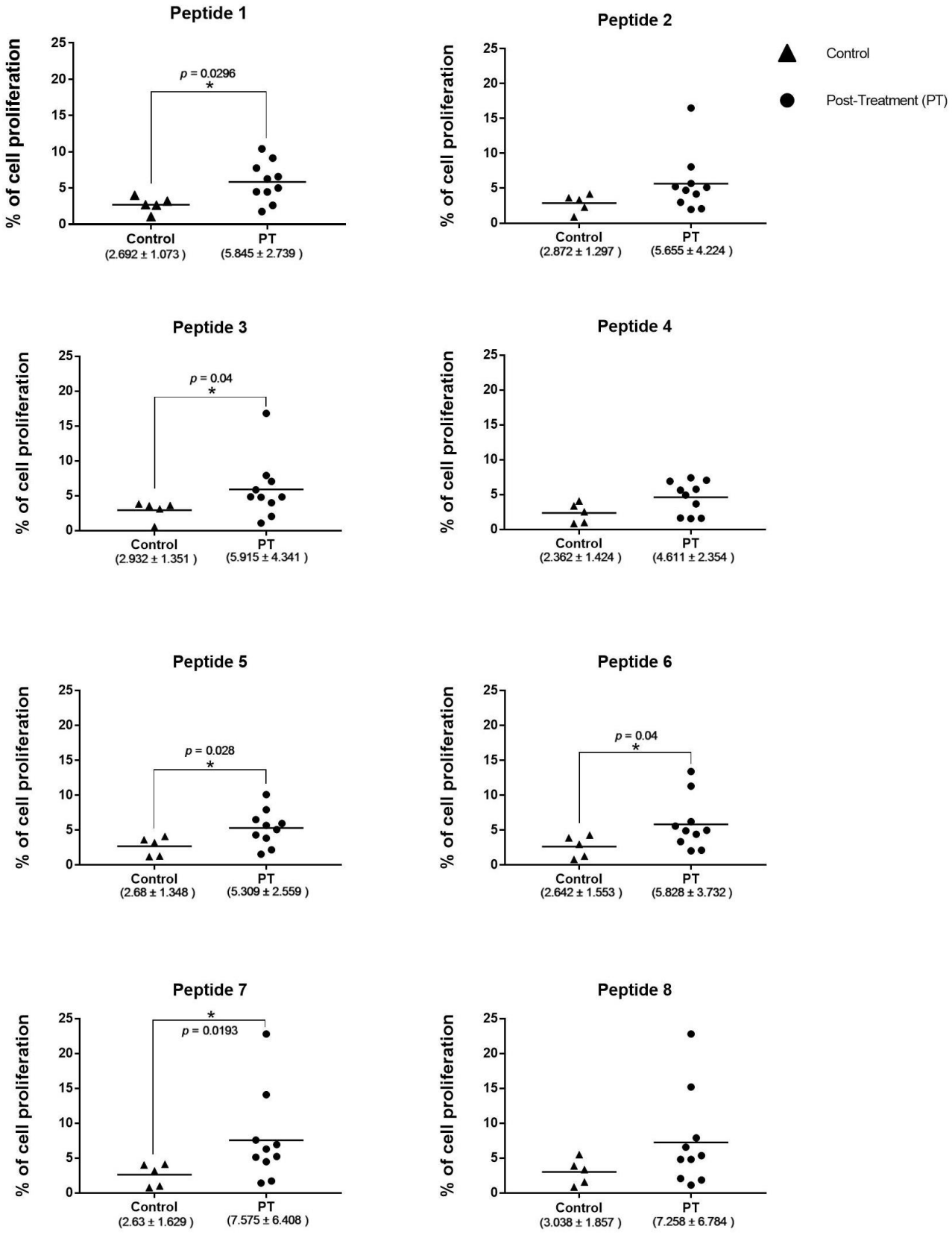
Next, the identity of the ten highest ranking epitopes was investigated and they were seen to derive from a total of four *L. braziliensis* proteins. The first of these, a conserved hypothetical protein, is encoded by a CDS localized to chromosome 34 and encompasses at least four potential epitopes. Interestingly, the protein segment encompassing the four 15-mer epitopes is 19 aa in linear length. Therefore, the four potential epitopes with high affinity for MHC I and MHC II molecules are found within this particular segment, with minor differences between each of these four epitopes but all producing high scores when analysed by the approaches described above. Another conserved hypothetical protein located on chromosome 1 encodes at least three epitopes. Here the three epitopes were found in a window 18 aa in length. The third protein, found in chromosome 14, encodes two 15-mer epitopes located in a window of 16 aa. Finally, the last and largest protein, also hypothetical, encodes only one 15-mer epitope. All the genes encoding for those proteins are syntenic with other genes from

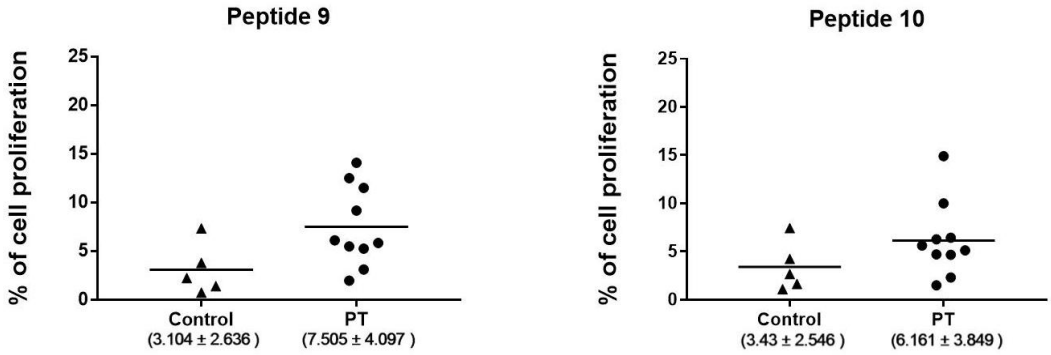
trypanosomatids. This indicates that these proteins have evolved in the same genetic loci from diverse trypanosomatids and the potential epitopes may induce cross-protection against other pathogenic species from the same family.

2.7 Validation of peptide epitopes

In order to have an experimental validation of the results derived from the bioinformatic approach, peptides corresponding to the ten highest ranking 15-mer epitopes described above were commercially synthesized. These were then used for an evaluation of their ability to induce proliferation of Peripheral Blood Mononuclear Cells (PBMC) derived from human patients afflicted with cutaneous leishmaniasis and cured after treatment. These PBMC were capable of proliferating when stimulated with total antigen from *L. braziliensis* and non-stimulated cells had minimal levels of proliferation (data not shown). The results from the assays carried out with the synthetic peptides are presented as the mean percentage of proliferation, with its standard deviation, calculated with the data from the proliferation of PBMCs derived from ten patients, when exposed to the individual peptides. Peptides 2, 4, 8, 9 and 10 were capable of stimulating proliferation of many of the PBMC derived from the afflicted patients. However, no significant difference was observed when the mean proliferation values obtained for these peptides with the PBMC from the ten patients was compared with the mean values derived from the data with PBMC from the control group, consisting of five healthy volunteers. In contrast, significant statistical differences were observed for the mean PBMC proliferation results between samples from treated patients and control volunteers for the assays carried out with peptides 1, 3, 5, 6 and 7 (Fig. 7). These five peptides are derived from three of the four *L. braziliensis* proteins described above, confirming that the positive response is not associated with one specific protein. The possibility that the improved results from some peptides might also be associated with a stronger response from a reduced number of individuals was also investigated. The PBMC from two patients did respond better to a greater number of peptides, four in all, but these include peptides included among both groups described above. In all, the PBMC proliferation data validates the computational approaches used for epitope selection and confirm the potential for some of the synthetic peptides tested to stimulate a protective immune response.

Figure 7: Comparison of the proliferation data from PBMC derived from the cutaneous leishmaniasis post treatment (PT) patients with the PBMC proliferation data from the control group, in response to different peptides. The asterisk indicates significant differences ($p<0.05$) between patients and control group. The horizontal bars represent the mean values for each group. The median percentage levels and the corresponding standard deviation for each group tested are described below.


Peptide 1
% of cell proliferation
p = 0.0296
*
Control
(2.692 ± 1.073)
PT
(5.845 ± 2.739)
Peptide 2
Control
(2.872 ± 1.297)
PT
(5.655 ± 4.224)
Control
Post-Treatment (PT)
Peptide 3
p = 0.04
Control
(2.932 ± 1.351)
PT
(5.915 ± 4.341)
Peptide 4
Control
(2.362 ± 1.424)
PT
(4.611 ± 2.354)
Peptide 5
p = 0.028
Control
(2.68 ± 1.348)
PT
(5.309 ± 2.559)
Peptide 6
p = 0.04
Control
(2.642 ± 1.553)
PT
(5.828 ± 3.732)
Peptide 7
p = 0.0193
Control
(2.63 ± 1.629)
PT
(7.575 ± 6.408)
Peptide 8
Control
(3.038 ± 1.857)
PT
(7.258 ± 6.784)



3 Discussion

So far, different approaches have tried to address the lack of an anti-*Leishmania* vaccine capable of being effectively used against the leishmaniasis. However, most of the vaccines under development have failed in very early assays, due to factors such as poor antigen response, absence of good animal models, and lack of standardization [30]. An ideal vaccine should also be capable of stimulating a promiscuous response against different *Leishmania* species, but generally only one species has been considered at a time. Moreover, only a few papers have described predictions of T cell epitopes from *Leishmania* spp. proteomes [31, 32, 33, 34, 35]. Most of these have focused on epitopes which binds to MHC I, with a focus on CD8$^+$ T cell response and not considering the induction of a response mediated by CD4$^+$ T cells [31, 32, 35, 36]. CD8$^+$ T cells have a major role in protecting against cutaneous leishmaniasis, but evidence has been provided that they may also exacerbate and compromise the disease outcome [37]. In this context, we have searched for natural epitopes that would stimulate both CD4$^+$ and CD8$^+$ T cells, in order to establish a more balanced response that could favour the prevention of disease progression. Development of CD4$^+$ T cell predicted epitopes however is still a challenge, since we do not fully understand the epitope motifs that bind to the MHC II groove, most of the peptides may not be naturally processed by APCs and the positional alignment is critical for the prediction [38, 39]. To overcome these issues, the epitopes predicted here were tested through different criteria in order to define a final set of peptides. One of these criteria was the capacity to bind with high affinity to both MHC I and MHC II, as demonstrated by the high scores of the complexes and the elevated number of hydrogen bonds, for example. Thus, it is expected that, during natural processing by APCs, these peptides could bind either to MHC I or MHC II or be able to bind both molecules.

In terms of target MHC molecules, this work has crucially focused in simulating peptide epitopes with different allele supertypes expressed by different human populations. Allele selection considered their distribution across the globe and other important parameters, such as the promiscuity of the selected peptides to which they bind and PDB crystalized structures. To do so, most of the work was based on human MHC alleles and in order to reduce bias due to MHC multiplicity, allele supertypes were used [40, 41, 42]. Supertypes share specific residues at some anchor positions, thus, they are capable of binding to overlapping groups of peptides [43]. It is important to consider the human MHC molecules, since the purpose of the work is to develop a vaccine that would be applied to humans. Previous works have already helped the search for potential epitopes that could bind to murine MHC, since it is the most used experimental model for pre-clinical assays testing potential vaccines against

leishmaniasis [23, 36]. Most of the *in silico* developed peptides based on the murine model, however, may fail to translate good results to humans, due to the huge differences between human and mouse immune responses. All the computational efforts performed here were therefore dedicated to simulate peptides with human MHC alleles.

One of the major problems of bioinformatics these days is the huge amount of data generated. Linear epitope predictions using predicted proteomes provides a large number of potential epitopes which cannot be possibly be tested. Therefore, this large number must be reduced into a feasible number of epitopes which can be experimentally tested for their immunogenicity. Thus, the option here for molecular modeling protocols to solve this issue, mainly through the application of molecular docking approaches, adding another layer of strength to the data. Here, it has been shown that the best ranked peptide epitopes are clearly those which establish the highest number of molecular interactions (like hydrogen bonds and hydrophobic contacts, for example) with the chemical groups in the MHC groove.

Based on the results presented so far, it seems that the proposed combination of approaches is consistent enough to be applied in cases of reverse vaccinology, when there is a large quantity of candidate epitopes to be tested. The strategy of distributed computing (computational grid) alongside the filtering algorithms has turned an unpractical problem, into a feasible task, done in weeks. Moreover, in the context of leishmaniasis, the results of this research identified peptide epitopes with high potential to stimulate the immune system to develop a protective response.

4 Material and Methods

4.1 Linear epitope prediction

4.1.1 Proteome retrieval and conservancy

The available proteomes (from *L. braziliensis*, *L. major*, and *L. infantum*) were downloaded from TriTrypDB [44] and used to perform, in parallel, different bioinformatics analyses. Only protein sequences from L. braziliensis with more than 60% conservancy with other Leishmania species verified through BLAST protein alignment were considered for epitope analysis. This parameter was taken into account since an ideal vaccine should be capable to induce protection in individuals against as many species as possible.

4.1.2 MHC Class I and MHC Class II prediction tools and binding affinity prediction

NetMHC and NetCTL tools were used for a MHC I predictions while MHC II predictions were made using the NetMHCII tool for the most prevalent allele supertypes [45, 46, 47]. Both NetMHC and NetCTL are epitope predictors, however NETMHC just predicts the epitope while NETCTL also considers other predictions, such as the transport efficiency prediction mediated by the Transporter Associated with Antigen Processing (TAP) protein and the C-terminal proteasomal cleavage prediction. The cut-off score defined to select peptides with high affinity for those tools was ≥ 1 in order to maximize the number of true positive predictions.

4.1.3 Similarity and biological features of protein candidates

In order to exclude protein candidates with high degree of similarity with proteins of humans and mice, the BLAST sequence alignment tool [48] was used to compare parasite protein sequences against host protein sequences. Proteins with degree of similarity equal or higher than 40% with human or mice proteins were excluded from the next steps. Moreover, transmembrane helix and sub-cellular localization predictions were performed using the TMHMM tool [49] and WoLF PSORT [50], respectively.

All the data obtained after running the methods described above were deposited in a relational data base, which is managed using MySQL as a Data Base Management System (DBMS). Parsers and algorithms in PERL and SQL languages were developed in order to access and integrate the results deposited in the databank.

4.1.4 Clusterization

An in house algorithm based on BLAST alignment results was developed in order to group the data with high similarity. A threshold of 60% of identity and 100% of coverage between any two epitopes were used in order to cluster them in the same group. In addition, group selection was performed based on selective criteria: peptide epitopes with high affinity predicted for at least three different allele of either MHC Class I or MHC Class II, or peptide epitopes derived from at least three different proteins.

4.2 Molecular modeling approach

4.2.1 Preparing MHC structures from PDB

The structures from the 33 different alleles of MHC I (21 PDB structures: 2HJL, 3C9N, 3HCV, 3KPP, 3L3D, 3RL1, 3VCL, 3VFS, 3X11, 4F7M, 4G8G, 4HWZ, 4JQX, 4MJ5, 4MJI, 4NQV, 4O2C, 4QRR, 4QRU, 4WU5, 4XXC) and MHC II (12 PDB structures: 1A6A, 1BX2, 1H15, 1S9V, 1UVQ, 1YMM, 2NNA, 2Q6W, 3C5J, 3LQZ, 3PL6, 3WEX) were downloaded from RCSB Protein Data Bank [51]. These structures were then prepared by removing the water molecules, ligands and duplicated residues or alleles. Furthermore, using the PyMol [52] software in build mode, the co-crystallized small peptide chain, for each structure, was modified to have the same length as the predicted peptides used in this work (9 residues for MHC I and 15 residues for Class II). In addition, each non canonical amino acid found in the co-crystallized peptide was manually mutated to alanine.

An in-house developed software named GriDoMol was then used to prepare and submit into a computational grid environment all the in silico procedures required to combine the MHC structures to the predicted epitopes and to compile the results obtained at each step into formatted datasheets. This computational grid environment was assembled, in our laboratory, by using eight computers, each containing 2x Intel Xeon quadcore (total of 8 cores per computer) chipset and 16GB RAM memory.

4.2.2 Producing MHC-epitope complexes

The sequence of steps on which the Rosetta framework [53] protocols were used can be found in Figure 2. For the replacement of the co-crystalized peptides for each one of the predicted epitopes, the Rosetta's FixBB protocol, available within the Rosetta framework, was used. However, the Rosetta's FixBB protocol does not move the backbone atoms, and thus the replacement of the co-crystalized peptide for a new one may produce unstable final conformations. Therefore, the Rosetta's Relax protocol was

used, right after the FixBB protocol, in order to energetically stabilize each one of the new epitopes. Moreover, all the MHC receptor's residues have been locked unmovable to prevent conformational changes on the receptor side, while only the epitope's residues were allowed to move and rotate towards a more stable conformation in the chemical neighbourhood.

In order to quantify the binding affinity between receptors and predicted epitopes, at this particular step, the score function named Interface score (Isc) was chosen from the Rosetta's FlexPepDock protocol [54], which is the sum over the energetic contributions of the interface residues on both the receptor and the predicted epitope. Hence, all the 32,658 structures obtained by the application of the Rosetta's Relax protocol were only re-scored using the Rosetta's FlexPepDock protocol, in order to obtain the Isc values, but keeping the same structure obtained by Relax protocol (i.e., without any change on the atomic coordinates). In other words, the re-score procedure just re-compute the energy (with a better scoring function) at the same geometry, without perturbing the chemical system.

4.2.3 Scoring potential epitopes through molecular docking

While each run of the FixBB or Relax protocols takes a few seconds to generate the result, each run of the FlexPepDock protocol, using the default setup (100 docked solutions obtained as result), takes hours to complete. Thus, a filtering strategy had to be adopted in order to select the most promising predicted epitopes, based on their Isc scores (obtained by re-scoring, as mentioned above) with receptors and the frequency of affinity observed along different MHC receptor structures.

The Rosetta's FlexPepDock protocol, using the refinement approach, was applied to perform the molecular docking, allowing the full flexibility for the predicted epitope and the side chain flexibility for the residues at the receptor's interface. This procedure searched for the predicted epitopes with the best binding affinities for MHC's alleles. For each predicted epitope, the best docking solutions were selected according to the Interface score (Isc). At the end, the predicted epitopes were ranked by the average Interface score of their solutions along the MHC's alleles, granting an overall view of each epitope's average affinity. From the list of hundred pairs previously described, the first 10 pairs of predicted epitopes with the best average Isc among the alleles have been selected for an enhanced run of Rosetta's FlexPepDock protocol, increasing the number of generated structures from 100 to 500. The computational cost of such calculation (500 docked solutions) was about five times greater than the default 100 docked solutions.

4.3 Validation of peptide epitopes

4.3.1 Synthetic peptides and storage

Peptides corresponding to the top 10 ranked 15-mer peptides were synthesized (Genome Biotechnology, Brazil). Linear peptides were purified through a high performance liquid chromatography (HPLC) approach with a final purity greater than 95%. All the synthetic peptides were individually ressuspended in DMSO and stored at -80°C until use.

4.3.2 Sampling and isolation of peripheral blood mononuclear cells

All individuals included in this research signed a written informed consent before blood collection, following recommendations of the Ethics Committee from the Centro de Pesquisas Aggeu Magalhães (CPqAM-FIOCRUZ, Project: 522.964). From each individual, a total of 30 mL of peripheral blood was collected by venipuncture in sodium-heparin tubes (Vacuette, USA). The blood was diluted (1:1 v/v) with phosphate-buffered saline (PBS, pH 7.2) and deposited onto the Ficoll Paque PLUS density (1.077 g/mL) gradient (GE Healthcare, USA) and centrifuged. Subsequently, the PBMC layer was individually removed and washed twice with PBS.

4.3.3 CFDA-SE labelling and cell culture

About 4 x 10^6 cells were ressuspended in 1 mL of PBS containing 2 μM of carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, Invitrogen, USA) and incubated at 37°C for 10 min. The CFDA-SE concentration was previously titrated in order to prevent inhibition of cell proliferation or cell death. After incubation, cell labelling was quenched with 1 mL of ice-cold (4°C) RPMI 1640 containing 2mM of L-glutamine, 50mg/L of gentamicin sulfate and 2mg/L of amphotericin B, supplemented with 10% fetal bovine serum (both Cultilab reagents, Brazil). The cells were pelleted and washed with PBS followed by resuspension in 1mL of RPMI 1640 supplemented at a density of 2 x 10^6 cells/mL. The PBMC were plated in 96-well U bottom plates (BD Falcon) at a density of 2 x 10^5 cells/well with 20 μg/mL of each peptide. These cells were then incubated at 37°C with 5% CO_2 for 96 hours. For each patient or control volunteer tested, non-stimulated and phytohemagglutinin (PHA)-stimulated cells were evaluated as intra-experimental controls.

4.3.4 Flow cytometry analysis

The analyses were performed on a FACScalibur flow cytometer (Becton Dickinson Company, USA) equipped with an argon laser (wavelength 488 nm). Fluorescence of 20,000 lymphocyte gated events, based on scatter parameters of size and granulosity, was acquired. The data was analyzed and treated with FlowJo v10.1 (Tree Star Inc., USA). Non stimulated cells were used during the analysis for setting quadrant parameters and to set the basal level of lymphocyte proliferation. For the statistical analysis, the data were analysed with non-parametric Mann-Whitney U-test. Differences were considered statistically significant when $p < 0.05$.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

LF is the recipient of a doctoral fellowship received from the "Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco" (FACEPE). MZ, OP and VR acknowledge research fellowships received from the "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq).

Author Contributions

Conceived and designed the methods: RF, MZ, AM, VR. Performed the *in silico* approaches: RF, LF, MZ, AM. Performed the epitope validation: RF, VR, MEF, BC, AAS. Analyzed the data: RF, LF, MZ, OP, AM, VR, MEF, BC, AAS. Wrote the paper: RF, LF, MZ, OP, AM, VR.

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5.2 Capítulo II

Immunogenicity of potential CD4$^+$ and CD8$^+$ T cell epitopes derived from the proteome of *Leishmania braziliensis*

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Abstract
Background Up to today, there is no safe and effective vaccine against *Leishmania* to be applied in humans. This work tested the immunogenicity of ten different CD4$^+$-CD8$^+$ 15-mer epitopes from the proteome of *Leishmania braziliensis* with PBMC from cutaneous leishmaniasis (Active disease-AD; Post-treatment-PT) and resistant (RT) individuals. **Research design and methods** Cells were labelled with CFDA-SE prior to culture for 96h in the presence of 20μg/mL of each peptide, individually. Cell supernatant was stored for Th1, Th2, and Th17 cytokines measurements, and the cells were processed for flow cytometry. T-bet expression was assessed in CD4$^+$ and CD8$^+$ T cells. **Results** A significant high level of cell proliferation for the RT group was found for all peptides tested. T-bet was significantly up-expressed in CD4$^+$ and CD8$^+$ T cells from PT group after stimulation with six peptides. Significant levels of IFN-γ, TNF, and IL-6 were observed for the cells from the PT group stimulated with four tested peptides. High levels of the same cytokines were also observed for the AD group. IL-10 was also present in PT and AD group, and very low levels of IL-4. **Conclusions** The peptide epitopes have the potential to stimulate human cells from AD, PT patients and RT individuals, suggesting its immunogenicity.

Keywords: Neglected Diseases, Cutaneous Leishmaniasis, *Leishmania braziliensis*, Immunogenicity, CD4$^+$ CD8$^+$ T cell epitopes

1 Introduction

The Leishmaniasis are an important group of neglected tropical diseases (NTDs) [1,2] caused by protozoan from the genus *Leishmania*. It is endemic in 98 countries and territories, with annual incidence of 1.5 to 2 million cases, and threatening at least 350 million people living in areas with risk of transmission. Clinical manifestation may occur under different forms, such as visceral (VL), mucocutaneous (ML), and cutaneous (CL) [3]. Among human populations, VL is more limited in terms of number of cases, although it has been implicated with more lethal forms caused by *Leishmania donovani* (Old World) and *Leishmania infantum* (New World) for e.g. In other hand, CL is

widespread and responsible for a high number of cases, and may cause high morbidity by inducing disfigurement and loss of productivity [4]. New and Old World *Leishmania* species are involved in the development of CL, such as *Leishmania (Viannia) braziliensis* (*L. V. braziliensis*) and *Leishmania Leishmania major* (*L. major*), respectively. Currently alternatives to control these diseases are limited to vector control measures and the use of old and toxic antimonial drugs for which there is an increasing number of resistant parasites [5]. Thus, there is an urgent need for a safe and effective vaccine against leishmanisis to be applied in humans.

Even though there is a high number of cases until now, only three vaccines against leishmaniasis have been approved for use, two are whole-parasite vaccines for use in humans, and one is a recombinant vaccine for dogs [6,7]. Evidences have shown that the development of a vaccine for CL is feasible since the recovery from *L. major* and, in some level, from *L.V. braziliensis* infection may induce protection against reinfection [8,9], demonstrating that natural infection is capable to induce memory immunity capable of preventing reinfection [10]. However, there are missing points which might be implicated with this failure in developing a CL vaccine. One of them is that most of the acquired data comes from murine models of *L. major* infection, and the New World specie *L.V. braziliensis* has a different response pattern in which data from *L. major* cannot supply. Another point is that we do not fully understand the immune response against *Leishmania* infection in humans, how to generate memory against reinfection and what are the best correlates of protection to look for [10]. Moreover, one of the main challenges faced to develop a vaccine is to find the best type and combinations of antigens to correctly induce immunity against *Leishmania*, since traditional whole parasite vaccines have major safe concerns.

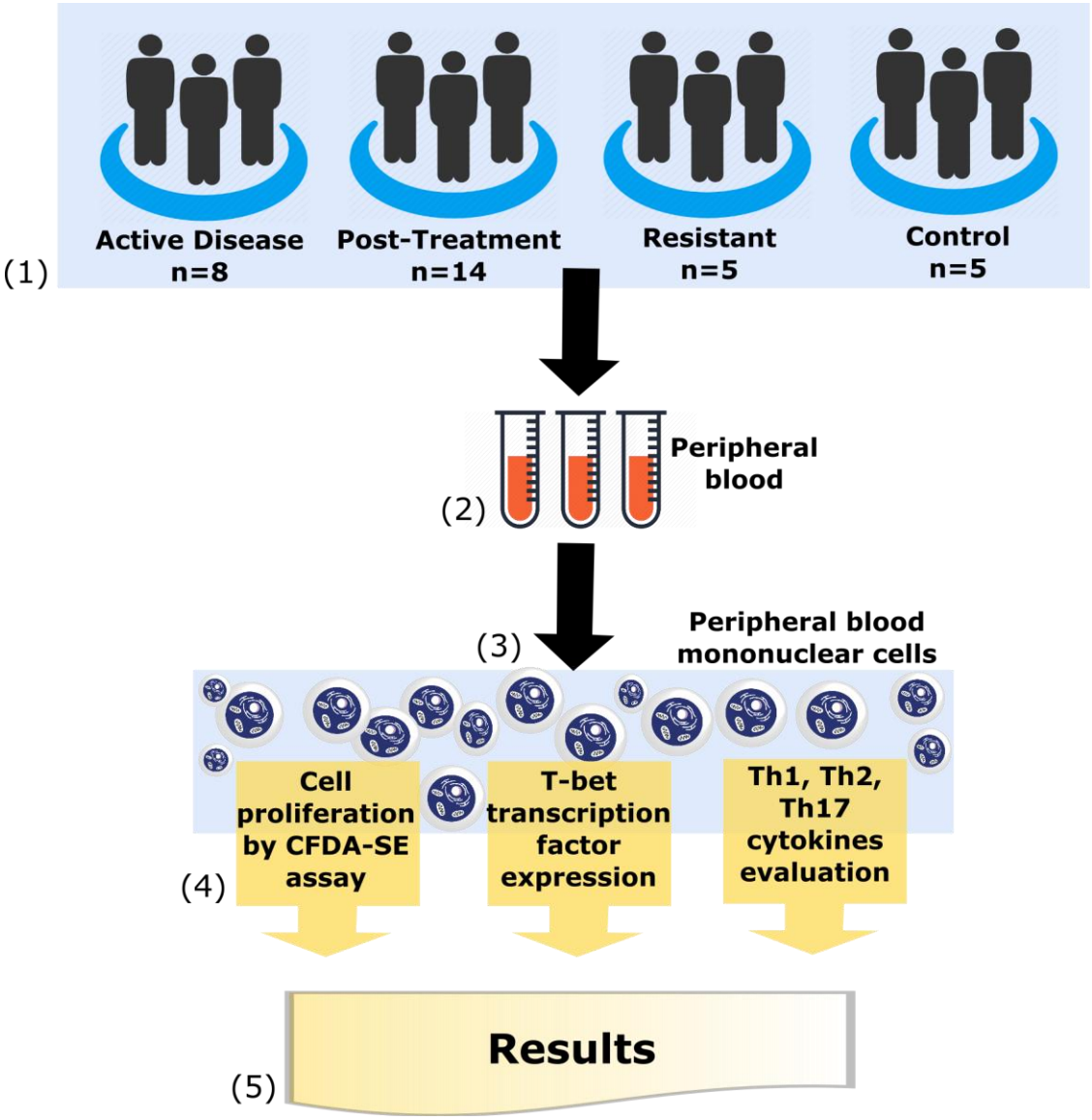
The immune response against *Leishmania* infection is orchestrated through different cell subsets and molecules, and current data shows that such immunity is achieved through the development of T CD4$^+$ and T CD8$^+$ lymphocytes producing key cytokines [11]. For murine models, the T CD4$^+$ Th1 profile is implicated with parasite clearance and disease control, and the Th2 profile with high parasite burden and disease progression [12]. T-bet (encoded by *Tbx21*) is a transcription factor which acts as the master regulator of Th1 profile commitment [13]. However, for humans the response is more complex and there is an important contribution from different profiles counter interacting to achieve a protective response. For example, the Interleukin (IL)-17 has been implicated with a massive influx of inflammatory cells, and disease exacerbation [14], and the IL-10 with strong immunosuppression and exacerbated pathology [15]. Therefore, there should be a balance between cytokines from Th1 and Th2 profiles, such as the Interferon (IFN)-γ and Tumor Necrosis Factor (TNF) for the first, and IL-6 for the second, acting together to induce a protective immune response [16,17].

The cellular immune response against *Leishmania* is initiated through the priming of naïve T lymphocytes by dendritic cells (DCs) which are specialized antigen presenting cells (APCs) capable of presenting *Leishmania* peptides bound to major histocompatibility complex molecules (MHCs). In addition, DCs co-stimulate, produce and secrete cytokines to mount a T cell response [18]. It is estimated that each DC can express 10^6-10^7 MHC Class II (MHC II) and 10^5 MHC Class I (MHC I) molecules [19]. The activation of CD8$^+$ T cells is a result of the specific engagement of 9-mer-peptide to MHC I proteins (9-mer-p-MHC Class I), while CD4$^+$ T cells are activated by 15-mer-peptides bound to MHC II (15-mer-p-MHC Class II). Previous data have shown that whole lysed antigen from *L.V. braziliensis* was capable to stimulate the secretion of IFN-γ *in vitro* by cells from infected patients [20,21].

Thus, regarding the potential of the *L.V. braziliensis* antigens and the lack of vaccines and approaches to develop it, we have described, in a previous work, the use of a combination of robust *in silico* methods in the search for potential CD4$^+$ and CD8$^+$ T cell epitopes in the proteome of *L.V. braziliensis*. Therefore, based on this methodology, we have identified a set of 15-mer peptides with an intrinsic potential to both bind MHC I and MHC II molecules from the main alleles present in human populations. The top 10 high-ranked peptides were capable to significantly stimulate the proliferation of immune cells from post-treatment CL patients [22]. The peptide epitopes were restricted to Human Leukocyte Antigen (HLA) supertypes (A1, A2, A3, A24, A26, B7, B27, B44, B58, DPA, DPB, DQA, DRB), and found at least in four different hypothetical highly conserved proteins. In this context, the aim of this work was to analyze the *in vitro* immunogenicity aspects induced by the top 10 high-ranked peptides over cells from post-treatment (PT), active disease (AD) and resistant (RT) patients (no clinical disease, but skin delayed-type hypersensitivity positive reaction).

2 Patients and Methods

Figure 1: Methodology flowchart used in this work. Individual volunteers from different groups were recruited to participate after signature of the "Term of free and informed consent" (1). From each individual, peripheral blood was collected by venipuncture (2). After, blood was deposited onto Ficoll-Paque solution for isolation of peripheral blood mononuclear cells (PBMC) (3). Cells were used for cell proliferation assay, T-bet transcription factor expression, and Th1, Th2, Th17 cytokine evaluation (4), and ultimately generating the results (5).



2.1 Study design and ethics statement

Individuals from both genders at median age were selected from the city of Moreno, Pernambuco state, Brazil. Three different groups were studied: active disease (AD-*n*=8), post-treatment (PT-*n*=14), and resistant (RT-*n*=5) patients. The last group is composed of individuals living in endemic region, positive for Leishman Skin Test (LST) and with no history of clinical disease. One group (*n*=5) of healthy individuals living in non-endemic region for leishmaniasis were used as control. The protocol of this research was evaluated by the Human Research Ethics Committee at CPqAM/FIOCRUZ (Protocol number 522.964). All individuals have signed the "Term of free and informed consent".

2.2 Synthetic peptides and storage

Molecules corresponding to the top 10 ranked 15-mer peptides described in previous work were commercially synthesized (Genome Biotechnology, Brazil). Linear peptides were purified through a high performance liquid chromatography (HPLC) approach with a final purity greater than 95%. All the synthetic peptides were individually resuspended in dimethyl sulfoxide (DMSO) and stored at -80°C until use.

2.3 Isolation and culture of human PBMC

From each individual enrolled in this research, 20 ml of blood was aseptically collected. Then, the blood was diluted at 1:1 (v/v) with phosphate buffered saline (PBS, pH 7.2); afterward it was deposited onto the Ficoll-paque PLUS (GE) gradient solution and centrifuged for 35 min at 400 x g. Then, peripheral blood mononuclear cell (PBMC) layer was removed and washed twice with PBS (pH 7.2). Cells were resuspended in roswell park memorial institute (RPMI) 1640 medium containing 2 mM of L-glutamine, 50 mg/L of gentamicin sulfate, supplemented with 10% fetal bovine serum (both Cultilab reagents, Brazil). The resuspended cells were counted and their concentration was adjusted to 10^6 cells/mL prior to culture. 2 x 10^5 cells were deposited in 96-wells U-bottom plates, and each well individually stimulated with 20 μg/mL of each peptide. This was performed separately for each tested peptide. Cell plates were incubated at 37°C with 5% CO_2 for 4 days. After this period, plates were centrifuged for 10 min at 400 x g and cell supernatant was collected and stored at -80°C for cytokine measurement by flow cytometry.

2.4 CFDA-SE labelling and cell culture

Cells from AD, RT, and the control group were labelled with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, Invitrogen, USA) in order to assess the level of cell proliferation induced by the peptides. For this, 4 x 10^6 PBMC cells were resuspended in 1 mL of PBS (pH 7.2) containing 2 μM of CFDA-SE and incubated at 37°C for 10 min. The CFDA-SE concentration was previously titrated in order to prevent inhibition of cell proliferation or cell death. After incubation, cell labelling was quenched with 1 mL of ice-cold (4°C) RPMI 1640 containing 2 mM of L-glutamine, 50 mg/L of gentamicin sulphate, supplemented with 10% fetal bovine serum (both Cultilab reagents, Brazil). The cells were pelleted and washed with PBS followed by resuspension in 1mL of RPMI 1640 supplemented at a density of 2 x 10^6 cells/mL. The PBMC were plated in 96-well U bottom plates (BD Falcon, USA) at a density of 2 x 10^5 cells/well with 20 μg/mL of each peptide. Cells were then incubated at 37°C with 5% CO_2 for 96 hours. After this period, cells were removed from the plates, deposited in polystyrene tubes, washed with PBS (pH 7.2), and analysed by flow cytometry. For each patient or control, non-stimulated and 20 μg/mL phytohemagglutinin (PHA)-stimulated labelled cells were evaluated as intra-experimental controls to set the level of cell proliferation during flow cytometry data analysis.

2.5 Anti-T-bet cell labelling

For this approach, cells from four PT individuals and four controls were possible to evaluate the levels of T-bet expression after stimuli with peptides. For this, cells were then washed with a solution called PBS-W which is composed of PBS with 0.5% bovine serum albumin (Sigma, St. Louis, MO) and 0.1% sodium azide (Sigma, St. Louis, MO); then cells were centrifuged (400g, 10 min, RT) and transferred to polystyrene tubes. Monoclonal antibodies anti-CD4 and anti-CD8 were added to the tubes, both conjugated with allophycocyanin (APC) and fluorescein isothiocyanate (FITC), respectively (BD Bioscience, San Jose, CA). Following, the cells were incubated for 30 min at 25°C. Afterwards, cells were fixed with 1% paraformaldehyde (PFA) in PBS, washed by centrifugation with PBS-W (400g, 5 min) and permeabilized with PBS plus 0.5% of saponin. Subsequently, the cells were washed with PBS-W by centrifugation (400g, 5 min), and then they were incubated with anti-T-bet-phycoerythrin (PE) conjugated (BD Bioscience, San Jose, CA) for 30 min, RT. After

this time, they were washed by centrifugation with PBS-W (400g, 5 min) and resuspended with 1% PFA in PBS.

2.6 Flow cytometry analysis of CFDA-SE and T-bet labelled cells

All flow cytometry analyses were performed on a FACScalibur flow cytometer (Becton Dickinson Company, San Jose, USA) equipped with an argon laser (wavelength 488 nm) and using the software CELLQuestPro^{TM} (BD Bioscience, San Jose, CA) for acquisition. Fluorescence of 20,000 lymphocyte gated events, based on scatter parameters of size and granularity, was acquired. The data was analyzed and treated with FlowJo v10.1 (Tree Star Inc., USA). For CFDA-SE assay, non-stimulated cells were used during the analysis for setting quadrant parameters and to set the basal level of lymphocyte proliferation. For T-bet assay, labelled cells from control individuals were used to set the quadrant standards.

2.7 Th1, Th2, Th17 cytokine measurements

Cell supernatants from AD, PT and control group were thawed and cytokine measurement of IFN-γ, IL-2, IL-10, IL-4, TNF, IL-6, IL-17a was performed using kits for human Th1, Th2, Th17 cytometric bead arrays (CBA) (Cytometry Bead Array kit, BD Bioscience). All methods were carried out according to manufacturer's recommendation. Afterwards, samples were analysed by flow cytometry on a FACScalibur flow cytometer (Becton Dickinson Company, San Jose, USA). For each sample, 2,100 bead-events were acquired as recommend by the fabricant. The standard curve for the CBA assay was determined using 9 dilutions and the data was analyzed with FCAP Array software provided by BD.

2.8 Statistical analysis

The statistical analysis was performed using the GraphPad Prism Software v.7, the data were analyzed with non-parametric Mann-Whitney U-test. Differences were considered statistically significant when $p \leq 0.05$.

3 Results

3.1 CFDA-SE cell proliferation assay

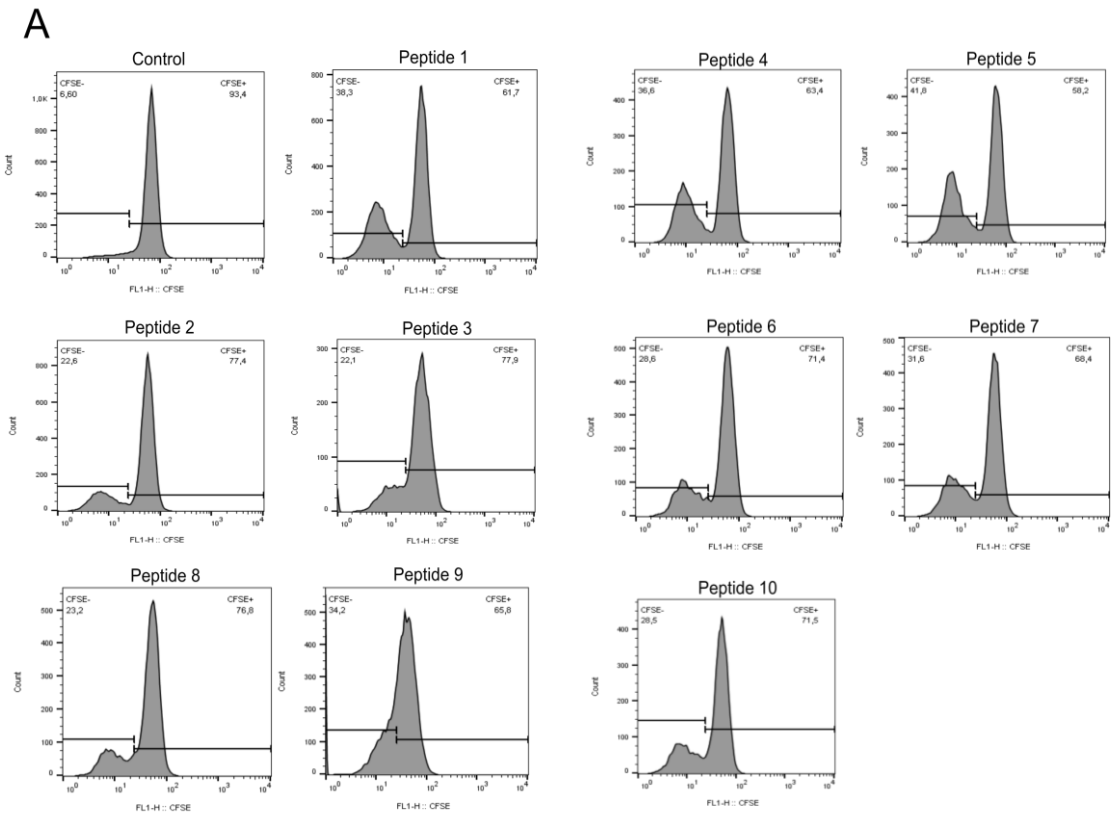
In order to evaluate whether the peptide epitopes would stimulate PBMC from active disease (AD) and resistant (RT) individuals, we have performed the CFDA-SE proliferation assay. These PBMC were also capable of proliferating when stimulated with total antigen from *L. braziliensis* and non-stimulated cells had minimal levels of proliferation (data not shown). The representative histograms for the flow cytometry analysis of one RT individual are represented in Fig. 1 A. Histograms for the analysis were performed as represented in Fig. 1 A, the intra control labelled and non-stimulated samples was carefully used to set the gates which were applied for all the other samples tested for the peptides. This step was performed for each individual tested.

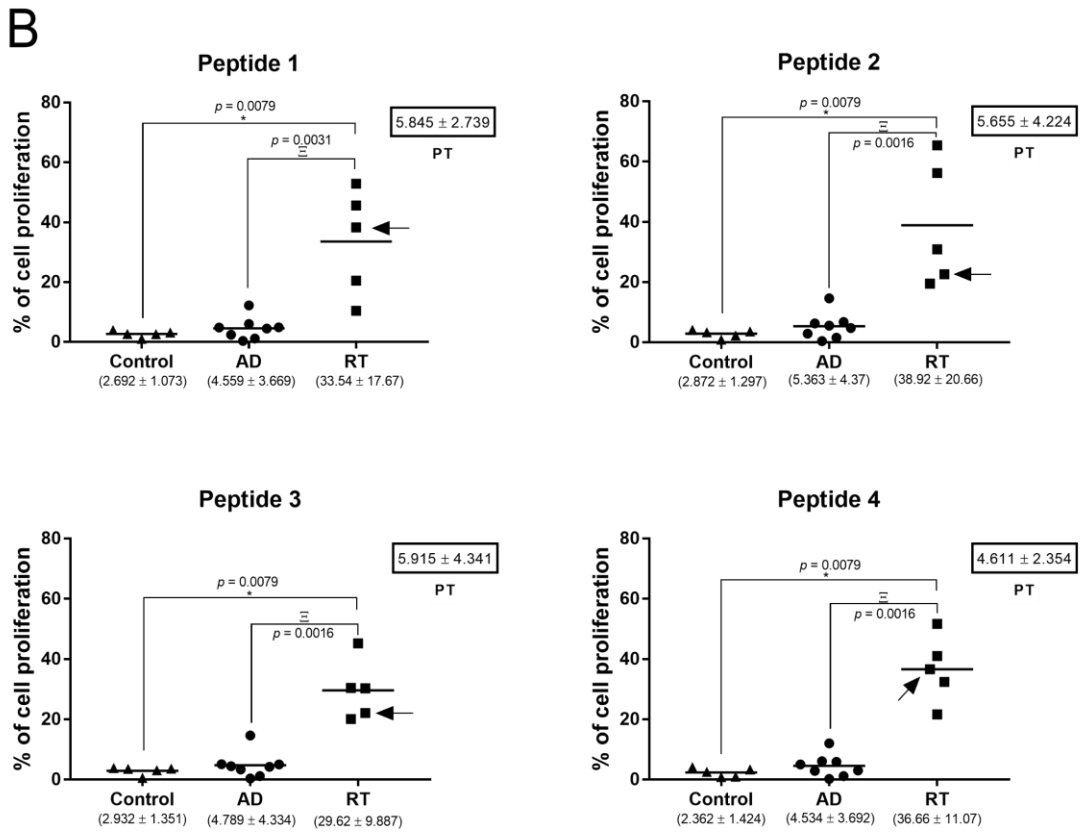
The results from the assays carried out with the synthetic peptides are presented as the mean percentage of proliferation, with its standard deviation, calculated with the data from the proliferation of PBMCs derived from eight AD and five RT individuals, when exposed to the individual peptides (Fig. 1 A). Comparisons between RT vs. control, and RT vs. AD, were performed. For AD individuals, none of the peptides were capable to significantly stimulate the cell proliferation when comparing to the healthy control and RT, and the medium values were slightly higher than the control. In contrast, for the RT group, all peptides were capable to significantly induce high levels

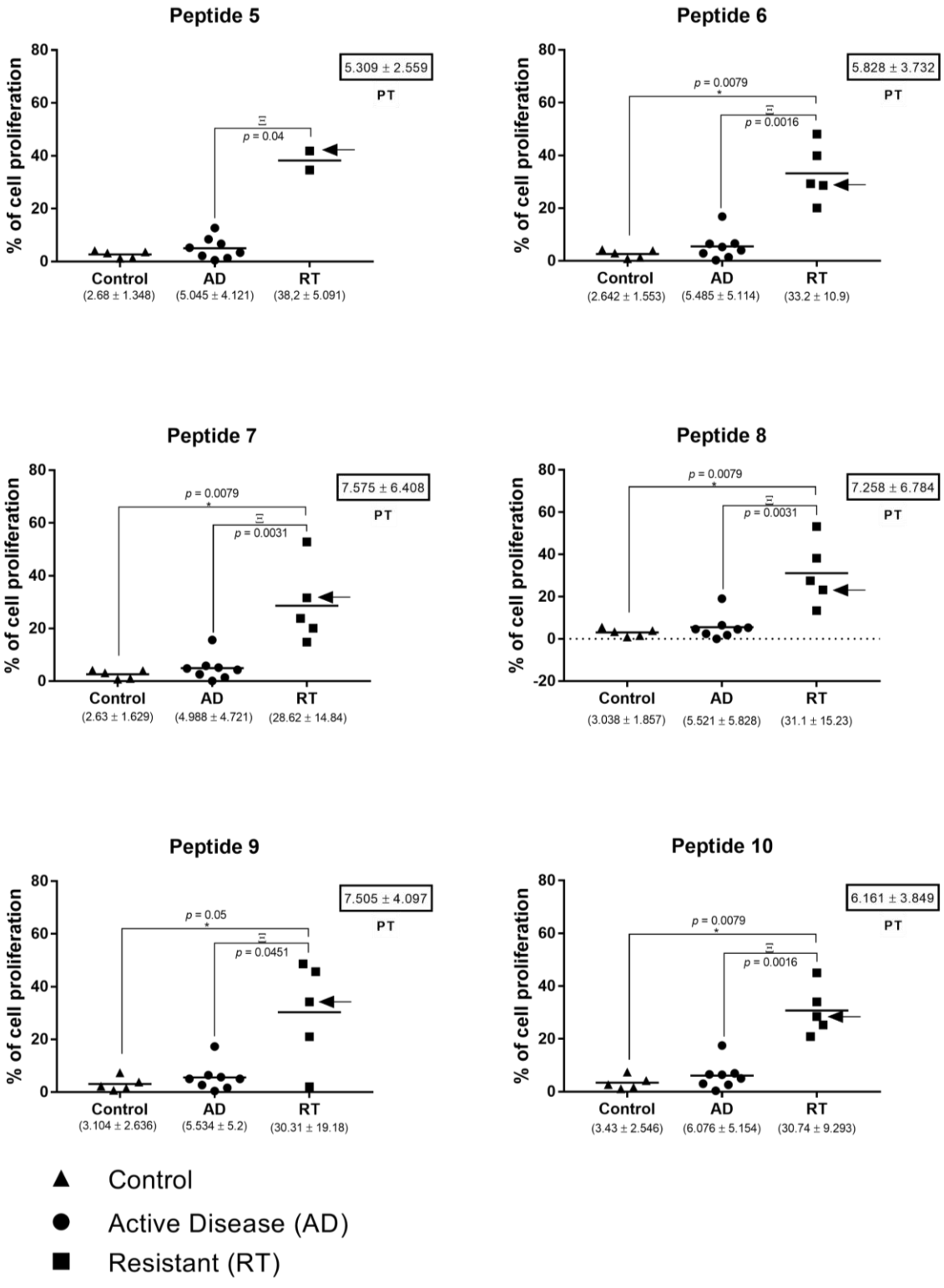
of PBMC proliferation. The PBMC proliferation medium values for the RT individuals were also significantly different from the AD individuals in this study, and different from the PT group performed in a previous work. It is possible to observe that the results obtained for the five RT individuals were improved due to high level of PBMC proliferation from almost all individuals tested. Additionally, for one RT individual (a female girl of 13 years old) it was observed a stronger proliferation response for five peptides (1, 2, 6, 8, and 10). The medium values for the RT group ranged from 28.6% up to 38.9%, in contrast with the AD group which ranged from 4.5% up to 6%.

For this work, a high level of significance between RT and the other groups was found for almost all peptides, except peptides 5 and 9. For peptide 5 the analysis was only possible with two individuals. In the previous work, five peptides have shown statistically significance between PT and Control group, and peptide 7 was the most significant evaluated.

Figure 2: The CFDA-SE cell proliferation assay evaluation of healthy control group and *Leishmania* active disease (AD) and resistant individuals (RT). In A, there are representative histograms from the analysis of one RT individual showing how it was performed. In B the horizontal bars represents the medium values, and its standard deviation, calculated from the evaluation of eight AD, five RT, and five control individuals. The values are presented bellow each graph and the black dots represent individual values. The black arrows indicate the values obtained with the analysis represented in A. The medium values and its standard deviation for the post-treatment (PT) group performed are presented in small boxes for comparison. The asterisk represents the significant differences between RT and control group, and the Greek letter Ξ (ksi) shows the significances between RT and AD groups. Significant differences were considered when $p \leq 0.05$.


A
Control
Peptide 1
Peptide 4
Peptide 5
Peptide 2
Peptide 3
Peptide 6
Peptide 7
Peptide 8
Peptide 9
Peptide 10
Count
FL1-H :: CFSE


B
Peptide 1
% of cell proliferation
p = 0.0079
p = 0.0031
5.845 ± 2.739
PT
Control
AD
RT
(2.692 ± 1.073)
(4.559 ± 3.669)
(33.54 ± 17.67)
Peptide 2
p = 0.0079
p = 0.0016
5.655 ± 4.224
PT
Control
AD
RT
(2.872 ± 1.297)
(5.363 ± 4.37)
(38.92 ± 20.66)
Peptide 3
p = 0.0079
p = 0.0016
5.915 ± 4.341
PT
Control
AD
RT
(2.932 ± 1.351)
(4.789 ± 4.334)
(29.62 ± 9.887)
Peptide 4
p = 0.0079
p = 0.0016
4.611 ± 2.354
PT
Control
AD
RT
(2.362 ± 1.424)
(4.534 ± 3.692)
(36.66 ± 11.07)


Peptide 5
% of cell proliferation
5.309 ± 2.559
PT
p = 0.04
Control
AD
RT
(2.68 ± 1.348)
(5.045 ± 4.121)
(38,2 ± 5.091)
Peptide 6
5.828 ± 3.732
p = 0.0079
p = 0.0016
(2.642 ± 1.553)
(5.485 ± 5.114)
(33.2 ± 10.9)
Peptide 7
7.575 ± 6.408
p = 0.0031
(2.63 ± 1.629)
(4.988 ± 4.721)
(28.62 ± 14.84)
Peptide 8
7.258 ± 6.784
p = 0.0031
(3.038 ± 1.857)
(5.521 ± 5.828)
(31.1 ± 15.23)
Peptide 9
7.505 ± 4.097
p = 0.05
p = 0.0451
(3.104 ± 2.636)
(5.534 ± 5.2)
(30.31 ± 19.18)
Peptide 10
6.161 ± 3.849
p = 0.0016
(3.43 ± 2.546)
(6.076 ± 5.154)
(30.74 ± 9.293)
Control
Active Disease (AD)
Resistant (RT)

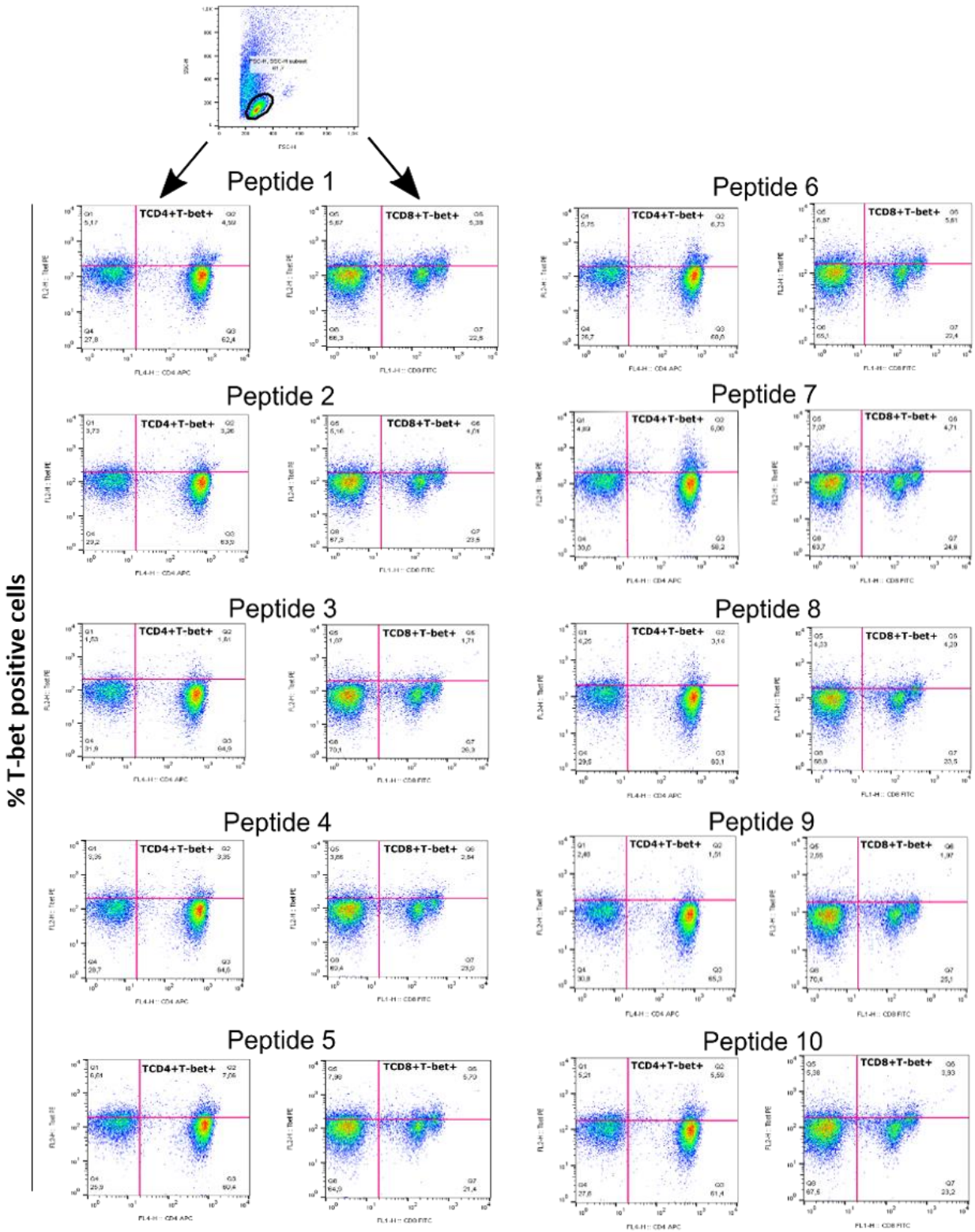
3.2 T-bet+ cell expression

In this investigation, the T-bet expression levels of T CD4$^+$ and T CD8$^+$ cells was possible to analyse by flow cytometry after peptide stimulation of PBMC from four post-treatment individuals enrolled in this research. The Fig. 2 A represents the dot-plots for the analysis carried out for the T-bet expression assay with cells from one PT individual. The gates were settled with the control labelled samples just above the main population of cells, for each individual analysed.

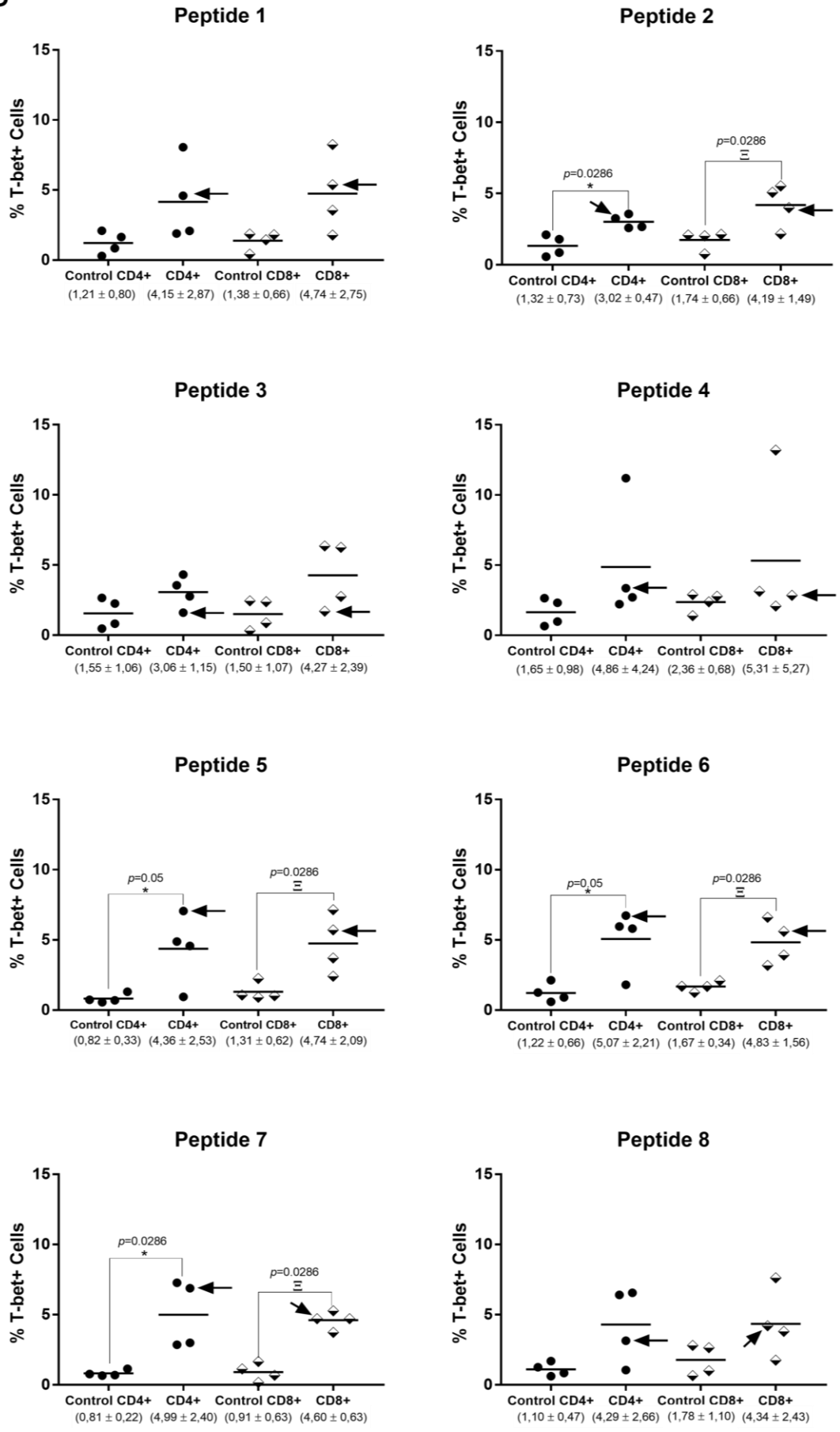
A significantly increased level of T-bet expression in both T CD4$^+$ and T CD8$^+$ cells stimulated with the peptides was observed for peptides 2, 5, 6, 7, 9 and 10. Minimal levels were achieved for control counterparts. For the PT group, the medium levels of T-bet ranged from 2.58% to 5.07% for T CD4$^+$, and from 3.32% to 5.31% for T CD8$^+$ cells. For the control group, the medium levels of T-bet ranged from 0.8% to 1.65% for T CD4$^+$, and from 0.9% to 2.36% for T CD8$^+$ cells. It demonstrates that T-bet was up-regulated for both types of lymphocytes.

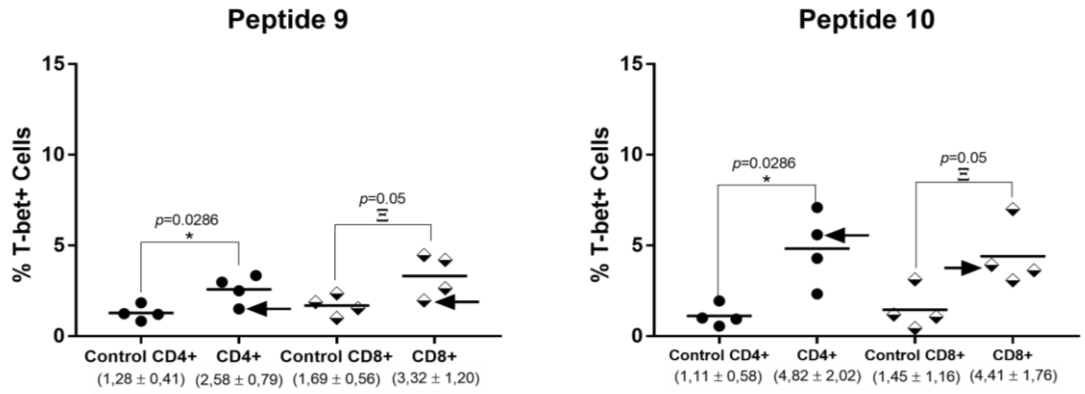
Figure 3: Evaluation of T-bet expression in CD4$^+$ and CD8$^+$ T cells from post leishmania treatment (PT) individuals. In A, it is represented the dot-plot graphs utilized for the analysis performed for one individual. In B the horizontal bars represent the mean values calculated for each group. The black arrows indicate the values obtained with the analysis represented in A. The asterisk represents the significant differences between T CD4$^+$ Control and T CD4$^+$ from RT group, and the Greek letter Ξ (ksi) shows the significances between T CD8$^+$ Control and T CD8$^+$ from PT group. The median percentage levels and the corresponding standard deviation for each group tested are described below. Significant differences were considered when $p \leq 0.05$.

A


Peptide 1
Peptide 2
Peptide 3
Peptide 4
Peptide 5
Peptide 6
Peptide 7
Peptide 8
Peptide 9
Peptide 10
TCD4+T-bet+
TCD8+T-bet+
% T-bet positive cells

B


Peptide 1
% T-bet+ Cells
Control CD4+ CD4+ Control CD8+ CD8+
(1,21 ± 0,80) (4,15 ± 2,87) (1,38 ± 0,66) (4,74 ± 2,75)
Peptide 2
p=0.0286
*
p=0.0286
Ξ
Control CD4+ CD4+ Control CD8+ CD8+
(1,32 ± 0,73) (3,02 ± 0,47) (1,74 ± 0,66) (4,19 ± 1,49)
Peptide 3
Control CD4+ CD4+ Control CD8+ CD8+
(1,55 ± 1,06) (3,06 ± 1,15) (1,50 ± 1,07) (4,27 ± 2,39)
Peptide 4
Control CD4+ CD4+ Control CD8+ CD8+
(1,65 ± 0,98) (4,86 ± 4,24) (2,36 ± 0,68) (5,31 ± 5,27)
Peptide 5
p=0.05
*
p=0.0286
Ξ
Control CD4+ CD4+ Control CD8+ CD8+
(0,82 ± 0,33) (4,36 ± 2,53) (1,31 ± 0,62) (4,74 ± 2,09)
Peptide 6
p=0,05
*
p=0.0286
Ξ
Control CD4+ CD4+ Control CD8+ CD8+
(1,22 ± 0,66) (5,07 ± 2,21) (1,67 ± 0,34) (4,83 ± 1,56)
Peptide 7
p=0.0286
*
p=0.0286
Ξ
Control CD4+ CD4+ Control CD8+ CD8+
(0,81 ± 0,22) (4,99 ± 2,40) (0,91 ± 0,63) (4,60 ± 0,63)
Peptide 8
Control CD4+ CD4+ Control CD8+ CD8+
(1,10 ± 0,47) (4,29 ± 2,66) (1,78 ± 1,10) (4,34 ± 2,43)



3.3 Th1, Th2, Th17 cytokine measurements

The levels of seven cytokines from the Th1, Th2, and Th17 profiles (namely IL-17a, IFN-γ, TNF, IL-10, IL-6, IL-4, and IL-2) were measured, in order to evaluate the type of cytokines secreted in the cell culture supernatant after stimuli of PBMC with the peptides. The results are expressed as the mean, and its standard deviation, calculated with the data from the measurements of cytokines in the cell supernatants from six AD and ten PT individuals when stimulated with the peptides (Fig. 3).

Low levels of IL-17a were seen for the control group, and a slightly increase of these levels in the PT group for peptides 1, 3, 4, 5 and 9. This increase has been also seen in the AD group for peptides 4, 5, 8 and 9. However, these increased levels were not statistically significant. In other hand, a statistically significant decrease of this cytokine in AD patients was seen when comparing with the healthy control for peptide 1. With all peptides tested, the levels of IL-10 were higher among the PT and AD group when comparing with the control, and greater levels of this cytokine were seen at the PT group. However, most of these increased levels of IL-10 were not statistically, only in the PT group tested with peptide 8.

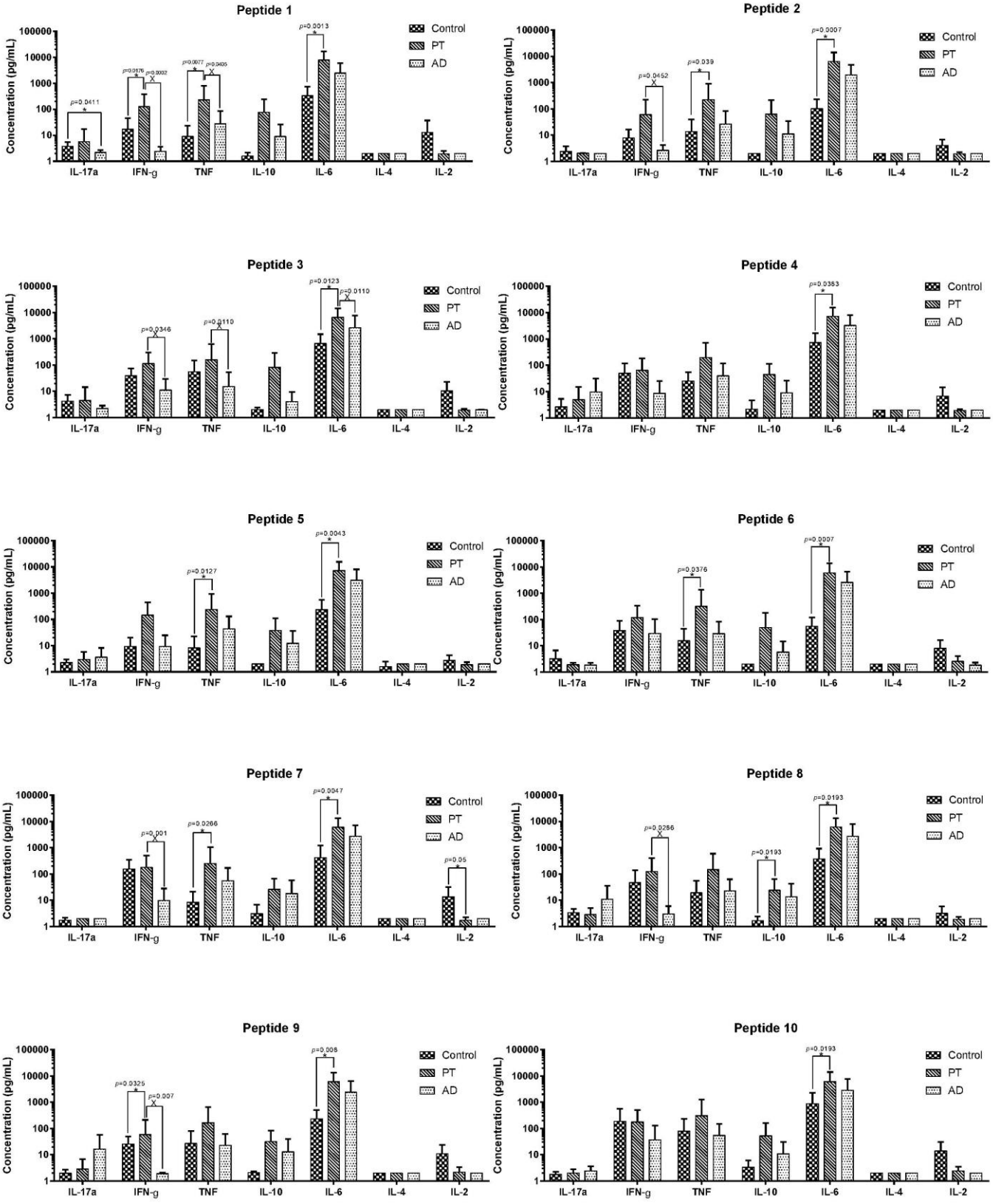
IL-4 was the only cytokine for which the levels were low or even undetectable for all peptides tested for the different groups. In other hand, the levels of IL-2 were higher for the control group for all peptides tested, and low levels were measured for the PT and AD groups. However, statistically significant increase of IL-2 was only marked for the control group of peptide 7.

The most interesting results were achieved for two cytokines from the Th1 profile, IFN-γ and TNF, and for IL-6 which belongs to the Th2 profile. The levels of IFN- γ were higher in the PT group for almost all peptides tested, except for peptides 7 and 10 in which the levels of this cytokine were approximately the same between PT and control. The increased levels of IFN- γ in the PT group were statistically significant for 60% of the peptides tested (1, 2, 3, 7, 8, and 9). In contrast, low levels of IFN- γ were found in the AD group for all peptides tested.

Regarding the TNF levels, an important increase in its production was found for the PT individuals. This difference was statistically significant for peptides 1, 2, 3, 5, 6, and 7. Differences were statistically significant between PT and control, and for peptides 1 and 3 between PT and AD. Differently from the IFN-γ in which the AD group secreted low levels, the AD group secreted higher levels of TNF when comparing with the control, although not statistically significant.

Among the relevant cytokines produced, IL-6 was the only which had its production and secretion stimulated by all peptides investigated in this work. Higher levels of IL-6 were found for the PT group when comparing with the control and AD group. Statistically significant levels of IL-6 were observed for PT individuals when comparing to controls and AD for peptide 3. Finally, a relevant production of the three main cytokines found in this study, IFN-γ, TNF, and IL-6, was demonstrated for the PT group stimulated with the peptides 1, 2, 3 and 7. 80% of the peptides tested produced statistically significant levels of at least two of the main cytokines found, except the peptides 4 and 10.

Figure 4: Measurement of IL-17a, IFN-γ, TNF, IL-10, IL-6, IL-4, and IL-2, in the supernatant of cells from active leishmaniasis (AD) and post-treatment (PT) patients. The bars represent the medium values and its standard deviation which were calculated from the data of cell supernatant from fourteen PT, eight AD, and five healthy control individuals.


Peptide 1
Peptide 2
Peptide 3
Peptide 4
Peptide 5
Peptide 6
Peptide 7
Peptide 8
Peptide 9
Peptide 10
Concentration (pg/mL)
Control
PT
AD
IL-17a
IFN-g
TNF
IL-10
IL-6
IL-4
IL-2

4 Discussion

The development of safe and effective vaccines against intracellular pathogens like *Leishmania* is one of the major challenges for biomedical scientists. Until now, such ideal vaccine against human leishmaniasis was neither developed nor under clinical tests, and there is a limited number of studies dealing with this question. An ideal anti-*Leishmania* vaccine is expected to correctly activate the immune system so that it can induce protection through T CD4$^+$ and T CD8$^+$ cells counter-interacting and promoting parasite clearance and memory cell induction. In this study, an immunogenicity screen of the ten best results for peptide epitopes searched in the proteome of *L.V. braziliensis* by using a robust combination of *in silico* approaches was performed. The assays were concentrated to investigate how the cells from individuals with cutaneous leishmaniasis, post-treatment, and individuals resistant from getting the disease, would react when stimulated with the synthetic peptides.

The results showed that PBMC from patients with active cutaneous *Leishmania*sis did not significantly proliferate when stimulated with the peptides tested. On the other hand, PBMC from resistant individuals showed high levels of proliferation when stimulated with the epitope peptides. The results also highlight that the RT group was the most responsive which indicates that those individuals have T memory cells capable to recognize the epitopes presented to them in this study. It is in agreement with previous results (Fig. 1 B) obtained with PBMC from PT patients which showed greater levels of proliferation when compare with the control. Another work investigating the T cell response of resistant individuals and those ones with active disease have shown higher levels of cell proliferation after stimuli with leishmanial total antigen for the AD group when comparting with the RT [23]. The degree of cell proliferation observed might be a direct consequence of the number of T cell clones present in the periphery. We suggest that the degree of cell proliferation observed for the RT group is a consequence of the number of circulating experienced T cells present in these individuals, since it is supposed that they are exposed to different booster through the persistence of parasites in lymphoid organs and re-infections [24]. Moreover, the expansion of T cells is a consequence of how these antigens are presented and how the cells are activated [25]. The development of memory T cells is one of the most important aspects desired for a vaccine [26], and previous works have suggested that induction of memory T cells is a critical step for the development of an anti-*Leishmania* vaccine [27,28].

One important limitation of recent studies dealing with the development of peptide epitopes towards an anti-*Leishmania* vaccine is the validation method. Most of the works shows results from peptides derived from known proteins or which were previously evaluated as whole antigen, thus narrowing the possibilities to find new potential antigens [29,30]. Another limited aspect is the validation process which is performed with murine models and not with human samples and this may not fully reflect what happens with human cells [30–32]. Therefore, it reinforces the importance of performing validation approaches using human samples which may translate with more confidence what would be expected in the human body. In this sense, Mou *et al.* [33] have showed promising results with human samples stimulated with a naturally processed and conserved *Leishmania* peptide. The cell proliferation values observed for the RT group is strongly suggesting that there is a potent T cell presentation and recognition with the samples from these individuals. Furthermore, the recognition of those epitopes might be correlated with the development of T cells capable to recognize and react against those peptides in *L.V. braziliensis*. However, further confirmation studies are needed in order to clarify this aspect.

T-bet is the master regulator of Th1 commitment, and its role in resistance against some intracellular pathogens was already confirmed [34]. Nevertheless, the role of T-bet in *Leishmania* infection is not yet clear, but the results from this study suggest that the peptide epitopes, especially for peptides 2, 5, 6, 7, 9 and 10; were capable to induce the expression of T-bet. In addition, since the peptides used in this work were structurally identified to stimulate both CD4$^+$ and CD8$^+$ T cells, this result confirms that both subsets of T cells have been directed towards a Th1 profile. T-bet is only expressed in the absence of negative stimulation such as that furnished by TGF-β [35], thus, this result may corroborate and signalize that some of the peptide epitopes may have a potential to be recognized and induce a Th1 cell response against *Leishmania* infection. Although limited, this type of result needs to be confirmed through the use of murine models of vaccination and challenge.

Regarding the investigated cytokines, the majority of the tested peptides evaluated were capable to induce the expression and secretion of two major cytokines for the Th1 immune response, which are IFN- γ and TNF. Since a long time, it has been stated that IFN-γ and TNF may act together in order to promote a Th1 profile which induces macrophage activation with nitric oxide (NO) production, culminating with parasite killing [36,37]. Although the Th1 immune profile has an important influence to control *Leishmania* infection, it has also been implicated in its pathogenesis. This link has been seen in murine models of infection and also in humans, since high levels of TNF is associated with more severe forms of CL and chronicity, and the use of TNF inhibitors along with antiparasitic drugs have shown more enthusiastic disease outcomes [38–41]. In humans, IL-10 is one of the main cytokines which has been associated with immunosupressiona and pathology of CL [42,43]. This fact may reinforce the important contribution of cytokines from other profiles to counterbalance the immune response.

In this regard, the levels of IL-6 induced by the peptides may have a dual role as pro-inflammatory cytokine and also preventing regulatory T cell profiles which might be associated with disease exacerbation [44,45]. Besides the main cytokines involved with the Th1 cell profile, the absence or the low levels of the other cytokines from the Th2 and Th17 cell profile are an indicative that the peptides, mainly the 1, 2, 3, and 7, have the potential to promote an immune response which would culminate with protection against CL. It is expected for an ideal vaccine against CL to induce long last memory T cells, and an anti-*Leishmania* response mediated by a Th1/Th2 immunobalance [46,47]. Our results indicate that circulating memory T cells were activated to produce and secrete the cytokines appointed by this study. One of the main roles of memory T cells during reinfection is to produce the key cytokines necessary to combat the pathogen [48]. The differences observed for the responses of the peptides tested might be related to their intrinsic capacity to bind the MHC molecules expressed on the surface of APC, or even the APC process and presentation to T cells [18,49]. Further investigation is needed to clarify this question.

5 Conclusion

In this context, it can be concluded that most of the peptides which were tested have the capacity to induce the proliferation of cells from resistant individuals. Also, most of the peptides were capable to stimulate the expression of T-bet by CD4$^+$ and CD8$^+$ T cells, and the secretion of IFN-γ, TNF, and IL-6 in the supernatant of cell culture from post-treatment and active disease individuals.

Conflict of interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments
LF and BC are recipients of a doctoral fellowship from the "Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco" (FACEPE). MZ, OP and VR acknowledge research fellowships received from the "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq).

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5.3 Capítulo III

Evaluation of the effect of potential CD4$^+$ and CD8$^+$ T cell epitopes from the proteome of *Leishmania braziliensis* on bone marrow and splenic dendritic cell subsets

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Abstract
Dendritic cells (DCs) are specialized antigen presenting cells (APCs) with a key role initiate and maintain an immune response and activation of these cells is important for vaccine approaches. In a previous work, we reported the identification of potential CD4$^+$ and CD8$^+$ T cell epitopes from the proteome of *Leishmania braziliensis*. The aim of this work was to evaluate the effect of the peptide epitopes on murine bone marrow (BMDC) and spleen dendritic cells and in other cell subsets. Bone marrow cell precursors were stimulated for 10 days with granulocyte macrophage colony stimulating factor (GM-CSF). Then, BMDC were stimulated with 20 μg/mL of each peptide for 24h and 48h. After, cell supernatant was stored for cytokine measurements and cells were labelled with monoclonal antibodies (anti-CD11c/MHCI/MHCII/CD40) for flow cytometry. Groups of five BALB/c mice were weekly immunized in the left ear with 20 μg of each peptide plus 10 μg of CpG ODN for three weeks. After, spleens from each group were collected, pooled, and cells stimulated with 20 μg/mL of each respective peptide for 48h. Cells were labelled with monoclonal antibodies (anti-CD11b/CD8a/CD103/IL-12p70/CD317/CD205/MHCII) for flow cytometry. The results did not demonstrate any significant change in the expression markers for BMDC, and in the cytokine levels. Although increased levels of MHCII and CD40 were verified for BMDC CD11c$^-$ stimulated with peptides, and high levels of TNF and IL-6 were seen after 48h. For splenic cells, high levels of CD11b$^+$ DCs were verified after stimulation with peptides 1 and 6, and also consistent levels of MHC II. Additionally, cell subsets expressing CD103$^+$ IL-12p70$^+$ and CD205$^+$ were observed after stimulation with peptide 1. The results suggest that some of the peptides evaluated have a potential to stimulate an immune response against *Leishmania* based on the DCs and cell subsets which were activated.

1 Introduction

The leishmaniasis are diseases caused by the infection of protozoans from the genus *Leishmania* transmitted to humans or animals (which can act as important parasite reservoirs), through the bite of infect phlebotomine sand flies ("WHO | Leishmaniasis", 2015). These diseases are endemic in more than 98 countries and territories, and new epidemics are occurring in endemic and free areas due to migration, tourism, and military activities (OKWOR; UZONNA, 2016a). The main clinical forms are the visceral (VL) and cutaneous leishmaniasis (CL) with an estimated number of 0.2 to 0.4 million and 0.7 to 1.3 million cases per year, respectively. VL is more fatal if left not treated, and CL has been more implicated with high morbidity due to disfigurement and economic impact through the loss of productivity (BACON *et al.*, 2013; GILLESPIE *et al.*, 2016). Two important species are involved with the development of CL, in the Old World the *Leishmania Leishmania major* and in the New World the *Leishmania (Viannia) braziliensis* (COSTA, C. H. N. *et al.*, 2011b). Besides the current strategies to control, there is no vaccine available or in the pipeline against human leishmaniasis, and a limited number of clinical trials (REZVAN, HOSSEIN; MOAFI, 2015). Therefore, it is of great need the development of effective vaccines against human leishmaniasis that could help in the control and limit the use of toxic drugs.

Desired anti-*Leishmania* vaccines are intended to correct stimulates the host's immune response in order to achieve the optimal level of protection. For that, the immune response against these pathogens and the role of different cell subsets and molecules has been investigated (DE LUCA; MACEDO, 2016a). *Leishmania* parasites are intracellular pathogens and most of the data acquired in the last years comes from *L. major* infection of BALB/c mice. It indicates that an effective anti-*Leishmania* immune response involves mainly a pro-inflammatory response with CD4$^+$ and/or CD8$^+$ T cells recognizing peptide epitopes derived from *Leishmania* proteins, and secreting interferon (IFN)-γ to activate infected cells to produce nitric oxide (NO) and other reactive species to kill the parasites. Antigen presenting cells (APCs) are cell types which express high levels of Class I and Class II major histocompatibility complex (MHC) molecules capable of presenting epitopes to CD8$^+$ and CD4$^+$ T cells, respectively (INABA *et al.*, 2000).

In this regard, dendritic cells (DCs) are specialized APCs with the unique capacity to prime naïve T cells and thus induce activation through the engagement of MHC-peptide with T cell receptor (TCR), cross-presentation, delivery of co-stimulatory signals, and production and secretion of key cytokines to induce different T cell profiles (FREITAS-SILVA; BRELAZ-DE-CASTRO; PEREIRA, 2014). CD40 is a co-stimulatory molecule and triggering of signals through it induces changes in the DC, such as upregulation of other co-stimulatory molecules and of MHC II (MA; CLARK, 2009). In addition, IL-12p70 is the biological active form of IL-12, and it is produced and secreted by DC after encounter of pathogens and it acts on T cells supporting a Th1 profile with production of IFN- γ. IL-12 secreted by DC can modulate protection against *L. major* infection in murine models (SCHWARZ *et al.*, 2013), and molecular defined antigens can stimulate DC production of IL-12 in vaccination models against *L. major* (BERBERICH *et al.*, 2003). Up to today, distinct methods are available to *in vitro* investigate DCs, such as the generation of high amounts of murine bone marrow dendritic cells (BMDC) by stimulating bone marrow precursors with granulocyte macrophage colony stimulating factor (GM-CSF) (LUTZ *et al.*, 1999) or with Fms-like tyrosine kinase 3 (FLT-3) (BRASEL *et al.*, 2000). This process can also be done for humans, by stimulating peripheral monocytes with a combination of GM-CSF and interleukin (IL)-4 (TEDDER; JANSEN, 2001).

Furthermore, different DC subsets present in the tissues of humans and animals can be phenotypically characterized by the expression of functional markers, such as CD11c, CD11b, CD8α, CD103, CD317, and CD205, for e.g. (HEATH; CARBONE, 2013). CD8$^+$ DCs are positive for CD8α and negative for CD11b and their main functions are cross presentation of antigens, induction of Th1 cells, production of IL-12, and capture of antigens from migratory DCs or from blood. In other hand, CD11b$^+$ DCs are positive for CD11b and negative for CD8α and have the ability to capture soluble antigens from blood or lymph nodes and present them via MHC II (SHORTMAN; LIU, 2002). Other cell markers are functionally present on DCs and other cell types, such as the integrin alpha E (known as CD103) that can also be found in T cells and T regulatory cells (LEFRANÇOIS *et al.*, 1994); the CD205 which is a glycoprotein present on DCs and it is important to antigen presentation and induction of T cell immunity (BONIFAZ, L. C. *et al.*, 2004); the plasmacytoid DC antigen-1 (PDCA-1/BST2/CD317) which is an specific marker for plasmacytoid DC present in less than 0.5% of splenocytes and it is upregulated after stimuli that triggers and IFN response (BLASIUS *et al.*, 2006).

Considering the potential of the different types of DC to activate an immune response, a number of recently vaccination approaches relies on the target of these cells to evaluate DC activation and vaccine efficacy for *L. major* infection (Remer et al., n.d.) (MATOS *et al.*, 2013; SCHWARZ *et al.*, 2013). In a previous work, the biological activity of potential CD4$^+$ and CD8$^+$ T cell epitopes from the proteome of *L.V. braziliensis* was demonstrated with human peripheral blood mononuclear cells (PBMC). The top-10 ranked epitopes are 15-mer peptides derived from at least four hypothetical highly conserved proteins with an intrinsic capacity to both bind MHC I and MHC II from the main allele supertypes present in human populations. In this context, the aim of this work was to evaluate the effect of the peptide epitopes selected on murine bone marrow and spleen dendritic cells and in other cell subsets.

2 Material and Methods

2.1 Ethics statement

BALB/c mice were bred and feed at the animal facility of Aggeu Magalhães Research Centre (CPqAM-FIOCRUZ), Recife, Brazil. The protocol used in this work was previously approved by the Animal Ethics Committee at CPqAM/FIOCRUZ (Protocol number 47/2013).

2.2 Synthetic peptides and storage

Molecules corresponding to the top 10 ranked 15-mer peptides described in previous work were commercially synthesized (Genome Biotechnology, Brazil). Linear peptides were purified through a high performance liquid chromatography (HPLC) approach with a final purity greater than 95%. All the synthetic peptides were individually resuspended in dimethyl sulfoxide (DMSO) and stored at -80°C until use.

2.3 Induction of bone marrow derived dendritic cells (BMDC)

The protocol for generation of BMDC was adapted from Lutz et al. (LUTZ *et al.*, 1999). For each experiment performed with BMDC, five males of BALB/c mice were euthanized in a CO_2 chamber and femurs and tibiae were carefully removed. Intact bones were left in 70% ethanol for 1 min and washed with roswell park memorial institute (RPMI) 1640 media containing 2 mM of L-glutamine, 50 mg/L of gentamicin sulfate, supplemented with 10% fetal bovine serum (both Cultilab reagents, Brazil). Then, both ends were cut and the marrow washed with RPMI 1640 supplemented using

a syringe with 0.45mm diameter needle. Bone marrow clusters were removed by vigorous pipetting followed by passage through a 0.45mm pore filter. After, cells were deposited in 6-well plates and left resting overnight. After, cell concentration was adjusted and 2 x 10^6 cells were deposited per well in 6-well plates. At day 0, 3, 6 and 8 cells were replenished with recombinant granulocyte macrophage colony stimulating factor (rGM-CSF, Sigma, USA) at final concentration of 20ng/mL. At day 10 cells were completely differentiated and non-adherent cells were harvested to use. Cell morphology was confirmed by optical and fluorescence microscopy.

2.4 BMDC expression of CD11c, MHC I, MHC II, and CD40 after stimulation with peptides

BMDC concentration was adjusted and 2 x 10^5 cells were deposited in 96-well U-bottom plates, and each well individually stimulated with 20 μg/mL of each peptide. This was performed in duplicates, separately for each tested peptide. Cell plates were incubated at 37°C with 5% CO_2 for 24h and 48h. After each period, plates were centrifuged for 10 min at 400 x g and cell supernatant was collected and stored at -80°C for cytokine measurement by flow cytometry. Then, cells were harvested with phosphate buffered saline (PBS, pH 7.2), transferred to polystyrene tubes and labelled with anti-CD11c fluorescein isothiocyanate (FITC) conjugated (Clone N418), anti-MHC I phycoerythrin (PE) conjugated (Clone AF6-88.5.5.3), anti-MHC II allophycocyanin (APC) conjugated (Clone M5/114.15.2), and anti-CD40 conjugated with APC (Clone 1C10) (all antibodies from Affymetrix eBioscience, USA) for 30 min at room temperature. After, cells were washed (400 x g, 10 min) and resuspended in 400 μL of paraformaldehyde (PFA) 1% until flow cytometry acquisition.

2.5 Mice immunization and *in vitro* cell stimulation

Immunization experiments were carried out in groups of 5 female BALB/c mice. Five peptides which had shown significant results with human cells in a previous work were selected for immunization and cell subsets evaluation. Mice were inoculated three times at one-week interval in the left ear with 20 μg of each peptide plus 10 μg of CpG ODN 1688 5'-tccatgacgttcctgatgct-3' (InvivoGen, USA) as adjuvant in a total volume of 20 μL. Control groups were immunized either with PBS (pH 7.4) or with CpG ODN only. Two weeks after the last immunization 5 animals per group were euthanized to collect the spleen. Spleens were aseptically removed, pooled and macerated. Clusters were removed by vigorous pipetting followed by passage through a 0.45mm pore filter. After, cell concentration was adjusted to 10^6 cells/mL and 10^6 cells were deposited per well and stimulated with 20μg/mL of each peptide. This was performed in duplicates, separately for each tested peptide. Cell plates were incubated at 37°C with 5% CO_2 for 48h. After 48h, cells were harvested for labelling with monoclonal antibodies.

2.6 Labelling of spleen cell subsets

For this, cells were then washed with a solution called PBS-W which is composed of PBS with 0.5% bovine serum albumin (Sigma, St. Louis, MO) and 0.1% sodium azide (Sigma, St. Louis, MO); then cells were centrifuged (400g, 10 min) and transferred to polystyrene tubes. Monoclonal antibodies anti-CD11b-FITC, anti-CD8a-PE, anti-MHC II-APC, anti-CD103-FITC, anti-CD317-PE, anti-CD205-Alexa Fluor (AF) 488 were added to the tubes (all antibodies from Affymetrix eBioscience, USA). Following, the cells were incubated for 30 min at 25°C. Afterwards, cells were fixed with 1% paraformaldehyde (PFA) in PBS, washed by centrifugation with PBS-W (400g, 5 min). Cells labelled for IL-12p70 were previously permeabilized with PBS

plus 0.5% of saponin. Subsequently, the cells were washed with PBS-W by centrifugation (400g, 5 min), and then they were incubated with anti-IL-12p70-PE conjugated (Affymetrix eBioscience, USA) for 30 min at 25°C. After this time, they were washed by centrifugation with PBS-W (400g, 5 min) and resuspended with 1% PFA until flow cytometry acquisition.

2.7 Flow cytometry analyses

All flow cytometry analyses were performed on a FACScalibur flow cytometer (Becton Dickinson Company, San Jose, USA) equipped with an argon laser (wavelength 488 nm) and using the software CELLQuestProTM (BD Bioscience, San Jose, CA) for acquisition. Fluorescence of 20,000 BMDC and splenocytes gated events, based on scatter parameters of size and granularity, was acquired. The data was analyzed and treated with FlowJo v10.1 (Tree Star Inc., USA). For splenocytes, boolean gates were drawn in order to access the levels of MHC II expression for the different subsets analysed.

2.8 Th1, Th2, and Th17 cytokines measurements

Cell supernatants from BMDC were thawed and cytokine measurement of IFN-γ, IL-2, IL-10, IL-4, TNF, IL-6, IL-17a was performed using kit for mice Th1, Th2, Th17 cytometric bead arrays (Cytometry Bead Array kit, BD Bioscience). All methods were carried out according to manufacturer's recommendation. Afterwards, samples were analysed by flow cytometry on a FACScalibur flow cytometer (Becton Dickinson Company, San Jose, USA). For each sample, 2,100 bead-events were acquired as recommend by the fabricant. The standard curve for the CBA assay was determined using 9 dilutions and the data was analyzed with FCAP Array software provided by BD.

2.9 Statistical analysis

The graphs and statistical analysis were performed using the GraphPad Prism Software v.7, the data were analyzed with non-parametric Kruskal-Wallis and Mann-Whitney U-tests. Differences were considered statistically significant when $p \leq 0.05$.

3 Results

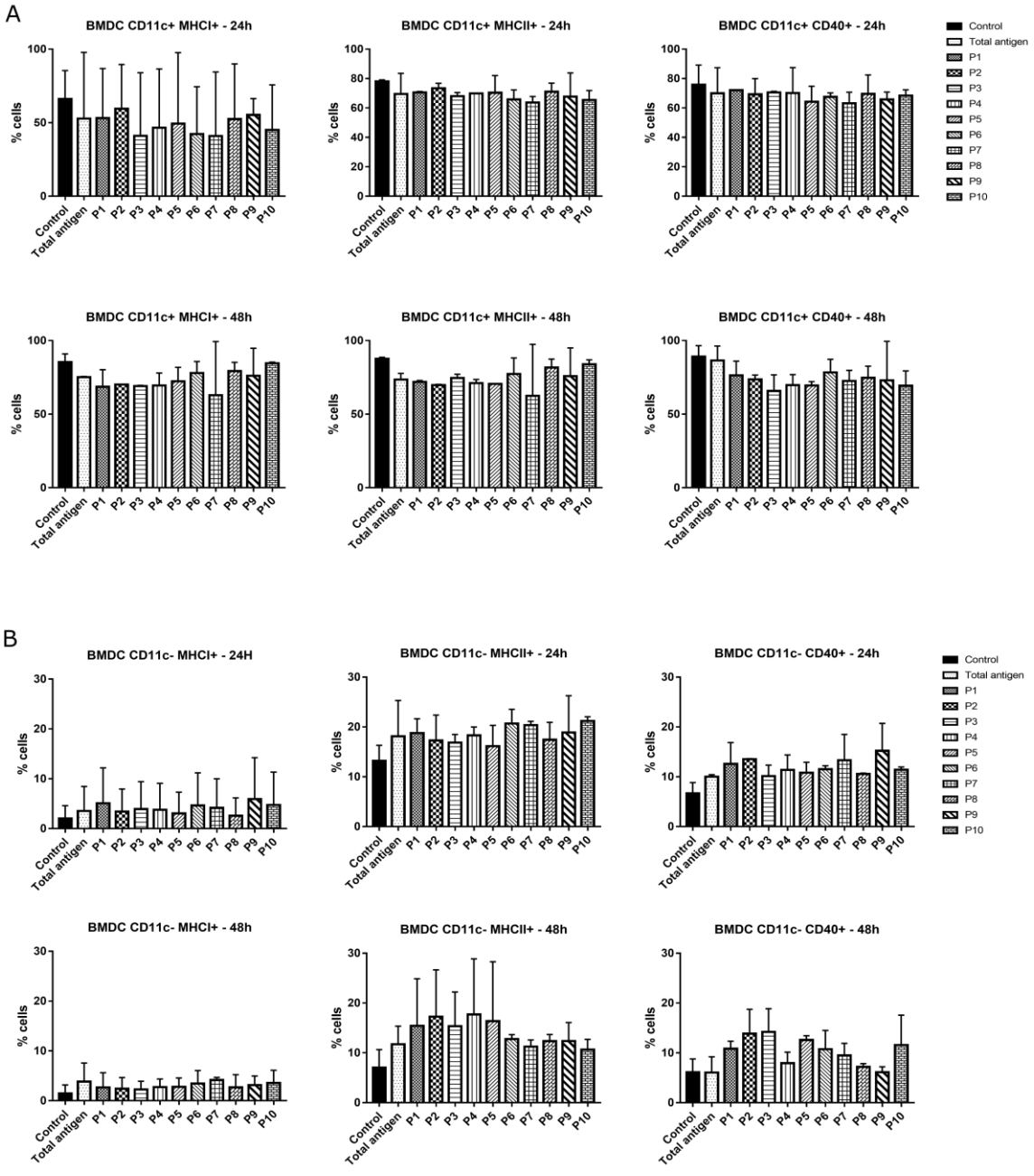
3.1 BMDC expression of MHC I, II, and CD40

For this assay, BMDC were, independently, stimulated with the different peptides investigated for 24h and 48h. After each period, BMDC were harvested and labelled with monoclonal antibodies for flow cytometry. During analysis, two populations of cells were distinguished, one CD11c$^+$ and another CD11c$^-$, based on this, the levels of MHC I, II and CD40 were measured in both BMDC sub-populations (Fig 1). The results are expressed as the mean and its standard deviation calculated from two independent experiments.

Fig 1 A shows the expression of MHC I, MHC II, and CD40 for CD11c$^+$ BMDC. High levels of these markers were found among the controls for this cell type, with 60% - 80% of the CD11c$^+$ cells expressing the molecules investigated. However, a decrease in the levels of expression of MHC I, MHC II, and CD40 was observed among the CD11c$^+$ BMDC stimulated with the peptides for 24h and 48h.

In general, for the population of CD11c$^-$ BMDC, low levels of the markers were found for non-stimulated and stimulated cells with the peptides for both time periods. However, the levels of MHC II and CD40 for the cells stimulated with the majority of the peptides were higher than the control non-stimulated for both time periods.

Figure 1: Evaluation of bone marrow dendritic cells (BMDC) markers MHC I, MHC II, and CD40 stimulated with 20 µg/mL of each peptide for 24h and 48h. The values are expressed as mean and its standard deviation calculated from two independent experiments. In A, it is presented the values for the BMDC CD11c$^+$, and in B it is presented the values for the BMDC CD11c$^-$.

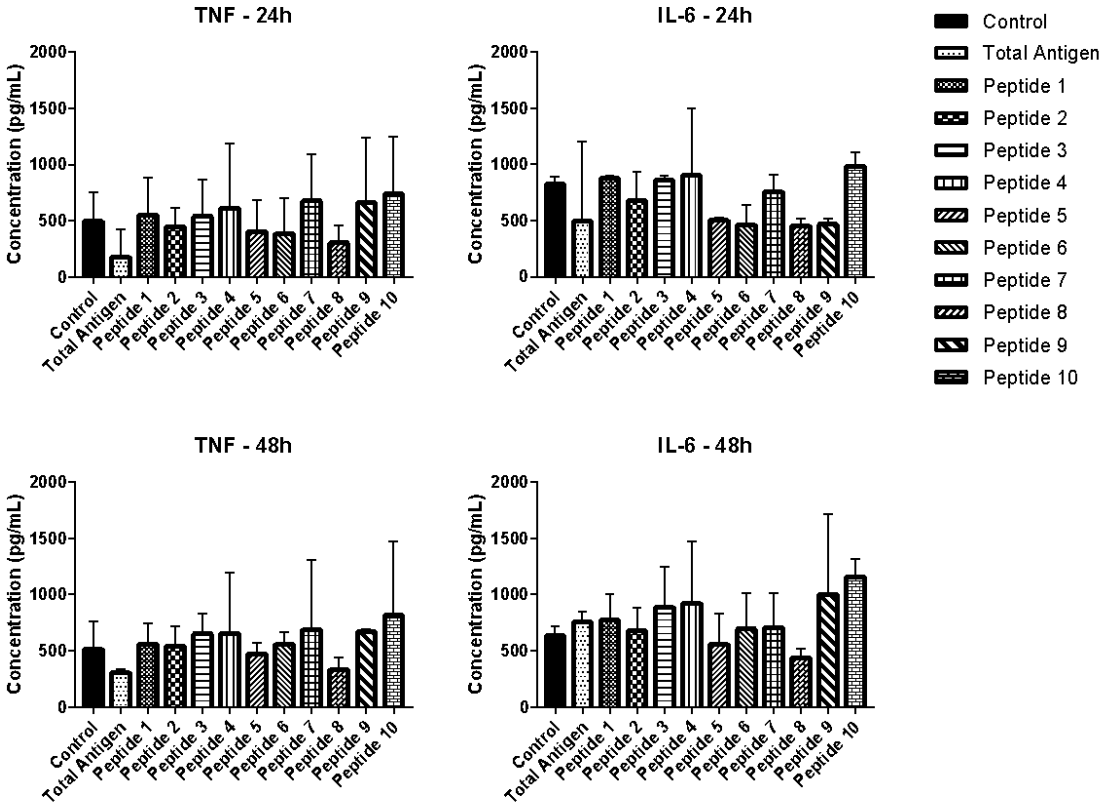


3.2 Cytokine levels in the BMDC supernatant

Fig. 2 shows the cytokine levels found in the supernatant of the BMDC stimulated, individually, with the peptides investigated in this work. The results are expressed as the mean and its standard deviation calculated with data from two independent experiments.

Low or undetectable levels of IL-17a, IFN-γ, IL-10, IL-4, and IL-2 were found in the samples. Fig. 2 represents the two main cytokines, TNF and IL-6, measured in the supernatant of the BMDC stimulated with the peptides. It might be remarked a slightly high level of these cytokines in the supernatant of cells stimulated with the peptides 1, 3, 4, 7, 9 and 10 when comparing with the control non-stimulated. Among the peptides evaluated, peptides 5, 6, and 8 were the molecules which induced the lowest levels of the TNF and IL-6 when comparing with the control. Low levels of the cytokines were found for cells stimulated with the total antigen of *L. braziliensis*, except for IL-6 after 48h in which the levels were slightly higher than the control.

Figure 2: Levels of two cytokines, tumour necrosis factor (TNF) and interleukin (IL)-6, in the supernatant of BMDC stimulated with 20 µg/mL of each peptide for 24h and 48h. The values are expressed as mean and its standard deviation calculated from two independent experiments.



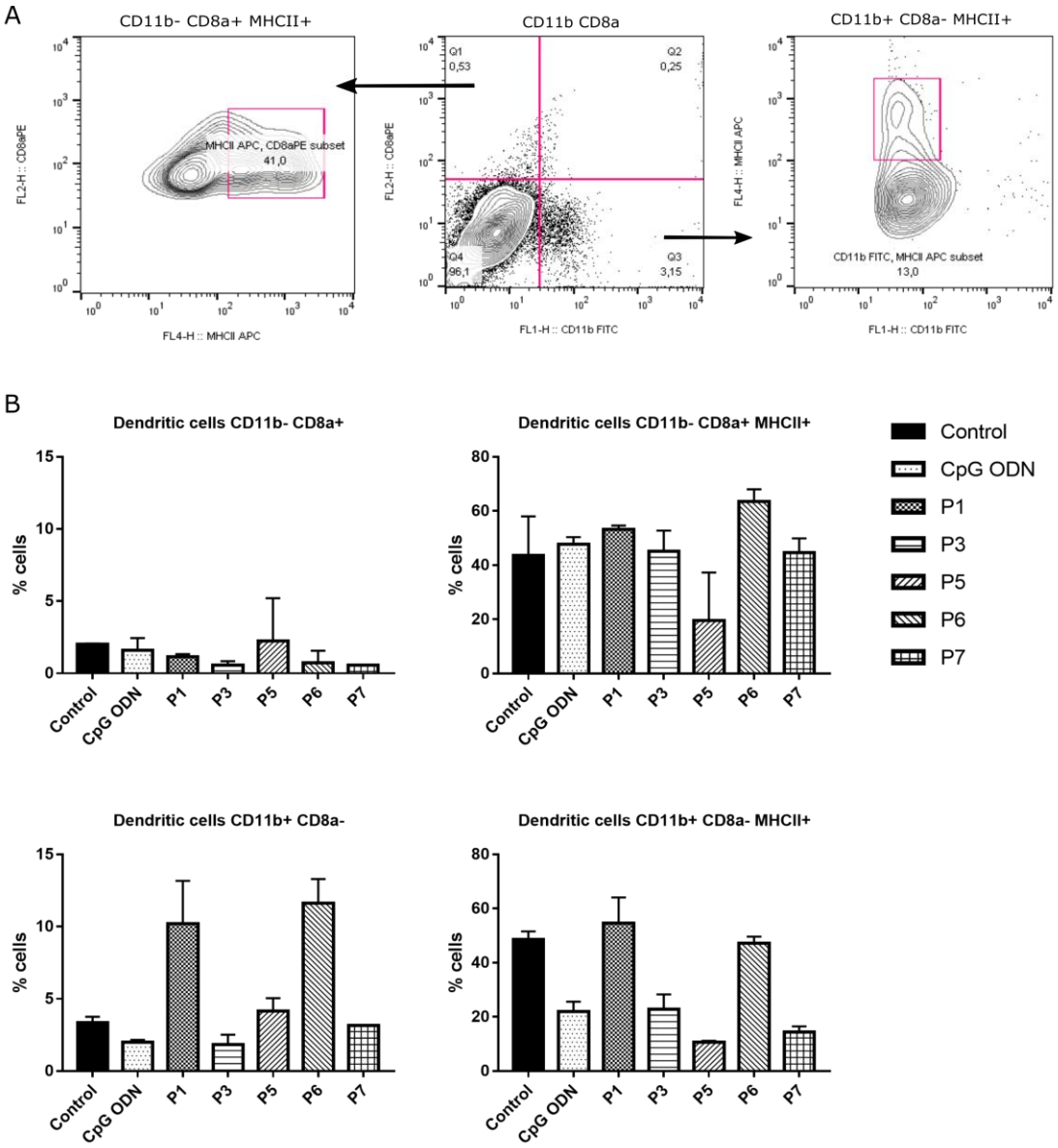
3.3 Levels of CD11b and CD8a dendritic cells

For this assay, the percentage levels of CD11b$^+$ CD8a$^-$ and CD11b$^-$ CD8a$^+$ splenocytes and its levels of MHC II expression after stimuli with the peptides were evaluated. The dot-plots and gates drown for the analyses of the parameters are represented in Fig. 1 A. The results are represented in Fig. 3 B as the mean and its standard deviation calculated from two independent cell cultures. A low frequency of CD11b$^-$ CD8a$^+$ cells was found, and the medium levels of MHC II expression was higher or equal to 40%, except for cells stimulated with the peptide 5 which showed

lower levels of MHC II. For this subset, the highest levels of MHC II expression was found after peptide 6 stimulation.

In other hand, a higher frequency of CD11b+ CD8a- cells was found for samples stimulated with peptides 1 and 6 when comparing with the control. In addition, the levels of MHC II expression for these last samples was also high than the other peptides and equal to the control cells non-stimulated. Samples stimulated with peptides 3, 5 and 7 presented low levels of this cell subset and also of MHC II.

Figure 3: The levels of CD11b+ and CD8+ dendritic cells (DCs) and its MHC II in splenocytes of peptide immunized mice, and after in vitro stimulation with 20 µg/mL of the respective peptide for 48h. In A, representative dot-plots of how the analysis was performed to achieve the values. In B, the values are expressed as mean and its standard deviation calculated from two independent cell cultures.



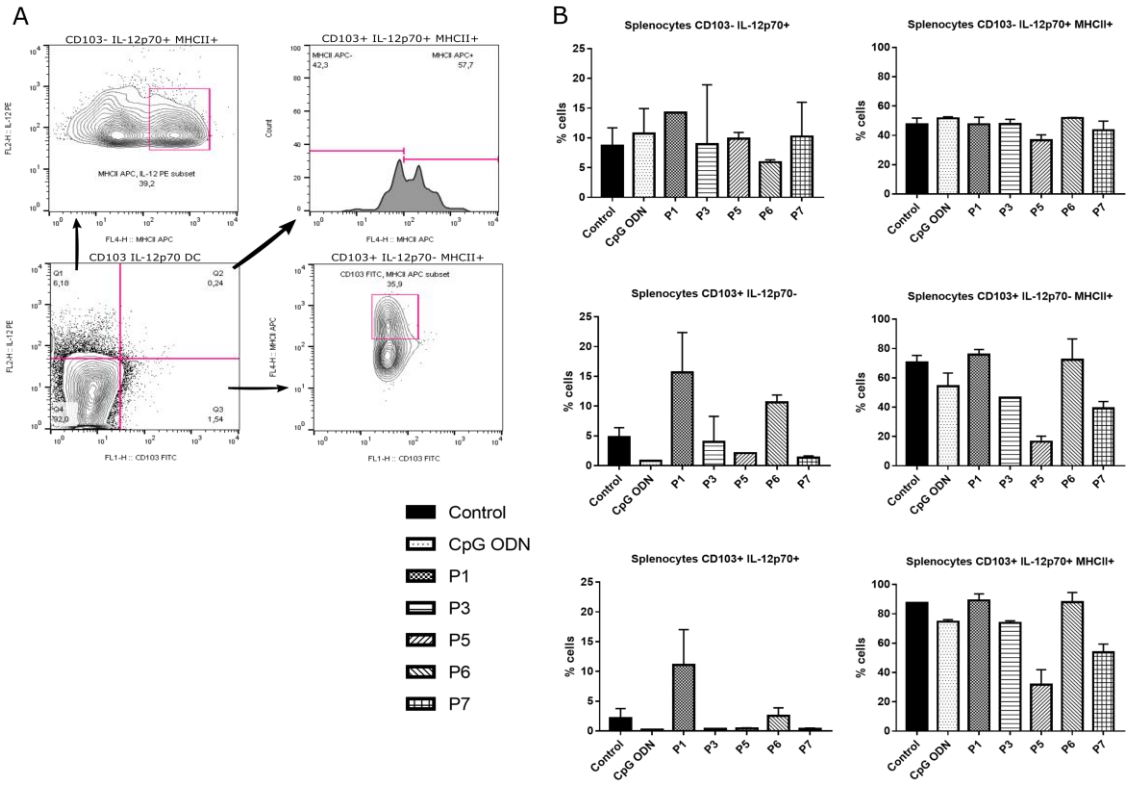
3.4 Levels of CD103 IL-12p70 MHC II cells

In this evaluation, the percentage levels of CD103$^+$ IL-12p70$^-$, CD103$^-$ IL-12p70$^+$, and CD103$^+$ IL-12p70$^+$ cells was assessed and its expression of MHC II. The dot-plost and gates drown for the analyses are represented in the Fig. 4 A. The results are expressed in Fig. 4 B as the mean and its standard deviation calculated from two independent cell cultures.

Regarding the percentage levels of cells, a slightly higher level of CD103$^-$ IL-12p70$^+$ cells was found for samples stimulated with peptide 1 when comparing with non- and peptide-stimulated cells. In other hand, increased levels of CD103$^+$ IL-12p70$^-$ cells were found in samples stimulated with peptides 1 and 6 when comparing with the controls. Low levels of this cell subset were seen for the samples stimulated with peptides 3, 5, and 7. Samples stimulated with peptide 1 also presented increased levels of double positive cells for CD103$^+$ IL-12p70$^+$. For peptide 6, these levels were the same for the control non-stimulated. Very low levels of these double positive cells were found for samples stimulated with peptides 3, 5, and 7.

In terms of MHC II expression, CD103$^-$ IL-12p70$^+$ cells expressed almost the same levels of MHC II among the samples. For the other two cell subsets analysed, same levels of MHC II were found for samples stimulated with peptide 1 and 6 when comparing with the control non-stimulated, and low levels of MHC II expression for samples stimulated with peptides 3, 5, and 7. The lowest levels of MHC II expression were found among the peptide 5 samples when comparing the different subsets and and the other peptide samples.

Figure 4: Levels of CD103, IL-12p70 and MHC II in splenocytes of peptide immunized mice, and after in vitro stimulation with 20 µg/mL of the respective peptide for 48h. In A, representative dot-plots of how the analysis was performed to achieve the values. In B, the values are expressed as mean and its standard deviation calculated from two independent cell cultures.



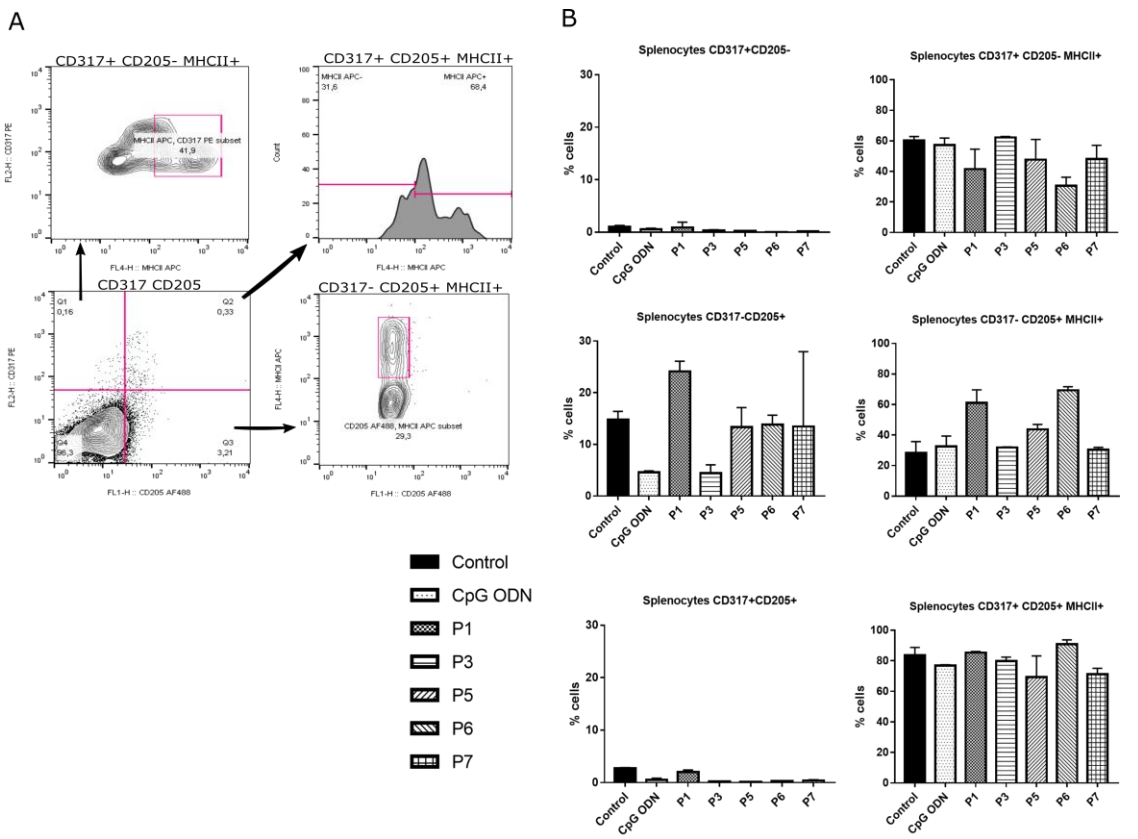
3.5 Levels of CD317 CD205 MHC II cells

In this assay, the percentage levels of $CD317^+$ $CD205^-$, $CD317^-$ $CD205^+$, and $CD317^+$ $CD205^+$ cells were assessed and its levels of MHC II expression. In Fig. 5 A it is represented the dot-plots and gates drown for the analyses performed. The results are expressed in Fig. 5 B as the mean and its standard deviation calculated from the data of two independent cell cultures.

Very low levels of $CD317^+$ $CD205^-$ and $CD317^+$ $CD205^+$ cells were found in all samples, including the cells non-stimulated with the peptides. Even tough, relevant levels of MHC II expression were found in these cell subsets. In other hand, high levels of $CD317^-$ $CD205^+$ cells were found in the samples stimulated with peptide 1, and low levels in samples stimulated with peptide 3 when comparing with the control. The percentage level of $CD317^-$ $CD205^+$ cells for peptides 5, 6, and 7 was the same as the control non-stimulated. Regarding the levels of MHC II expression, the highest levels were found for peptides 1 and 6 in this cell subset. For peptides 3 and 7 the levels of MHC II expression were the same as the control non-stimulated.

The differences found in this study were not statistical significant.

Figure 5: Levels of CD317, CD205 and MHC II in splenocytes of peptide immunized mice, and after in vitro stimulation with 20 µg/mL of the respective peptide for 48h. In A, representative dot-plots of how the analysis was performed to achieve the values. In B, the values are expressed as mean and its standard deviation calculated from two independent cell cultures.



4 Discussion

APCs, especially DCs, have an important role in developing a property immune response and several vaccines approaches available are intended to activate this cells type. For *Leishmania*, once activated the DCs can modulate the antigen presentation and induce a Th1 cell profile which is correlated with parasite elimination in murine models of infection. However, the murine models of infection and vaccine efficacy are not the best in translating the antigen efficacy, since there are several differences in the response triggered in humans. Therefore, in this work, potential $CD4^+$ and $CD8^+$ T cell peptide epitopes which were identified from the proteome of *L. braziliensis* and tested significantly with human cells have their effect evaluated on bone marrow and splenic dendritic cell subsets.

BMDC $CD11c^+$ have shown high levels of MHC I, MHC II and CD40 expression for the controls non-stimulated and lower levels for cells stimulated with peptides for both time points. However, the DC marker CD11c is down regulated in murine bone marrow and spleen DC after activation by Toll-like receptors (TLRs) 3, 4 and 9 (SINGH-JASUJA *et al.*, 2013). Therefore, it is possible that in this BMDC $CD11c^+$ population, the peptides could have induced cell activation and somehow the down regulation of the express markers MHC I, MHC II and CD40. On the other hand, increased levels of MHC II and CD40 were observed in the cell population BMDC $CD11c^-$ for both time points. The CD40 pathway is one of the main pathways that could trigger the production of IL-12 which is well known to stimulate Th1 profile with IFN-γ and resistance to *L. major* (OKWOR; UZONNA, 2016b).

Although the up-regulation of these makers expression can be used to evaluate cell activation, it is also important to consider that different levels of these markers can occur among the cell populations. Additionally, this evaluation is limited to the presence of the markers and does not consider whether the peptides used for stimulation

are present or not on the BMDC surface. Recently, Mou et al. have identified naturally processed and MHC-coupled peptides after *in vitro* infection of BMDC with *Leishmania* parasites (MOU *et al.*, 2015). Moreover, the infection by *L.V. braziliensis* is related with DC activation and consequently induction of a protective immune response (VARGAS-INCHAUSTEGUI, D. A *et al.*, 2009).

The cytokine environment of DCs is important for T cell activation and higher levels of IL-6 and TNF were found among the cells stimulated with peptides 3, 4, 7, 9 and 10, when comparing with the control non-stimulated. Considerable levels of these cytokines were also produced by the controls, mainly for IL-6. The enhanced production of pro-inflammatory cytokines such as IL-6 and TNF by BMDC has been reported after stimulation with TLR type 2 ligands, lipopolysaccharide (LPS), for e.g. (BOONSTRA *et al.*, 2006; GEISEL *et al.*, 2007). However, in this work the BMDC were not stimulated with LPS and presented this cytokine phenotype. Complex signalling cascades are activated after binding of TLR ligands which depend on the intracellular adaptor molecules myeloid differentiation primary response gene 88 (MyD88) and/or TIR-domain containing adaptor protein inducing IFN (TRIF), leading to production of pro-inflammatory cytokines, such as TNF (HIROTANI *et al.*, 2005; KAWAI; AKIRA, 2006). In human monocyte derived dendritic cells, the presence of IL-6 dampens the capacity of antigen presentation, reducing co-stimulation by CD86, production of IL-12p70 and MHC II expression (OHNO *et al.*, 2016). Because of the difficulties for the generation of this cell type, the observation that different peptide epitopes tested would trigger the production of IL-6 and TNF is of interest.

In terms of spleen DCs, a slightly higher level of CD8α$^+$ MHC II$^+$ DCs was found after stimulation with peptide 6 when comparing with the controls and the other peptides. This cell type is particularly efficient in cross-presenting antigens and produce IL-12, and during intracellular infections they promote antigen presentation to CD8$^+$ T cells (SHORTMAN; HEATH, 2010b). Differently from the result with CD8$^+$ DCs, a higher frequency of CD11b$^+$ DCs was observed after stimulation with peptides 1 and 6, and also high levels of MHC II expression. The CD11b$^+$ DC subpopulation is heterogeneous and it remains less characterized than the CD8$^+$ DC subpopulation. Most functions of CD11b$^+$ cells are still not clear and, conventionally, they do not have the same functions as CD8$^+$ DCs. They are inefficient to cross-present antigens and produce cytokines like IL-12 (MILDNER; JUNG, 2014). However, compared to the CD8$^+$ DCs, the CD11b$^+$ DCs are superior at inducing CD4$^+$ T cell immunity due to its high capacity to express MHC II (DUDZIAK *et al.*, 2007; LEWIS *et al.*, 2011). Thus, it is suggestive that the peptides could stimulate the more frequency of this cell type in the spleen of vaccinated mice.

In this work, increased levels of a CD103$^+$ cell subset were found for the cells stimulated with peptide 1 and 6. In addition, CD103$^+$ IL-12p70$^+$ cell subsets were also up-regulated after peptide 1 stimulation. These results suggest a role for migratory DC producing considerable levels of IL-12p70 after stimulation which may trigger Th1 protective immune responses. The presence of high levels of MHC II on this cell subset confirms its function as APC. CD103 is a typical marker found in CD103$^+$ DC and in some T cells, and the DC subset is particularly important as a migratory DC and an important inducer of CD8$^+$ T cell responses (BELZ; NUTT, 2012)(BROZ *et al.*, 2014; RUFFELL *et al.*, 2014). CD103$^+$ DCs producing IL-12 may act as important antagonists of a Th2 immune response which is not desired for an anti-*Leishmania* vaccine (EVERTS *et al.*, 2016).

Low levels of CD317$^+$ DC were found in all samples analysed in this study. CD317 is a typical marker of DCs with an important role in the innate immune system

due to its capacity to produce type I IFN (BIERLY *et al.*, 2008), mainly IFNα, and poor antigen presentation. Other cell subsets may also express the CD317, such as B cells and macrophages, which can also express MHC II. The CD205$^+$ cells presented increased levels in terms of frequency for peptide 1 and expression of MHC II for peptides 1 and 6. It suggests that these peptides may up-regulate the levels of these cell subsets. CD205 has been one of the main important receptors for targeting antigens in *in vivo* vaccination approaches for delivery of antigens to DCs in the body (PACK *et al.*, 2008). In murine models, targeting of antigens to CD205 improves at least 100-fold the process of antigen presentation (BONIFAZ, L. *et al.*, 2002; HAWIGER *et al.*, 2001).

In summary, these results indicate that there are no significant changes in the expression markers evaluated on BMDC after stimulation with peptide epitopes, but higher levels of MHC II and CD40 were found among BMDC CD11c$^-$. Together with the cytokine levels, the presence of pro-inflammatory cytokines may indicate triggering of downstream pathways that could stimulate cell activation. Considering the splenic cell subsets and the markers investigated, a higher proportion, even though not statistically significant, of CD11b$^+$ DCs and of CD103$^+$ IL-12p70$^+$ cells was observed after stimulation with peptide 1 and peptide 6. Probably due to the limited number of samples evaluated, the differences observed were not statistical significant. This result suggests that the peptides are capable of inducing activation of splenic APCs and the role of the different subsets in developing immunity against *Leishmania* is not yet understood.

Conflict of interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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6 CONCLUSÕES

- O presente trabalho permitiu identificar, de forma inovadora, potenciais epítopos vacinais de linfócitos T CD4$^+$ e T CD8$^+$ no proteoma de *Leishmania braziliensis*;
- As ferramentas e o protocolo de métodos *in silico* empregados, permitiram identificar epítopos peptídicos com alta afinidade de ligação às moléculas do MHC de Classe I e Classe II de diferentes alelos humanos;
- Alguns dos epítopos peptídicos foram capazes de estimular, significativamente, a proliferação de PBMC de indivíduos com leishmaniose tegumentar após cura clínica; contudo, não foram capazes de estimular a proliferação de PBMC de indivíduos com leishmaniose tegumentar ativa;
- Os epítopos foram capazes de estimular, significativamente, altos níveis de proliferação de PBMC de indivíduos residentes de áreas endêmicas para leishmaniose tegumentar;
- Alguns dos peptídeos investigados foram capazes de induzir a produção de citocinas dos perfis Th1 (IFN-γ, TNF) e Th2 (IL-6) no sobrenadante de cultura de células de indivíduos com leishmaniose ativa e após cura clínica;
- Verificou-se níveis significativos de expressão do fator de transcrição T-bet em linfócitos T CD4$^+$ e CD8$^+$ de indivíduos com leishmaniose tegumentar após cura clínica;
- Dentre os peptídeos testados, os identificados como peptídeos 1, 2 e 7, estimularam significativamente a produção e secreção de IFN-γ e TNF no sobrenadante de cultura de células dos pacientes, estimularam a expressão do fator de transcrição T-bet e a proliferação celular;
- Os peptídeos testados estimularam células dendríticas mielóides murinas e células dendríticas esplênicas e outras subpopulações desse órgão;
- Os resultados apresentados no presente trabalho indicam que os epítopos possuem um grande potencial de induzir uma resposta protetora contra a leishmaniose tegumentar.

7 PERSPECTIVAS

- Imunizar camundongos susceptíveis com os peptídeos e desafiá-los por meio da infecção com cepa infectiva de *L. braziliensis*;
- Analisar a resposta proliferativa induzida pelos peptídeos com células de pacientes com leishmaniose visceral causada por *L. infantum* e tegumentar causada por *L. amazonensis*;
- Imunizar camundongos susceptíveis com BMDC estimuladas com os peptídeos e desafiá-los por meio da infecção com cepa infectiva de *L. braziliensis*;
- Construir moléculas poli-epítopos no intuito de avaliar a imunogenicidade e a capacidade de proteger contra a leishmaniose tegumentar.

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APÊNDICE I – Capítulo de livro: *In Silico Approaches Against Trypanosomatids*

Tropical Diseases: Immunological and Molecular Tools Applied to the Epidemiology and Control, 2015,

CHAPTER 2

In silico approaches against trypanosomatids

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Abstract:

In the absence of an effective and safe vaccine for most trypanosomatid infections, the main strategies currently available for the control of these diseases are chemotherapy whit drugs limited by their cost, toxicity, long time of treatment, and by emergence of resistant parasites. Therefore, *in silico* methods have arisen in the last years as powerful tools to optimize the search for new drugs and targets and for potential antigens for vaccines. In this context, the aim of this chapter is to provide the reader with important tools in use for drug design, particularly for molecular docking and epitope prediction for vaccines, in order to make useful to put this concepts and approaches in practice. All these tools should be applied in the fight against trypanosomatids.

Keywords: *in silico*, trypanosomatids, target, molecular docking, drug design, vaccine design, epitope.

INTRODUCTION

The *in silico* term follows the trend from *in vitro* and *in vivo,* and refers to the silicon chip inside the computers. The term is widely used in medicinal chemistry literature to designate the use of computational methods to perform modeling studies, including the molecular modeling techniques. The importance of *in silico* approaches has been already perceived by the scientific community around the world, and by the

APÊNDICE II – Capítulo de livro: *Vaccines Against Trypanosomatids*

Tropical Diseases: Immunological and Molecular Tools Applied to the Epidemiology and Control, 2015,
CHAPTER 3

Vaccines against trypanosomatids

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Abstract: The control of diseases caused by trypanosomatids, such as those caused by *Leishmania* spp. and *Trypanosoma cruzi,* are focused on the use of drugs to treat infected patients which are limited by their high cost, high toxity, and safe concerns; in the absence of an effective and safe vaccine for use in humans. Therefore, new strategies for vaccines have emerged in the last years and it is expected better perspectives for the future. In this context, the aim of this chapter is to discuss with the reader the past, present and future of vaccines against *Leishmania* spp. and *Trypanosoma cruzi*. In addition, not less important, it will be pointed out important aspects for the development of anti-trypanosomatids vaccines.

Keywords: trypanosomatids; vaccines; epidemiology; immunology; antigens; challenges.

INTRODUCTION

Vaccines are the major achievement of modern medicine in the last years, and the development of vaccines to combat Neglected Tropical Diseases (NTDs) remains as one important challenge for this century. It is well known that for many bacterial and virus infections there are already prophylactic vaccines that can prevent infection, thus, maintaining the individual and the population protected (herd immunity). However, for

APÊNDICE III – Artigo publicado: *Leishmaniases diagnosis: an update on the use of immunological and molecular tools*

de Paiva-Cavalcanti *et al. Cell & Bioscience* (2015) 5:31
DOI 10.1186/s13578-015-0021-2

Cell & Bioscience

REVIEW Open Access

Leishmaniases diagnosis: an update on the use of immunological and molecular tools

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Abstract

Leishmaniases are caused by obligate intracellular protozoan parasites of the genus *Leishmania*. They cause a spectrum of diseases, most notably visceral (VL), cutaneous (CL), and mucosal (ML) leishmaniasis, which affect millions of people around the world, each year. Despite scientific advances, leishmaniases cases are expanding, constituting an important public health problem. Immunological and molecular diagnostic tools have been increasingly applied for the early detection of these parasitic infections, since the existence of limitations in clinical and parasitological examinations may provide false results, thus interfering in epidemiological research and diseases control. Although there is a great diversity of available immunological assays, important common deficiencies persist, which explains the current exploration of the molecular biology in research fields, especially the Polymerase Chain Reaction (PCR) and its variants, such as real-time quantitative PCR. However, in the last years, significant results have also been reached inside of immunological context (especially by Flow Cytometry), for humans and dogs, demonstrated by research works of the New and Old worlds. In spite of their potential to clarify and minimize the present global situation of the diseases, the implementation of molecular or immunological innovative reference assays for VL and CL at health services is still a challenge due to several reasons, including lack of standardization among laboratories and structural concerns. In this article we bring classical and current information about technological advances for the immunological and molecular leishmaniases diagnosis, their features, and applications.

Keywords: Visceral leishmaniasis, Cutaneous leishmaniasis, Diagnosis, Immunological tools, Molecular tools

Introduction

Leishmaniases are caused by obligate intracellular protozoan parasites of the genus *Leishmania*, capable of developing a spectrum of diseases, most notably visceral (VL), cutaneous (CL), and mucosal (ML) leishmaniasis [1, 2]. Global VL and CL incidences are approximately 0.2–0.4 million and 0.7–1.2 million cases occurring each year, respectively [3]. Despite the increasing number of infected individuals being registered in several countries of the Americas (New world) Europe, Asia and Africa (Old world), a huge number of cases are believed to be under-reported [4].

Leishmaniases are still ignored in discussions of tropical disease priorities, even being estimated to cause the ninth largest disease burden among individual infectious diseases [5, 6]. Despite scientific advances, leishmaniases constitute an important public health problem. Up to the moment, the leishmaniases diagnosis is performed by an association of clinical, epidemiological and laboratorial data. Particularly in relation to laboratorial methods, the lack of a gold-standard for human patients or animals is a limitation for the disease control, because the achievement of accurate epidemiological data is associated with the guidance of control measures, thus helping to increase their efficiency. Furthermore, false-negative results could delay early implementation of treatment, thereby contributing for maintenance of reservoirs, and as a consequence, the preservation of parasitological cycles in their environment.

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Rapid methods for diagnosis and species identification are urgently needed, along with therapies, prophylactics, and control measures that are effective, safe, affordable, and easily administered [2]. In this context, this article provides an overview on classical diagnosis and current information about technological advances for the immunological and molecular leishmaniases diagnosis, their features, applications, limitations and perspectives.

Immunological tools for visceral and cutaneous leishmaniasis diagnosis

The immunological procedures have been employed for screening and for the definitive diagnosis of diverse parasitological illnesses because of their easiness and accuracy. For leishmaniases, these methods are largely applied, being, in some routine protocols, the single diagnostic principle, before anti-*Leishmania* drug prescription [7]. Nonetheless, important limitations may carry to incorrect diagnostic interpretation, like cross-reactions, especially with phylogenetically closely related species, and loss of accuracy in immunosuppressed patients. The combination of different techniques, like immunochromatographic test (ICT) and rapid immunoenzyme assay, or the employment of different antigenic targets and the combination of distinct recombinant proteins in the same assay are strategies that may improve the global specificity and sensitivity, thus bringing a higher accuracy to the routine protocols [8–10]. The association with the molecular biology, mainly in reference centers, has become a common approach to the differential diagnosis of leishmaniases, especially when parasitology is also inconclusive [11, 12]. Even so, researchers around the world have been developing new protocols and technologies to the continuous enhancement of the immunological diagnosis, thus ensuring smaller risks to patients in the detection of leishmaniases.

Classical immunological tools

Montenegro skin test (MST) has been successfully used in the diagnosis of cutaneous forms, but is negative for recent lesions, in the diffuse form, and also in immunosuppressed patients [13]. The test is commonly positive in endemic areas due to the occurrence of subclinical infections. Furthermore, other characteristics may hamper the applicability of the method: cross-reactions; long time to retest, if necessary (at least two years); false-positive results caused by lack of patient cooperation, which itches the application site; subjectivity of the reading (especially when the diameter of the induration has between 4 and 5 mm) and so on [14]. In the diagnosis of CL the use of indirect immunofluorescence assay (IFA) associated with MST or a parasitological technique is recommended to provide a differential diagnosis. The limitation of IFA lies in the fact that it does not correlate the levels of circulating antibodies with disease staging. In addition, there is the possibility of cross reactivity with other trypanosomatids and fungi [15–17]. IFA is based on the detection of anti-*leishmania* antibodies by employing specific antigens (promastigote form, normally) and secondary antibodies (anti-immunoglobulin antibody) conjugated with a fluorescent dye [16, 17]. The technique is becoming less explored in routine not only for CL diagnosis, but also for canine VL (CVL) diagnosis mainly because of its low specificity, in contrast with the high sensitivity. Incompatibility or poor reaction between the secondary and primary antibodies or the antigen are also constraints associated with this indirect immunological assay.

The majority of the immunological techniques for detection of anti-*Leishmania* antibodies has been based on reactions like Enzyme-Linked Immunosorbent Assay (ELISA) [11, 18]. The sensitivity and specificity of ELISA depends on the antigen used [11]. In this context, several antigens with different molecular weights have been identified for potential use in the diagnosis. Recombinant *Leishmania* protein K39 (rK39), a very important and broadly employed antigen, showed 100 % specificity and 96 % sensitivity for the diagnosis of VL [19]. An interesting feature of this antigen is that it can be used in patients co-infected with HIV, in which anti-K39 antibody levels decline rapidly with the treatment success [11, 20, 21]. Other candidates for the diagnosis of several forms of leishmaniases are recombinant or purified membrane glycoproteins (gp): gp63, gp70, and gp72, and A2 protein, all of which are specific for the genus *Leishmania*. A2 protein is present in amastigotes, and studies suggest that is particularly useful for diagnosis of canine VL [20, 21]. The use of recombinant or purified gp63, gp72 or gp70 improved the sensitivity and specificity of ELISA. However, cross-reactions with other diseases caused by trypanosomes may occur [22, 23].

The numerous commercial kits of rapid diagnostic tests (RDTs) available are largely explored nowadays in routine. The easiness concerning its use and interpretation, as well as the quick results have contributed to its broad application, especially for screening. The high stability in a broad range of temperature (generally 4–30 °C) facilitates its employment in field for screening of CVL. RDTs are based on the detection of specific antibodies in serum or peripheral blood of the patient with VL. An important RDT, known as TRALd (Rapid Antibody Test *Leishmania donovani*) was developed using two recombinant proteins, the rK39 and K26, fixed on nitrocellulose paper. An initial clinical evaluation of TRALd observed 100 % of sensitivity and 98 % of specificity [11, 21, 23, 24]. Even when antigenic combinations are used, there is the possibility of cross-reactions with other trypanosomatids, being then indicated the application of a confirmatory

exam, normally of a distinct principle (e.g. IFA, ELISA etc.) or distinct antigenic composition.

New immunological tools

In relation to the new immunological tools, the use of flow cytometry (FC) in the diagnosis of canine VL through the detection of anti-fixed *L. infantum* antibodies has been increasingly explored [25]. Researchers demonstrated an excellent performance of an FC prototype in canine VL diagnostic, with high specificity, sensitivity, predictive values and accuracy, even when animals were infected with other pathogens (such as *Trypanosoma cruzi* and *Babesia canis*), and also absence of false-positive results in vaccinated dogs [26]. Recently it was demonstrated the potential of a magnetic microsphere coated with *Leishmania* recombinant antigens associated with FC as a viable tool for a highly sensitive laboratorial serodiagnosis of both clinical and subclinical forms of canine disease [27]. For human form, it was shown the detection of specific IgG antibodies against *L. chagasi* using FC for cure assessment [28].

FC to detect anti-live *L. braziliensis* antibodies has been first described by Rocha *et al.* [29], in which they demonstrated 93.6 % sensitivity in patients with active disease. Researchers working with live and fixed *L. braziliensis* showed that FC can be a useful serological technique to detect anti- *L. braziliensis* IgG antibodies, with the antigens displaying an 86 and 90 % sensitivity, respectively [30]. A good performance using fixed *L. amazonensis* promastigotes was also demonstrated [31]. Oliveira *et al.* [32] showed that FC had a better performance compared to IFA in the monitoring of specific post therapeutic cure of CL. Therefore, FC is becoming an increasingly useful tool in health care and research laboratories, due to its accuracy and reproducibility. Although there is still a substantial cost regarding the operational support in experiments involving FC, Shared Resource Laboratory models are enhancing the scope and quality of scientific research that applies the FC based methodologies [26, 33, 34].

The Table 1 summarizes the main aspects addressed in the immunological methods used in leishmaniases diagnosis.

Molecular tools for leishmaniases diagnosis

The limitations demonstrated by the conventional techniques, both parasitological and serological, have led the scientists to an increasing exploration of the molecular biology as a complement, as well as an alternative for the accurate diagnosis of leishmaniases. The practicality, safety and reliability of the molecular tools, in addition to the wide number of applications and the promising results have contributed to the continuous acceptance of these methods in routine and reference laboratories around the world.

The Polymerase Chain Reaction (PCR) technique and its variations, like Nested-PCR (nPCR), Seminested-PCR (snPCR) and Quantitative Real Time PCR (qPCR) have been largely employed for the optimization of new diagnostic assays, using different target regions and samples [35–37]. The follow-up of the treatment aiming the evaluation of a drug efficacy is a common approach [38] proportionated by the qPCR, with its capacity to estimate the parasitological burden in several specimen types [39,

Table 1 Advantages and limitations of immunological methods used in leishmaniases diagnosis

Method	Antigen	Advantage	Limitation
Montenegro skin test	Killed whole parasites	Low cost and detection of T cell immunity	May not detect cases of visceral leishmaniasis in some stages of the disease. Cannot differentiate between infection and disease, nor active and progressive disease. Risk of recurrence.
Enzyme-Linked Immunoabsorbent Assay (ELISA)	Recombinant molecules	Low cost and high sensitivity and specificity	Sensitivity and specificity is highly dependent on the antigen used
Immunofluorescence	Killed whole parasites	High sensitivity and specificity	Laborious process, time and cost consuming. Need of trained personnel to perform the test.
Flow cytometry	Recombinant molecules and/or killed whole parasites have been tested	Better sensitivity and specificity when compared with all other methods. Small amount of blood. Can differentiate between infection and disease, and cured patients.	Cost associated with reagents and equipment. Few studies yet.
Rapid Antibody Test (RAT)	Recombinant molecules	Low cost, small amount of blood, fast	Sensitivity and specificity is highly dependent on the antigen used
Direct Agglutination Test (DAT)	Killed whole parasites	Low cost, small amount of blood	Need of long incubation time, well-trained laboratory technicians, antigen cost, and quality controlled antigen

40]. Species characterization of *Leishmania* is also an important application of the PCR, and it has been strongly explored nowadays [41, 42]. Coupled with different methodologies, including gene sequencing and Restriction Fragment Length Polymorphism (RFLP) analysis, the studies have brought this kind of analysis with distinct goals: species confirmation in epidemiological researches [43, 44], specificity assessment of new optimized assays [11, 45] and comparative studies [46], for example. Ozensoy-Toz *et al.* [47] have used fluorescent dyes and the resulting melting temperature (Tm) interpretation as criterion for *Leishmania* species differentiation, to both CL and VL etiological agents, with success. The author, as others [43, 48], also performed phylogenetic analysis of species from isolates by gene amplification and sequencing. According to Grimaldi and Tesh [49], the correct identification of the causative *Leishmania* species is directly related to decision upon the appropriate treatment regimens and to design effective control programs.

Visceral leishmaniasis diagnosis

Between 2011 and 2013, conventional PCR protocols for VL presented a sensitivity variation from 53.7 to 97.78 % for humans and from 72.2 to 98.7 % for dogs, and the specificity varied from 61.82 to 100 % for humans and from 83.3 to 96.4 % for dogs [41, 43, 50, 51]. The high variability also occurs to qPCR for humans, with a specificity variation from 29.6 to 100 %. The range of sensitivity (91.3–100 %) [41, 51] presented short and with high values, thus demonstrating the applicability of the qPCR at situations in which a sensitive tool is pivotal, like disease monitoring to predict relapse. Results obtained by Solcà *et al.* [52] show the PCR as being more sensitive than qPCR in dog samples. This reinforces that some characteristics like the DNA target region, as well as the pair of primers used may be determinant.

As occurs in different clinical statuses, the parasitic burden in the various samples differs. Getting advantage on the specimen type, Pandey *et al.* [53] and Silva *et al.* [54] standardized PCR reactions (snPCR and qPCR, respectively) to detect the parasite DNA from Giemsa-stained smears, prepared from bone marrow aspirates. The first author's assay detected *L. donovani* DNA in 68.7 % of the human samples. The second author detected *L. chagasi* DNA in 100 % of the canine samples. Both assays presented superiority in relation to microscopy and culture. Other alternative samples, like urine and conjunctival swabs are being largely explored for humans [55, 56] and dogs [39, 57–60] with good accuracy. Although the small parasite DNA amount, they do not request invasive procedures for obtaining, thus bringing security and comfort to the patient [36, 57].

Despite these advantages, several *Taq* Polymerase inhibitors are found in these clinical specimens or they are commonly used for sample collection and DNA extraction, like EDTA, Proteinase K, Phenol and high salts concentrations [61, 62]. Recently, some authors have brought the strategy of the multiplex PCR to include endogenous controls altogether with the detection system of *Leishmania* sp., in the same reaction, for PCR [61] and qPCR [63] assays. Mohammadiha *et al.* [41] performed this multiplex format for humans and dogs, using TaqMan-based qPCR, having reached a very good sensitivity in both cases (93.9–100 %, respectively). Gonçalves-de-Albuquerque *et al.* [64] standardized a triplex PCR to CL capable to monitor not only the sample quality, but also small losses of DNA during the extraction process by using a plasmid as reporter.

Innovative molecular approaches, as the Nucleic Acid Sequence-Based Assay (NASBA), the Loop-Mediated Isothermal Amplification (LAMP), and the low-tech Oligo-TesT have been increasingly applied for *Leishmania* DNA or RNA detection. The NASBA have its variations, the quantitative (QT-NASBA) and coupled to oligochromatography (NASBA-OC) [65–69]. Vries *et al.* [65] have used QT-NASBA for the evaluation of the efficacy of a drug for VL treatment, by estimating the quantification of *L. infantum* parasites in blood. Basiye *et al.* [66] and Mugasa *et al.* [67] have applied NASBA-OC to diagnostic assays development, based on *L. donovani* RNA detection, reaching sensitivity of 79.80–93.30 % (respectively) and specificity of 100 % (both). The NASBA-OC uses a sensitized membrane of an oligochromatographic dipstick to detect the amplified RNA, in 5–10 min just with a pipette and a water bath, but without quantification capacity [68]. QT-NASBA has the inconvenience of the electrochemiluminescence as tool of detection, which involves more handling steps and procedure time than qPCR and Reverse transcription-qPCR (RT-qPCR) [69].

The LAMP, a promising diagnostic tool, has been adopted as an alternative technique to PCR, since it is a faster, sensitive and less expensive technology, which uses the turbidity of the sample as criteria of positivity. There is no need of a thermal cycler, just a water bath or a heat block, since the reaction is isothermal. Therefore, this is a tool suitable for field application [70, 71]. Verma *et al.* [72] developed a LAMP-based assay for *L. donovani* detection in humans with VL and Post-Kala-azar Dermal Leishmaniasis (PKDL) in which the sensitivity and specificity rates were good to both cases, achieving 96.4–98.5 % (in VL blood samples); 96.8–98.5 % (in tissue biopsy samples). Nevertheless, Chaouch *et al.* [73] developed a LAMP amplification for *L. infantum* detection in dogs, and the sensitivity reached was low (54.2 %), though having performed better than IF and PCR, statistically. The chosen target (cysteine Protease B gene - *cpb*) and the non-use of an internal quality control can in part explain this result.

Cutaneous leishmaniasis diagnosis

As presented for VL, molecular techniques are advancing in studies for increasing their sensibility and specificity. They have been increasingly recommended for CL diagnosis due to their accuracy and speed [74], when compared to conventional diagnostic methods [47, 74]. Mohaghegh *et al.* [75] used PCR for confirming negative direct stained smear, confirming the higher sensitivity of the molecular method in comparison with classical diagnosis (direct stained smear). As for VL, different biological samples can be used with the molecular technology: blood [76], smear [75], scarification of the edge lesion [77] and biopsy skin [78, 79].

Several authors have used the qPCR technology to detect the DNA from the etiological agents in varied samples from animals and human patients, allowing studies related to parasite load, host-parasite interaction, monitoring of therapy and post-treatment response in infected human patients [80, 81]. Another common approach to CL is the application for differentiation of *Leishmania* species [47, 81–83]. Authors using dyes or fluorescent probes have had great results. Paiva-Cavalcanti *et al.* [82] used skin samples from humans and blood samples from domestic animals; Ozensoy Toz *et al.* [47] used skin aspiration fluid, smear, and biopsy from human patients; Pita-Pereira *et al.* [83] used skin biopsy samples from patients living in areas with well-known occurrences of CL. They have differentiated *Viannia* and *Leishmania* subgenera through Tm. Nonetheless, the qPCR requires a laboratory with the technical capacity to perform it; thus, this technology is becoming available at Central Diagnostic Laboratories, in countries where leishmaniases are endemic [81].

The nPCR is commonly used for some researchers [78]. Shirian *et al.* [84] performed the technique by using scraped off of slides with impression smears from 20 suspect cases of ML, getting 18 positive samples (90 %). Only eight were positive through direct microscopy (44.4 %). The PCR based method was not only a useful and more precise diagnostic approach in the identification of ML cases with negative cytology, but also showed to be efficient in determining the species of the parasites. Azzi *et al.* [74] concluded that nPCR is a useful technique for studying the molecular epidemiology in the field.

The QT-NASBA assay is a useful instrument to monitor parasite load in skin biopsies of patients with CL after treatment and can help to predict clinical outcome [69]. qPCR, RT-qPCR and QT-NASBA were compared by Van der Meide *et al.* [69], and they concluded that RT-qPCR and QT-NASBA are the most sensitive assays, generating reproducible results. However, as described before, QT-NASBA is less convenient since the electrochemiluminescence detection involves more handling steps and procedure time.

Despite the variety of molecular tools (Pulsed Field Gel Electrophoresis – PFGE, the Multilocus Enzyme Electrophoresis – MEE, and more), PCR still represents one of the major advances on diagnosis and research of the leishmaniases [85], even presenting its deficiencies, which have been minimized due to the continuous effort of researchers, attempting to bring more viability to its application in health service.

Table 2 summarizes the main aspects addressed in the molecular methods used in leishmaniases diagnosis.

Table 3 gathers the clinical sensitivities and specificities of the immunological and molecular methods commented throughout the text.

Molecular targets

Several different genomic targets are used for *Leishmania* sp. detection as rDNA (ITS-1 and SSU rDNA), kinetoplastid minicircle DNA (kDNA), splice leader mini-exon (SMLE), tryparedoxin peroxidase gene and, heat-shock protein 70 gene (HSP70) [47, 77, 79, 82, 86–89]. This is one of the challenges to be surpassed for a future implementation of a gold-standard molecular methodology for CL and VL. Nevertheless, the choice of each target is related to the different applicability, i.e., with the objectives of each study, such as the case of HSP70 used by authors for species typing [45, 89, 90], while the kDNA is the main target used for diagnostic screening [79, 91–93].

The kDNA is largely elected for DNA amplification of *L. donovani* complex, because of the high number of copies (10,000) per parasite [94]. This target has been chosen for several applications, like for epidemiological studies in canine populations, took place in countries like Brazil and China [43, 95, 96], for species characterization [43, 97], for treatment follow-up and for assays development, with great results [72, 98].

Rocha *et al.* [99] compared different PCR protocols for *Leishmania* subgenus species detection, based on kDNA minicircle or mini-exon amplification. Concerning the kDNA minicircle, as long as the PCR protocol for *L. amazonensis* pointed out no specificity, since *L. infantum* was also detected, the PCR assay for *L. infantum* did not amplify DNA of any *Viannia* spp. or *L. amazonensis*, showing thus the importance also in selecting a specific and representative region of the target DNA, for primers design or selection from other authors. The mini-exon protocol presented inability to recognize any *Leishmania* subgenus species. Intraspecific variations of the utilized strains were considered to explain it. Roelfsema *et al.* [42] have compared the mini-exon and the Internal Transcribed Spacer-1 (ITS-1) rDNA in molecular typing of clinical samples. The mini-exon has performed better for typing species belonging to *L. Viannia* subgenus by sequencing, whereas

Table 2 Advantages and limitations of molecular methods used in leishmaniases diagnosis

Method	Advantage	Limitation
Conventional PCR (cPCR)	High sensitivity, specificity and accurate results. Many applications in molecular analysis. Easy diagnostic interpretation.	Unable to quantify the target DNA. Qualitative test. Time consuming. Limited detection range of some assays.
Quantitative real-time PCR (qPCR)	Higher sensitivity, specificity and security, quantitative capacity and speedy results. Possibility of species differentiation by melting temperature.	High cost due to equipment (thermocycler). Difficulty in interpreting the results, needing thus of a well-trained operator.
Nested-PCR (nPCR)	Higher specificity and sensitivity. Useful technique for studying the molecular epidemiology in the field.	Time consuming and higher cost. Unable to quantify the target DNA. Qualitative test.
Quantitative Nucleic Acid Sequence-Based Assay (QT-NASBA)	High specificity. It is based on an isothermal reaction and thus overcomes the need for a thermocycler; Ideal for lower-tech laboratories. Quantitative capacity. Indicated to detect active diseases; RNA detection.	It uses electrochemiluminescence as tool of detection, which involves more handling steps and procedure time. Assays developed only for RNA detection. . Few studies yet.
NASBA coupled with oligochromatography (NASBA-OC)	High specificity. Speedy results. There is no need of complex laboratorial structure. Simple dipstick format for the detection of amplification products. RNA detection.	Unable to quantify the target RNA. Assays developed only for RNA detection. Few studies yet.
Loop-Mediated Isothermal Amplification (LAMP)	High sensitivity. Low cost. Isothermal reaction, there is no need for a thermocycler. The temperature stability of the reagents enables its use in field conditions.	Unable to quantify the target DNA. Qualitative test. Few studies yet.

ITS-1 PCR has differentiated *L. infantum* and *L. donovani* after a Hae III RFLP analysis.

As kDNA, the ribosomal RNA small subunit gene (SSU rRNA), as well as the ITS-1 region have been explored for many objectives, including epidemiological researches in human and canine populations [40, 97, 100], assays optimization [35, 47, 70] and for studies involving HIV/VL co-infected patients, a situation in which the serological diagnosis of VL is limited, and the molecular diagnosis is highlighted [101, 102]. A qPCR system for CL created by Paiva-Cavalcanti *et al.* [82] using kDNA as target has applicability in population studies, etiological diagnosis and the monitoring of treatment efficacy of individual patients. The same work demonstrated the high concordance between kDNA and SSU rDNA, concluding that both may be used for American CL diagnosis;

Table 3 Clinical sensitivity and specificity of different immunological and molecular methods for diagnosis of leishmaniases

Method/clinical form	Specimen	Antigen/target	Sensitivity (%)	Specificity (%)	Reference (s)
Immunological tests:					
ELISA/VL	Human serum	rK39	96	100	[19]
TRALd/VL	Human serum	rK39, K26	100	98	[24]
FC-ALPA/CL	Human serum	Live *L. braziliensis* promastigotes	85.7–97.9	76.0–93.7	[29]
FC-ALPA-IgG/CL	Human serum	Live *L. braziliensis* promastigotes	86	78	[30]
FC-AFPA-IgG/CL	Human serum	Fixed *L. braziliensis* promastigotes	90	78	[30]
Molecular tests:					
cPCR/VL	Human blood	ITS-1, kDNA minicircle	53.7–97.78	61.82–100	[41, 51]
cPCR/VL	Canine blood	ITS-1, kDNA minicircle	72.2–98.7	83.3–96.4	[41, 43, 50]
qPCR/VL	Human blood	ITS-1, kDNA minicircle	91.3–100	29.6–100	[41, 51]
NASBA-OC/VL	Human blood	18S RNA; 18S DNA	79.8–93.3	100	[66, 67]
LAMP/VL	Human blood	kDNA minicircle	96.4	98.5	[72]
LAMP/PKDL	Human tissue biopsy	kDNA minicircle	96.8	98.5	[72]
LAMP/VL	Canine blood	cysteine Protease B (cpb)	38.2–69.5	65.2–89.5	[73]

ELISA Enzyme-linked immunosorbent assay, *TRALd* Rapid Antibody Test *Leishmania donovani. FC-ALPA/AFPA* flow cytometry anti-live/fixed promastigote antibody, *cPCR* conventional PCR, *qPCR* real-time quantitative PCR, *NASBA-OC* Nucleic Acid Sequence-Based Assay- oligochromatography, *LAMP* Loop-mediated isothermal amplification

altogether, the results indicate the best performance of the kDNA protocol. Summarizing, the kDNA target may be used for diagnosis due to its sensitivity [78, 79, 87, 103–105], whereas ITS-1 is more specific and may be used for species identification [78].

Regarding the advances in molecular biology, the choice of the target region to be imaged is the key point for the successful use of diagnostic PCR-based technologies.

Proposal of gold-standard diagnostic strategies for leishmnaniases detection in humans

Technological advances have contributed to raise new diagnostic guidelines not only in the parasitological field, but also in all areas regarding pathology. The technology has brought new analytical possibilities, with increasingly speed, accuracy and reliability. In microbiology, robotic-based equipments are helping to provide molecular assessments with safety and precision in both diagnostic routine and research. Regarding the leishmaniases, the advent of real-time PCR thermocyclers and flow-cytometric analysis (mainly) has contributed to the development of sensitive and specific approaches which have especially contributed to solve inconclusive cases, thus enabling implementation of early and adequate treatment. The establishment of a gold-standard immunological or molecular diagnosis is still a challenge, mainly because of a lack of standardization among laboratories and also structural/economic concerns. In fact, the complexity of leishmaniases also acts as an important barrier to be surpassed. Therefore, we propose a combination of different techniques for maximizing sensitivity and specificity, for a safe and reliable diagnosis: for CL, when MST is positive and parasitological analysis is negative (or is not performed), qPCR and/or FC could be employed as complementary investigation. For VL, when clinical evaluation is indicative (after differential diagnoses) and screening serological tests show negative results, qPCR could be employed as an alternative exam performed in reference centers, thus avoiding the painful and invasive bone marrow biopsy, especially for children. For immunosuppressed individuals such as HIV/VL co-infected patients, the monitoring of parasite load through qPCR is fundamental to predict relapses as well as treatment failures.

Conclusions

Immunological tools are options in leishmaniases diagnosis, highlighting the growth of FC as a more specific and sensitive alternative to overcome the limitations of these techniques and even as a method to assess clinical cure. Likewise, molecular diagnosis has become a potential strategy to provide the early detection and consequently the fast treatment implementation, as well as for species characterization, assessment of treatment efficacy and monitoring of relapses. The easiness, safety and high accuracy have made the molecular biology increasingly interesting as complement or as alternative for defining the definitive VL and CL diagnosis. The technological advance in this case has becoming an adjuvant to prevent the continuous spread of the disease and its social consequences, mainly at poor populations.

Competing interests
The authors certify that there are no competing interests with any financial organization regarding the material discussed in the manuscript.

Authors' contributions
MPC and VRAP contributed equally to the intellectual construction, writing and editing of the article. RCSM, RPS, LAMTS and SCGA contributed with research, analysis and interpretation of data regarding molecular tools. DHCT, MCABC and RFS contributed with research, analysis and interpretation of data regarding immunological tools. All authors read and approved the final version of this manuscript.

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Received: 16 March 2015 Accepted: 5 June 2015
Published online: 17 June 2015

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APÊNDICE IV – Artigo publicado: *Dendritic cell-based approaches in the fight against diseases*

frontiers in IMMUNOLOGY

OPINION ARTICLE
published: 26 February 2014
doi: 10.3389/fimmu.2014.00078

Dendritic cell-based approaches in the fight against diseases

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Keywords: dendritic cells, immunotherapy, vaccine, cancer, infectious diseases

DENDRITIC CELL ROLE IN THE IMMUNE SYSTEM AND ITS MANIPULATION

The immune system works to contain infections through activation of different molecules and cell types. Correct presentation of antigens by antigen-presenting cells (APCs) is a critical step necessary to initiate an immune response. APCs have the ability to take-up and process antigens, and express high levels of co-stimulatory and major histocompatibility complex (MHC) molecules bound to antigens (1).

Dendritic cells (DCs) are innate immune cells first characterized and reported by Ralph Steinman in 1973 (2). For their unique properties and features, DCs are the most important APCs acting at the interface of innate and adaptive immunity, which results in the activation of immune responses in the body. Distinct subsets of DCs are associated with lineage and receptor expression patterns (3).

Dendritic cells have different roles in the immune system, such as activation and regulation of adaptive immune responses, and other opposing functions in the induction of tolerance and anergy (4). During immune responses, DCs are crucial decision makers toward the development of naïve T cells to T helper type 1 (Th1) or type (Th2) profile (5).

Among the different families of molecules expressed by DC to aid in their function, one of them is the family of toll-like receptors (TLR). TLR which are expressed by different types of DCs, and bind to common molecules associated with pathogens. Once bound, molecules such as bacterial lipopolysaccharide and hypomethylated CpG DNA, can induce activation of biochemical cell pathways, resulting in overexpression of MHC, co-stimulatory molecules (CD80, CD86), and cytokines (6).

In this context, a number of methods have been available to manipulate DCs from diverse sites in the body resulting in activated cells for therapy. These methods include reinfusion of unloaded DCs; reinfusion of DCs co-cultured with peptides or proteins of interest; *in vivo* DC loading; DC transfection with antigen-encoding viruses or nucleic acids; and DC-derived exosomes (7, 8). After this, DCs might be ready to promote protection or treat specific diseases.

In this context, the availability of methods to manipulate DCs in laboratory, arise as an important tool for immunointerventions in different diseases. In this opinion article, we focused on the basis of DC approaches already available in the field of cancer currently in test for infectious diseases, and future interventions that are needed.

DENDRITIC CELL APPROACHES FOR CANCER

Since initial tests with murine models, activated DCs have been an attractive alternative to treat cancer as an immunostimulatory vaccine. This vaccine has the ability to induce effective cancer immunity by inducing Th1 cells and specific cytotoxic T lymphocytes to tumor antigens, as well as natural killer (NK) cells (9, 10). The potential of anti-cancer vaccines also lies on their capacity to stimulate long-lasting memory T cells against tumor antigens. Among the subsets of memory T cells, the presence of central memory (T_{cm}) cells has been associated with a better antitumor function than effector memory cells (11).

The first attempt of vaccination was performed with DCs derived from patients with non-Hodgkin's lymphoma who have failed current treatment. Immunoglobulin idiotype from the patient's tumor were used to load DCs *ex vivo* and then were reinjected into the patient's body – what resulted in the complete remission of the tumor (12).

To date, many clinical assays employing different methods to activate DCs have been in test for different types of cancers. Most trials were performed using autologous DCs pulsed *ex vivo* with tumor antigens or derived peptides, and administered to patients with or without chemotherapy or other immune agent (13). However, other types of interventions are in course in clinical trials, such as those using DCs engineered to express tumor antigens with or without molecules such as CD40 ligand, CD70, and TLR-4 (14, 15). Important results were shown in one trial performed by Tel et al. (16), who reported a strong immune-specific response against melanoma after administration of a particular subset of DCs, called plasmacytoid-DCs (pDCs) pulsed with melanoma specific antigens. pDCs have been seen as interesting players in this task, since once properly activated they are able to produce high levels of gamma-interferon (IFN-γ) and elicit a robust Th1 immune response.

Most clinical assays have used *ex vivo* manipulation of patient's peripheral blood monocytes cultured in the presence of interleukin (IL)-4 and recombinant granulocyte macrophage-colony stimulating factor (GM-CSF) to achieve DCs for therapy (17). In this way, a DC-based preparation of autologous cells expanded *ex vivo* in the presence of a prostatic acid phosphatase/GM-CSF fusion protein (sipuleucel-T, Provenge®) was approved by the US FDA and other international regulatory agencies for use in patients with advanced metastatic prostate

cancer (18). From trials initiated in 2012, sipuleucel-T is involved in at least seven trials against prostate cancer, combining sipuleucel-T with: different regimens of radiotherapy (19); administration of monoclonal antibody against cytotoxic T lymphocyte-associated protein 4 (CTLA-4) (20); administration of recombinant human IL-7 (21); and injection of DNA-based anti-cancer vaccine together with GM-CSF (22). Thus, it is expected that further results with sipuleucel-T will be disclosed in the next years.

However, *ex vivo* manipulation of DCs are limited by some factors, such as the high cost, the long time needed to handled in laboratory, and ultimately the high risk of infection to the patients (23, 24). The latter issue is clearly one of the most important, since cancer patients might be already immunocompromised and susceptible to diverse pathogen infections.

To overcome this issue, searching for new alternatives to *ex vivo* manipulation are in course, and many of them are being developed, such as activation and loading DCs with antigens *in vivo*. One good example is the use of specific peptides combined with GM-CSF to attract and activate DCs *in vivo*, which showed prospective clinical results (25). Other strategy is the use of cancer cells genetically modified to express GM-CSF, resulting in the attraction and activation of DCs (26). Another tactic is the delivery of oncolytic viruses, which preferentially infect and kill cancer cells (27).

One of the most promising approaches is the *in vivo* targeting of specific DC receptors using antibodies coupled with antigens (28, 29). It was verified that administration of this type of vaccine with DC activators such as TLR3, TLR-7-8, and CD40 agonists allows the establishment of immunity in diverse diseases settings, including infections [e.g., malaria and human immunodeficiency virus (HIV)] and cancer (30, 31).

Although prospective results are disclosed and expected, most clinical trials fail to go beyond Phase II due to a reduced success rate. This indicates that more studies are needed to fill gaps in the comprehension of the immune response necessary to eliminate cancer and explore this knowledge in DC cancer vaccines, such as the use of TLR agonists and the particular role of each DC subset. In parallel, work groups are dedicating efforts to identify better correlates of clinical efficacy to evaluate results from clinical trials more properly.

DENDRITIC CELL APPROACHES FOR INFECTIOUS DISEASES

Dendritic cell manipulation offers an interesting approach to fight against infectious diseases, and an alternative to prompt a protective immunity, since some treatments are ineffective or inexistent in those (32, 33). Previous studies have shown that DCs can induce protection against different pathogens, including protozoan, bacteria, and virus. DCs recognize microorganisms through TLR or C-type lectin receptors (34, 35). Vaccination works have reported protection against leishmaniasis (36, 37), Herpes simplex virus (38, 39), influenza virus (40), and *Candida albicans* (41), among other pathogens, such as HIV.

Human immunodeficiency virus has different mechanisms of evasion from the immune system, and nowadays the main source of treatment to infected patients is to follow combination antiretroviral therapy (cART) for life. However, attention was drawn to promising results obtained by the use of DC-based vaccine against HIV. Lu et al. (42) performed the first success clinical trial described, and found a significant reduction in plasma viral load (VL) after administration of autologous DCs loaded with inactivated autologous virus in HIV-1 infected patients. At least 12 studies have achieved interesting results, and evolved to clinical trials with HIV-1 infected patients and reported HIV-1 specific-immunological responses (43). Recently, García et al. (44) observed a significant decrease in VL in HIV-1 chronic infected patients who have interrupted cART treated with autologous monocyte-derived DCs pulsed with autologous heat-inactivated whole HIV. Previously, García et al. (45) also showed promising results with significant drop in VL in HIV-1 infected patients off-cART treated with the same vaccine preparation. Based on this, it is expected that in the next few years good results will be achieved, enhancing the chances to develop an immunointervention that could help infected individuals.

Although now it is possible to target vaccine antigens to DCs in T and B areas and to modulate their function with adjuvants, there is still no currently approved DC therapy for infectious diseases, and most experimental approaches are especially with animal models (46). One good example is leishmaniasis, which is one of the most important neglected diseases that cause deaths and morbidity in more than 88 countries. Current human anti-leishmania vaccines available are limited by their inefficiency to confer protection against the different species and also by their safety, which is contested. DCs approaches for leishmaniasis were proposed by different groups of research with remarkable results showing low levels of parasite burden and high levels of Th1 cytokines in animals treated (47, 48). However, results from studies with animal models might be difficult to translate the results to humans, and it will remain a goal for further investigations. DCs therapy for leishmaniasis and other infectious diseases would aid mainly refractory patients to current treatments due to high toxic drugs that are available for use or the increasing number of resistant pathogens. Furthermore, immunocompromised individuals, such as those with AIDS or grafted, would be benefited by more safety and effective treatments against different pathogens.

CONCLUSION

In the last couple of years, DC therapies approaches have been shown to be feasible and secure. Successful results were and are being obtained with cancer patients and animal models. DCs have an extraordinary capacity to orchestrate the host's immune response, which offers new perspectives for the development of vaccines and immunotherapeutic strategies against cancer and infectious diseases among others. However, due to the success that is been observed with cancer and also due to the efforts that is being put by many research groups in the development of antigens and adjuvants with good immunological stimulatory capacities, we believe that in a closer future DC therapies will be also a viable approach to treat and/or prevent infectious diseases.

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Received: 04 February 2014; accepted: 13 February 2014; published online: 26 February 2014.
Citation: Freitas-Silva R, Brelaz-de-Castro MC and Pereira VR (2014) Dendritic cell-based approaches in the fight against diseases. Front. Immunol. 5:78. doi: 10.3389/fimmu.2014.00078
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology.

APÊNDICE V – Artigo publicado: *Targeting dendritic cells as a good alternative to combat Leishmania* spp.

frontiers in IMMUNOLOGY

OPINION ARTICLE
published: 26 November 2014
doi: 10.3389/fimmu.2014.00604

Targeting dendritic cells as a good alternative to combat *Leishmania* spp.

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Keywords: dendritic cells, vaccine, neglected diseases, leishmaniasis

LEISHMANIASIS: GLOBAL BURDEN, CLINICAL FORMS, AND CURRENT STRATEGIES OF CONTROL

Leishmaniasis is an important group of neglected diseases caused by more than 20 spp. of protozoan from the genus *Leishmania*. It is transmitted by sandfly bite (1), and impacts populations by inducing disfiguration, loss of productivity, and a burden estimated at 2,357.000 disability-adjusted life years (DALY) (2). Ninety-eight countries have reported cases of leishmaniasis, and over 350 million people are living at risk, with 0.2–0.4 and 0.7–1.2 million cases of VL and CL annually, respectively (3). Three main clinical forms are known: visceral (VL, more lethal, e.g., *L. donovani* and *L. infantum*), cutaneous (CL, more common, e.g., *L. major*), and mucocutaneous (MCL). Strategies to limit these diseases are controlling the vectors and chemotherapy of affected individuals, but these approaches have a high cost and led to resistant parasites and vectors (4). Thus, there is an urgent need of vaccines and more effective therapies for leishmaniasis, otherwise the number of cases and resistant strains will probably continue to rise. Since they have a key capacity to initiate and maintain an immune response, dendritic cells (DCs) have been seen as an important target for the control of different diseases, such as leishmaniasis. Thus, in this article we present the principal strategies to efficiently induce activation of DCs in the context of leishmaniasis.

DENDRITIC CELLS AS THE MAIN TARGET OF VACCINES AGAINST *LEISHMANIA*

The major task for a vaccine is to correctly induce the immune system to develop a protective response against one specific pathogen. In the case of leishmaniasis, this protection comes from Th1 CD4$^+$ T cells producing IFN-γ, TNF, and IL-12, which have been associated with disease control, macrophage activation, and elimination of parasites (5). However, to initiate this response, antigen presenting cells (APCs), present in different tissues of the body, must be activated to induce a proper response to eliminate or control the parasite. DCs are highly specialized APCs of the immune system capable of priming naïve T cells, and mounting a T-cell response upon pathogen entry in the body. Distinct subsets of DCs are associated with lineages and receptor expression patterns (6), and they develop from hematopoietic stem cells stimulated with fms-like tyrosine kinase 3 ligand (Flt3L) or with granulocyte/macrophage colony-stimulating factor (GM-CSF) (7). The majority of DCs develop from myeloid precursors, whereas plasmacytoid DC (pDC) develops from lymphoid precursors and shares many features with B cells (8). To aid in their function, DCs express different toll-like receptors (TLR), which bind to common molecules associated with pathogens, and have been target for the development of new vaccine adjuvants. DCs express a large variety of receptors involved with uptake of molecules and pathogens, such as DC-SIGN (CD209) and DEC205 (CD205) (9). Immature DCs have a high endocytic capacity, which leads to pathogen or pathogen's antigens degradation, processing, and finally loading of major histocompatibility complex (MHC) molecules. Later, mature DCs lose their high capacity of endocytosis, and change efforts to up-regulate the expression of several receptors for cytokines, MHC, adhesion, and co-stimulatory molecules, such as CD80, CD86, and CD40. It is estimated that each mature DC expresses around 10^6–10^7 MHC Class II and 10^5 MHC Class I molecules, and "fix" a repertoire of peptides bounded onto MHC Class II to present to T cells (10). Eventually, DCs acquire capacity to activate a specific T-cell response against the pathogen that induced its activation (11). The last years were marked by an increase in the knowledge on the role and function of DCs in the immune system, and thus, the emergence of potential applications based on its manipulation. Starting on the field of cancer, which finally led to the US FDA approval of a DC-based vaccine against prostate cancer (Sipuleucel-T, Provenge®) (12), applications were quickly transferred to the field of infectious diseases with very encouraging clinical results against HIV (13). Although prospective results for infectious diseases were achieved, there is no DC vaccine or therapy for any infectious diseases that are currently available or in the pipeline (14, 15). Most of the results were gathered by

ex vivo manipulation of DCs, and *in vitro* assays by loading it with desired antigens.

EX VIVO ASSAYS WITH DCs

Different researches have shown interesting results with murine models vaccinated with DCs *ex vivo* loaded with leishmania lysate antigen (14, 16). These results were encouraging since they showed that animals presented low levels of parasite burden and high levels of protective cytokines from the Th1 profile after vaccination. DCs engineered to overexpress IL-12 were successfully used for both vaccination and immunotherapy of murine models with infections already established (17). Interestingly, it was also reported that different subsets of DCs may induce different responses upon activation. Vaccination of BALB/c mice with pDCs pulsed with *L. major* complete antigen showed that they were protected against subsequent infection (18). In parallel, the use of DCs pulsed with *L. donovani*-soluble antigen combined with chemotherapy with pentavalent antimonials was able to eradicate parasites from infected mice (19). Curiously, regarding viability of DCs present in the vaccine, Schnitzer et al. (20) have reported that fragments of DCs or exosomes derived from DCs that had been previously exposed to parasite lysate of *L. major,* conferred protection in susceptible mice. Although modulation of DCs activity is highly dependent on the specie of *Leishmania* causing the infection, some of these studies were performed using *L. major* that is responsible for cases of CL. Thereby, there is an outstanding importance of studies dedicated to evaluate the action of molecular defined antigens of *Leishmania* spp. in the activity of DCs as a target for vaccines. Furthermore, many antigens from the *Leishmania* spp. proteome have unknown function and could be key-molecules to induce protective immunity. The importance of using defined antigens is so expressive that DCs pulsed with peptide (154–169aa) from gp63 induced a Th1 protection in BALB/c mice infected with *L. major*, while stimulation with a second peptide (467–482aa) resulted in a Th2 shift and disease exacerbation (21). In this sense, computational immunology has been constantly increasing its value, and now diverse *in silico* approaches are available for identification of potential epitopes and antigens for vaccines, since experimental methods are difficult and time-consuming (22). In addition, the DNA sequencing techniques are getting cheaper, therefore, many parasite genome strains can be sequenced and their predicted proteomes can be assessed regarding their variability, an important feature for antigen candidates for vaccine development. Thus, sequence- and structure-based methods investigating the binding affinity of peptides to the MHC molecules Class I and II, and other parameters such as sequence diversity may aid in the search of new antigens (23). Taking advantages of these methods, Agallou et al. (24) have recently reported the construction of a multi-epitope peptide vaccine against leishmaniasis by analyzing four known proteins from *L. infantum.* In this last study, some of the proteins (histone H1 and KMP-11) have been previously tested in DCs vaccines against *L. infantum* (25, 26). Different studies for vaccines against *Leishmania* spp. have used defined molecules for classical immunization, without considering aspects of DCs activation, and maintenance of a Th1 response by memory cells. DCs have been implicated with the induction of a Th1 immune response through the production of IL-12, as seen in C57BL/6 and C3H resistant mice strains. On the other hand, BALB/c susceptible mice are more prone to produce a Th2 immune response with the presence of IL-4, IL-5, and IL-13; IL-$4^{-/-}$ BALB/c mice were capable to partially control the infection with some strains of *L. major* (27). It has been shown that IL-4 during the early phase of DC activation induces a potent Th1 response induced by IL-12 (28); and in the context of vaccination with DCs, it was reported that IL-4 might be an important adjuvant, since IL-4Rα signaling is key to promote a Th1 immune response (14). Studies have enrolled IL-10 with disease progression by means of suppression of the anti-leishmanial immune response in humans and mice (29–31). Recently, Schwarz et al. (16) have shown that T cell are the main source of IL-10 in early infection, however, BALB/c mice vaccinated with fragmented DCs that had been pulsed with *L. major* lysate and CpG oligodeoxynucleotides (CpG-ODN) were able to suppress IL-10 favoring the control of infection. One of the main concerns in the vaccine development is the use of adjuvants, in this regard, DC approaches in leishmaniasis studies have used, mainly, CpG-ODN, which is a TLR9 ligand (32). The effect of CpG-ODN is to induce DC activation and maturation, enhancing humoral responses, with Th1 indicator IgG2a antibodies, and activity of cytotoxic T lymphocytes (CTL) (33, 34). It has been shown that CpG-ODN induce the production of CXCL10, a chemokine with anti-leishmanial properties, regulation of parasitic load, and $CD4^+CD25^+$ regulatory T (Treg) cells in mice infected with *L. donovani* (35, 36). Some issues may concern this approach of *ex vivo* activation, such as the type of antigen that is used, the method itself, which is time-consuming and labor, and also the high risk of infection.

OVERALL ADVANTAGES OF *IN VIVO* ACTIVATION OF DCs

Toward a more rational and feasible DC-based vaccine against different pathogens, groups of research have endeavored to *in vivo* manipulate DCs, and thus, trying to understand the ways of induction a specific T cell response. In a very interesting work, Bonifaz et al. (37) have demonstrated that it is possible to manipulate DCs *in vivo* by directing antigen-conjugated antibodies against uptake receptors (DEC205). Other works followed the same strategy and achieved promising results for different pathogens, such as HIV, and also induction of immunity in distinct sites that are challenges for classical route immunization, such as mucosal sites (38, 39). For *L. major*, it was shown that *in vivo* targeting of DEC205 receptor of DCs with different antigen (LACK, LeIF, LmSTI1a)-conjugated antibodies can elicit a protective immune response with INF-γ and TNF-α in different mice strains (40). Therefore, based on these results collected during the last years, other uptake receptors could be a target to proper induce DCs maturation and activation. Nevertheless, as well as for other approaches, for a vaccine against *Leishmania* is not different, as there is a big gap in translating the research from murine models to humans due to environmental and genetic differences (41). Since murine models are difficult and do not always reproduce *Leishmania* infection, it might be interesting to test some of these approaches with cells from individuals affected or living in endemic regions,

like recently described for other types of vaccine (42). Another ongoing challenge to be considered is the number of DCs present *in vivo*. DCs *in vivo* are rarer than other leukocyte populations. To overcome this issue, it has been shown that using Flt3L *in vivo* through gene transfection, one can enhance the number of DCs *in vivo* (43). In this context, it is worth mentioning the importance of works designed for VL, due to its high mortality level. However, in terms of number of cases and morbidity, CL and MCL are much more expressive than VL, especially in developing nations, such as Brazil.

CONCLUSION AND PERSPECTIVES

The results of DCs-based vaccines against leishmaniasis are very encouraging; demonstrating that either *ex vivo* or *in vivo* target of DCs can elicit an effective immune response to combat *Leishmania* spp. However, a number of biological and methodological challenges should be overcome prior to the development of a DCs-based vaccine. It is possible that moving researches forward to animal, and then, clinical studies will better point out if the target of DCs is safe and effective for an anti-*Leishmania* spp. vaccine.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 17 August 2014; paper pending published: 29 October 2014; accepted: 10 November 2014; published online: 26 November 2014.
Citation: Freitas-Silva R, Brelaz-de-Castro MCA, Rezende AM and Pereira VR (2014) Targeting dendritic cells as a good alternative to combat Leishmania spp. Front. Immunol. 5:604. doi: 10.3389/fimmu.2014.00604
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology.

APÊNDICE VI – Material suplementar do capítulo 1.

Supplementary Material

Combination of *in silico* methods in the search for potential CD4$^+$ and CD8$^+$ T cell epitopes in the proteome of *Leishmania braziliensis*

Rafael de Freitas e Silva[1,2], Luiz Felipe Gomes Rebello Ferreira[3], Marcelo Zaldini Hernandes[3], Maria Edileuza Felinto de Brito[2], Beatriz Coutinho de Oliveira[2], Ailton Alvaro da Silva[2], Osvaldo Pompílio de-Melo-Neto[4], Antônio Mauro Rezende[4*¶], Valéria Rêgo Alves Pereira[2*¶]

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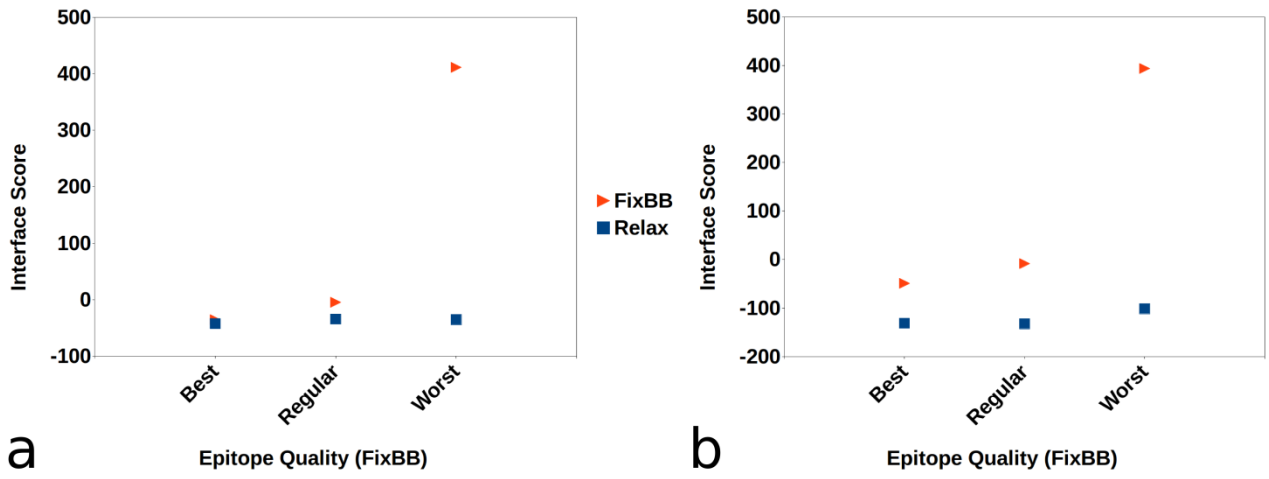
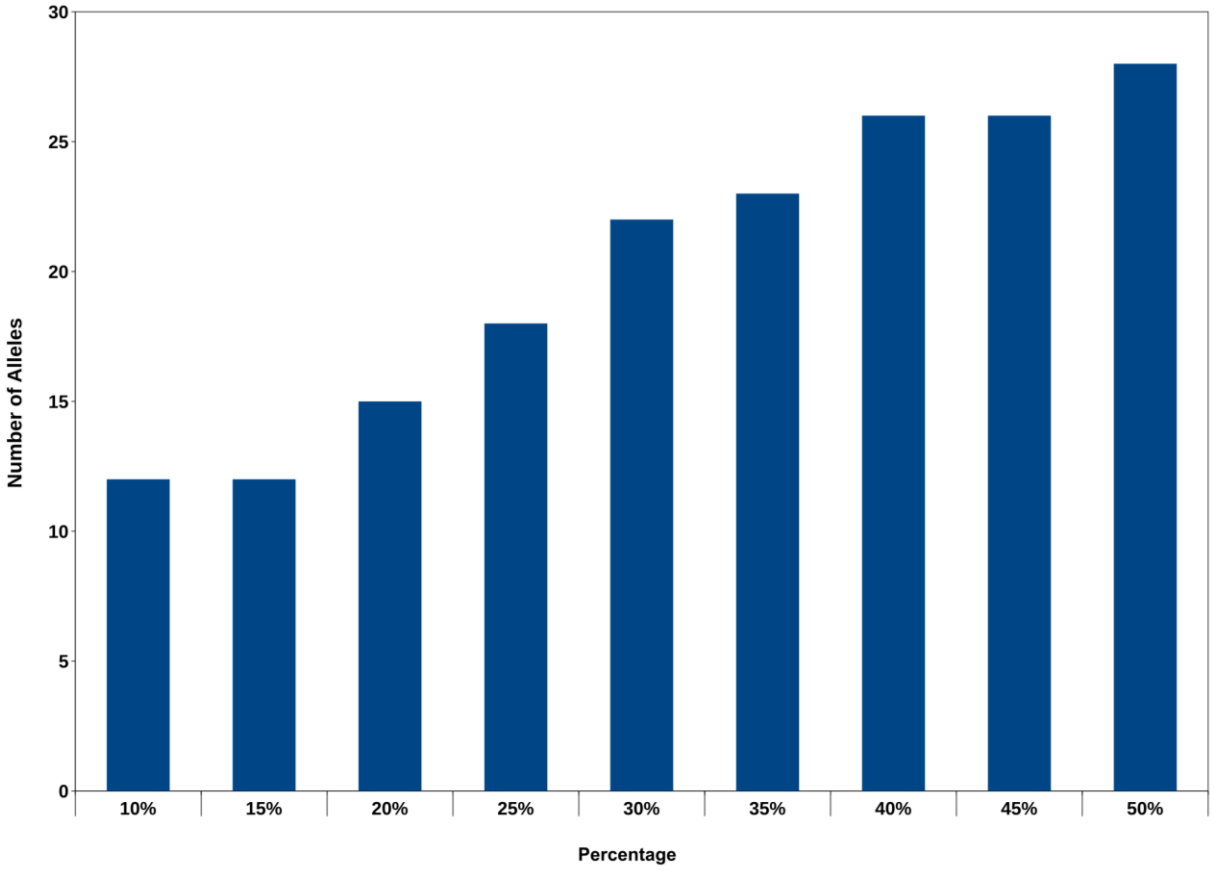
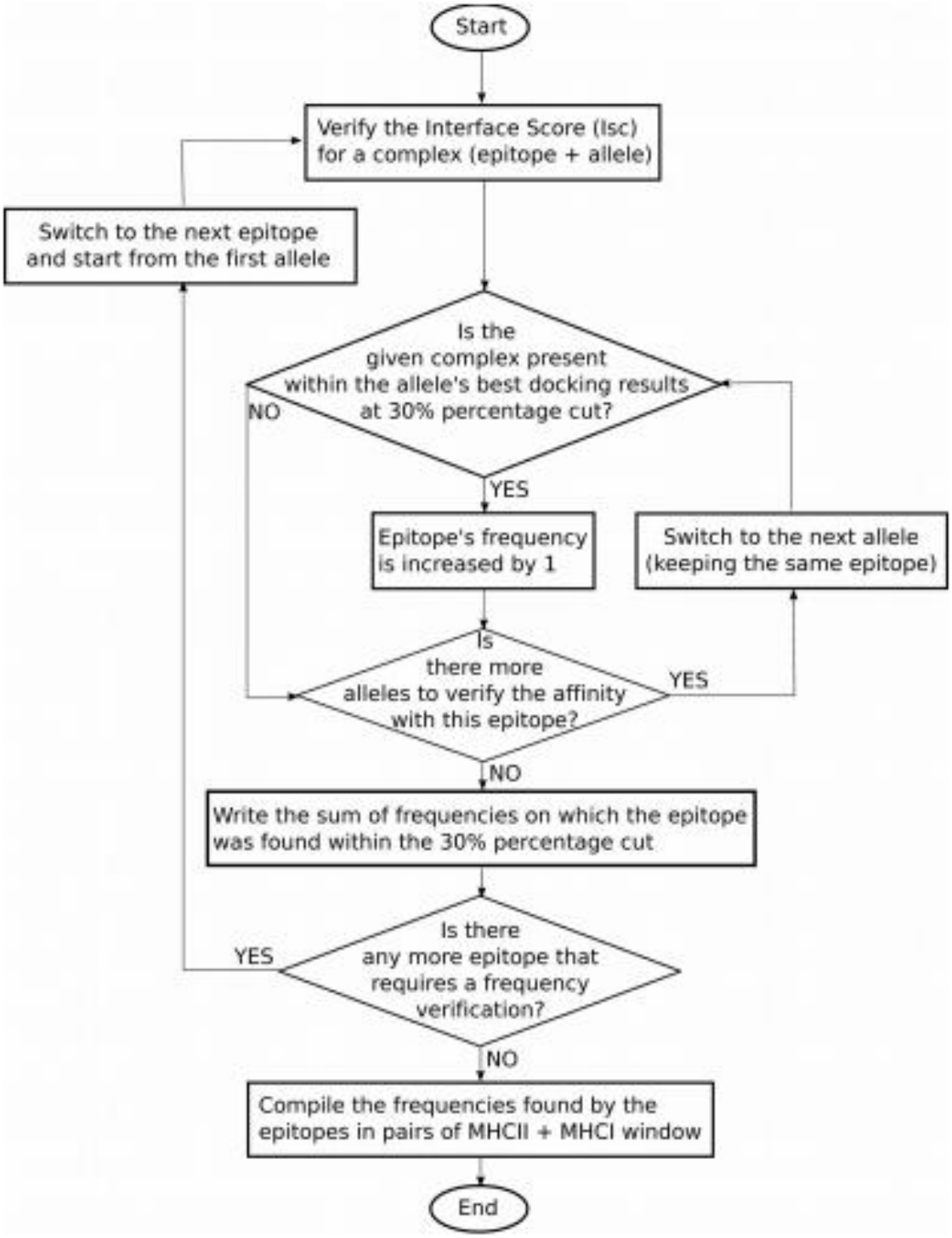


Supplementary Figure 1. Comparison of the Isc values found for the conformations obtained with FixBB (red triangles) and Relax (blue squares) protocols (Rosetta), for both MHCI (**a**) and MHCII (**b**). The predicted epitopes selected to this evaluation are classified as "best", "regular" and "worst" (see text for details).



Supplementary Figure 2. The highest sum of occurrences of the top epitope pair (MHC II + MHC I window) found for each cutoff (%).



Supplementary Figure 3. Filtering algorithm, using the example of 30% cutoff.

ANEXO I – Parecer do comitê de ética no uso de animais

Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz
Centro de Pesquisa Aggeu Magalhães

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificado de Aprovação

Certificamos que o projeto intitulado: **Avaliação de novas moléculas para tratamento e vacinação nas leishmanioses.** Protocolado sob nº 47/2013 pelo (a) pesquisador (a) Dra Valéria Rêgo Alves Pereira. Está de acordo com a Lei 11.794/2008 e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS do Centro de Pesquisas Aggeu Magalhães/Fundação Oswaldo Cruz (CEUA/CPqAM) em 27/02/2014. Na presente versão, este projeto está licenciado e tem validade até 28/fevereiro/2018.

Quantitativo de Animais Aprovados	
Espécie	**Nº de Animais**
Camundongo isogênico Balb-c macho	**1130**
Hamsters golden macho	**48**

We certify that project entitled **Avaliação de novas moléculas para tratamento e vacinação nas leishmanioses.** Protocol nº 47/2013, coordinated by Dra Valéria Rêgo Alves Pereira. Is according to the ethical principles in animal research adopted by the Brazilian law 11.794/2008 and so was approved by the Ethical Committee for Animal Research of the Centro de Pesquisas Aggeu Magalhães/Fundação Oswaldo Cruz on February, 27, 2014. In present version this project is licensed and valid until February, 28,2018.

Recife (PE, Brazil) February, 27, 2014.

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ANEXO II – Parecer do comitê de ética em pesquisa com seres humanos

Comitê de Ética em Pesquisa

Título do Projeto: "Avaliação In Vitro da Resposta Imunológica de Pacientes com Leishmaniose Utilizando Antígenos Recombinantes"
Pesquisador responsável: Valéria Rêgo Alves Pereira
Instituição onde será realizado o projeto: CPqAM/Fiocruz
Data de apresentação ao CEP: 10/01/2014
Registro no CAAE: 19252013.2.0000.5190
Número do Parecer PlatBr: 522.964

PARECER

O Comitê avaliou 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 05 de fevereiro de 2017.

Em caso de necessidade de renovação do Parecer, encaminhar relatório e atualização do projeto.

Recife, 13 de fevereiro de 2014.

Coordenadora do CEP/CPqAM

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ANEXO III – Carta de aceite do livro: *Tropical Diseases: Immunological and Molecular Tools Applied to the Epidemiology and Control*

Abstract Acceptance : eBook Entitled 'Tropical Diseases: Immunological and Molecular Tools Applied to the Epidemiology and Control' [Dr. Cavalcanti, Ref. No. 21-01-14-EBK1//EOI-35]

August 21, 2014

Dear Dr. Cavalcanti,

I am very pleased to inform you that we have received positive review reports from neutral referees and our review board for your proposed e-book entitled ***'Tropical diseases: immunological and molecular tools applied to the epidemiology and control'***

You may view the author's guideline at http://ebooks.benthamscience.com/manuscript-organization.php that will help you in the eBook preparation. I have also enclosed the ***sample chapter*** which will assist you to prepare the chapters as per our format.

Pleased note that we require all non-native English speaking contributors to have the eBook /chapter checked and certified by native English speakers for language and grammar.
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Sincerely!

Humaira Hashmi
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ANEXO IV – Carte de aceite do Artigo 1: "*Combination of in silico methods in the search for potential CD4$^+$ and CD8$^+$ T cell epitopes in the proteome of Leishmania braziliensis*"

Gmail Rafael Silva <rafa.freitass86@gmail.com>

José Mordoh via Frontiers: Provisional acceptance of your manuscript

Frontiers <noreply@frontiersin.org> Mon, Aug 15, 2016 at 6:46 PM
Reply-To: José Mordoh <jmordoh@leloir.org.ar>
To: rafael.silva@upe.br

José Mordoh has sent you a message. Please click 'Reply' to send a direct response

Dear Dr e Silva,

I am pleased to inform you that your manuscript Combination of in silico methods in the search for potential CD4+ and CD8+ T cell epitopes in the proteome of Leishmania braziliensishas been provisionally accepted for publication in Frontiers in Immunology, section Immunotherapies and Vaccines. Please note that your manuscript is in the final validation stage while the Frontiers Editorial Office is verifying that production can be initiated. During this stage, you may be contacted should issues be identified that require re-activation of the review process to resolve the concerns. Until this stage is complete your submission has not been definitely accepted. You will receive a notification of final acceptance when the manuscript has been approved.

Article type: Original Research
Title: Combination of in silico methods in the search for potential CD4+ and CD8+ T cell epitopes in the proteome of Leishmania braziliensis
Authors: Rafael de Freitas e Silva, Luiz Felipe Gomes Rebello Ferreira, Marcelo Zaldini Hernandes, Maria Edileuza Felinto De Brito, Beatriz Coutinho De Oliveira, Ailton Alvaro Da Silva, Osvaldo Pompilio De Melo Neto, Antonio Mauro Rezende, Valéria Rêgo Alves Pereira
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Thank you for submitting this paper to Frontiers.

With best regards,

José Mordoh
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