FUNDAÇÃO OSWALDO CRUZ

CENTRO DE PESQUISAS GONÇALO MONIZ

FIOCRUZ

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

TESE DE DOUTORADO

PERFIL DA RESPOSTA IMUNE CELULAR EM PACIENTES INFECTADOS PELO HIV COM LEISHMANIOSE OU TUBERCULOSE

LUANA LEANDRO GOIS

Salvador - Bahia

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LUANA LEANDRO GOIS

Tese de doutorado apresentada ao Curso de Pós-graduação em Biotecnologia em Saúde e Medicina Investigativa para a obtenção do grau de Doutor.

Orientadora: Profª Dra. Maria Fernanda Rios Grassi

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Dedico esta tese aos pacientes que aceitaram voluntariamente participar deste estudo, na esperança que de alguma forma este trabalho possa ajudá-los.

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"O sábio não é o homem que fornece as verdadeiras respostas; é o que formula as verdadeiras perguntas."

Claude Lévi-Strauss

GOIS, Luana Leandro. Perfil da resposta imune celular em pacientes infectados pelo HIV com leishmaniose ou tuberculose. 107 f. il. Tese (Doutorado) – Fundação Oswaldo Cruz, Centro de Pesquisas Gonçalo Moniz, Salvador, 2015.

RESUMO

A infecção pelo HIV promove a redução do número de linfócitos T $CD4^+$ e, consequentemente, o surgimento de doenças oportunistas. A leishmaniose visceral e a tuberculose são comumente reconhecidas como doenças oportunistas importantes e associadas ao óbito de indivíduos infectados por HIV. Ambos os patógenos, *Leishmania* e *Mycobacterium tuberculosis* (Mtb) infectam cronicamente macrófagos. A imunidade protetora associada a estas infecções envolve linfócitos Th1 produtores de IFN-γ. O prejuízo na resposta imune celular causado pelo HIV perturba a resposta imune contra estes patógenos. Não são bem determinadas quais alterações imunológicas causadas pelo HIV promovem o prejuízo na resposta imune específica contra a *Leishmania spp.* e Mtb, induzindo o desenvolvimento de formas atípicas e graves destas infecções. Deste modo, esta tese teve como objetivo descrever o perfil da resposta imune celular aos antígenos de *Leishmania spp.* ou Mtb em pacientes infectados com HIV. Para tal., foram recrutados pacientes infectados por HIV e com diagnóstico de leishmaniose (HIV/LV) e tuberculose (HIV/TB). Indivíduos não infectados por HIV e diagnóstico de leishmaniose (LV) ou tuberculose (TB) forma incluídos como controles. Foram avaliadas a linfoproliferação e a frequência das subpopulações de memória dos linfócitos T $CD4^+$ em resposta aos antígenos solúveis de *Leishmania spp.* (SLA). Igualmente, foram avaliadas a linfoproliferação, a frequência das subpopulações de memória dos linfócitos T $CD4^+$ e $CD8^+$, o perfil de funcional de linfócitos T $CD4^+$ e $CD8^+$ produtores de citocinas e a atividade citotóxica de linfócitos T $CD8^+$ e células NK em resposta ao purificado protéico derivado (PPD) do *M. bovis*. Duas revisões sistemáticas da literatura que abordam a associação entre estas infecções e a síndrome inflamatória de reconstituição imune em indivíduos infectados por HIV após terapia antiretroviral foram realizadas. Os linfócitos T $CD4^+$ e $CD8^+$ dos pacientes HIV-LV não apresentavam resposta proliferativa ao SLA e houve redução na frequência de subpopulações de linfócitos T $CD4^+$, a qual foi restaurada após o tratamento para leishmaniose visceral. Nos pacientes HIV-TB foi igualmente observada ausência de resposta proliferativa de linfócitos T $CD4^+$ e $CD8^+$ e de células produtoras de IFN-γ em resposta ao PPD. Além disso, o HIV promoveu a redução da atividade degranulativa de células NK, o que contribui para o descontrole da infecção e desenvolvimento de TB ativa. Nos pacientes HIV-TB, HAART foi capaz de induzir uma recuperação parcial de células específicas produtoras de IFN-γ, bem como da proliferação em resposta ao PPD. Em conjunto, os resultados desta tese sugerem que a infecção pelo HIV induz alterações na resposta celular de memória central e efetora contra patógenos intracelulares oportunistas. Essas alterações são parcialmente restauradas no curso da HAART.

Palavras-chaves: HIV, Coinfecção; Leishmaniose; Tuberculose; Resposta imune celular

GOIS, Luana Leandro. Profile of cellular immune response in HIV-infected patients with leishmaniasis and tuberculosis. 107 f. il. Tese (Doutorado) – Fundação Oswaldo Cruz, Centro de Pesquisas Gonçalo Moniz, Salvador, 2015.

ABSTRACT

The HIV-infection promotes reduced number of $CD4^+$ T-lymphocytes and manifestation of opportunistic diseases. Visceral leishmaniasis and tuberculosis are commonly known as main opportunistic infections and are associated with mortality in HIV-infected individuals. Both pathogens, *Leishmania* and *Mycobacterium tuberculosis* (Mtb), infect macrophages. The protect immune response involve T-lymphocytes help 1 (Th1) and producing of IFN-γ. The impairment of cellular immune response caused by HIV disrupts the immune response against these pathogens. It is unclear which immunological alterations caused by HIV infection promote the damage in specific cellular immune response against *Leishmania* and Mtb and induces the development of atypical and severe forms. Thus, this thesis aimed to describe the profile of the cellular immune response to *Leishmania* antigens or Mtb in HIV infected patients. To this end, were recruited HIV infected patients with visceral leishmaniasis (HIV/VL) and HIV infected patients with active tuberculosis (HIV/TB). Moreover, HIV uninfected individuals with VL or TB were also included as controls. Lymphoproliferation and frequency of memory $CD4^+$ T-lymphocyte subsets in response to soluble *Leishmania* antigen (SLA) were evaluated. Also were evaluated lymphoproliferation, frequency of memory $CD4^+$ and $CD8^+$ T-lymphocyte subsets, functional profile of cytokines producing $CD4^+$ and $CD8^+$ T-lymphocytes and cytotoxic activity of $CD8^+$ T-lymphocytes and NK cells in response to purified protein derivative (PPD) of *M. bovis*. Two systematic reviews of the literature concerning the association between leishmaniasis and tuberculosis with the inflammatory immune reconstitution syndrome (IRIS) in HIV-infected individuals after antiretroviral therapy were done. The absence of proliferative response to SLA of $CD4^+$ and $CD8^+$ T-lymphocytes and the reduced frequency of memory $CD4^+$ T-lymphocyte subsets were observed in HIV/VL patients. The frequency of memory $CD4^+$ T-lymphocyte subset was restored after treatment for visceral leishmaniasis. In HIV/TB patients was also observed absence of proliferative response to PPD of $CD4^+$ and $CD8^+$ T-lymphocytes and of PPD-specific IFN-γ producing cells. In addition, HIV infection promotes the reduction in degranulative activity of NK cells which contributes to the survival of Mtb into macrophages and development of active TB. In HIV/TB patients, HAART was able to induce a partial recovery of PPD-specific IFN-γ producing cells and of lymphoproliferation in response to PPD. Taken together, the results suggest HIV infection induces changes in cellular immune response of central and effector memory against opportunistic intracellular pathogens. These alterations are partially restored in the after HAART.

Keywords: HIV, Co-infection; Leishmaniasis; Tuberculosis; Cellular immune response

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LISTA DE ABREVIATURAS

AIDS	*Acquired immunedeficiency syndrome* (Síndrome da imunodeficiência adquirida)
APC	Aloficocianina
APC-Cy7	Aloficocianina-Cy7
BAAR	Bacilo ácido-álcool resistente
BSA	Albumina sérica bovina
CBA	*Cytometric bead array* (ensaio com esfera de citometria)
CEP	Comitê de ética em pesquisa
CFSE	Carboxifluoresceína succinidil ester
CM	Linfócitos T $CD4^+$ de memória central
CPqGM	Centro de Pesquisa Gonçalo Moniz
CTL	Linfócitos T citotóxicos
DC	Células dendríticas
EM	Linfócitos T de memória efetora
FIOCRUZ	Fundação Oswaldo Cruz
FITC	Isotiocianato de fluoresceína
FSC-A	*Forward Scatter* (Dispersão para frente - parâmetro correspondente ao tamanho celular)
HAART	Highly active antiretroviral therapy (Terapia antiretroviral altamente ativa)
HCM	Hospital Couto Maia
HEOM	Hospital Especializado Otávio Mangabeira
HIV-1	Vírus da imunodeficiência do tipo 1
HIV-LV	Pacientes infectados por HIV com diagnóstico de leishmaniose visceral
HIV-TB	pacientes infectados por HIV com diagnóstico de tuberculose
HUPES	Hospital Universitário Professor Edgar Santos
IBIT	Instituto Brasileiro para Investigação da Tuberculose
ID	Índice de divisão
IFN-γ	Interferon-gama
IL	Interleucina
IQR	Intervalo interquartil
IRIS	Síndrome inflamatória de reconstituição imune

LT	Leishmaniose tegumentar
LV	Leishmaniose visceral
mL	mililitros
mm^3	metro cúbico
Mtb	*Mycobacterium tuberculosis*
NA	Não se aplica
ND	Não determinado
NK	Células *Natural Killers*
º C	Graus Celsius
OMS	Organização Mundial da Saúde
PBMC	Células mononucleares do sangue periférico
PBS	Tampão fosfato salino
PE	Ficoeritrina
PE-Cy5	Ficoeritrina-Cy5
PE-Cy7	Ficoeritrina-Cy7
PHA	Fitohemagutinina
PPD	Purificado protéico derivado de *Mycobacterium bovis*
SFB	Soro fetal bovino
SSC-A	*Side Scatter* (Dispersão para o lado - parâmetro correspondente ao granulosidade celular)
TB	Tuberculose
TB-IRIS	Tuberculose associada à Síndrome inflamatória de reconstituição imune
TCLE	Termo de consentimento livre e esclarecido
Th1	Linfócitos T $CD4^+$ *helper* (auxiliar) do tipo 1
Th2	Linfócitos T $CD4^+$ *helper* (auxiliar) do tipo 2
TLR	Receptores semelhantes ao *Toll*
TNF	Fator de necrose tumoral
Treg	Linfócitos T regulatórios
µg/mL	Micrograma por mililitro
µL	Microlitro

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1 REVISÃO DE LITERATURA

1.1 O VÍRUS DA IMUNODEFICIÊNCIA HUMANA E A SÍNDROME DA IMUNODECIÊNCIA ADQURIDA

Segundo a Organização Mundial da Saúde (OMS, 2014) a síndrome da imunodeficiência adquirida (AIDS), causada pelo vírus da imunodeficiência humana tipo 1 (HIV-1), atinge cerca de 37 milhões de pessoas em todo o mundo. A epidemia da AIDS se estabilizou nas últimas décadas, tendo em vista a redução na incidência e na mortalidade relacionada à AIDS. Em 2012, foram estimados 2,6 milhões de novos casos de infecção por HIV, o que representa uma redução de 20% no número de casos novos em relação a 2001. A mortalidade por causas relacionadas à AIDS tem diminuído desde o final dos anos 1990, devido à terapia antiretroviral altamente ativa (HAART). Em 2012, 1,7 milhões de pessoas foram a óbito por causas relacionadas à AIDS, o que representa uma queda de 24 % na mortalidade em relação a 2005 (UNAIDS, 2012). Atualmente, no Brasil, a HAART é recomendada para todos os pacientes infectados pelo HIV-1, independente da contagem de linfócitos T $CD4^+$ (BRASIL, 2014). Apesar deste quadro de redução, a epidemia continua crescendo e atingindo populações mais vulneráveis que não têm acesso aos cuidados médicos, sobretudo nos países em via de desenvolvimento. Mais de 95% das infecções causadas pelo HIV-1 concentram-se em países em desenvolvimento e 68% do total ocorrem na África subsaariana (UNAIDS, 2010).

O HIV-1 é um retrovírus envelopado que pertence à família Retroviridae, gênero *Lentivirus* (BARRE-SINOUSSI et al., 1983; POPOVIC et al., 1984). O HIV-1 infecta células que expressam a molécula CD4, como os linfócitos T, monócitos/macrófagos e células dendríticas. A infecção causada pelo HIV-1 é caracterizada pela perda progressiva de linfócitos T $CD4^+$, que conduz ao surgimento de infecções oportunistas (CLERICI et al., 1989).

O curso clínico de infecção pelo HIV-1 inclui três estágios: infecção primária, latência clínica e AIDS. Nas primeiras semanas após a infecção, ocorre o aparecimento de sintomas agudos e inespecíficos, com extensiva viremia e elevado número de linfócitos T $CD4^+$ infectados. A produção de anticorpos específicos contra o HIV-1 e

ativação de linfócitos T $CD8^+$ citotóxicos (CTL) efetores conseguem conter a viremia inicial do HIV-1, conduzindo para uma fase de latência clínica que é caracterizada pela presença de uma carga viral baixa e reduzido número de células infectadas. Durante a latência clínica, que varia de 8 a 10 anos, continua a ocorrer replicação viral, sobretudo nos tecidos linfóides. Gradualmente ocorre a redução do número de linfócitos T $CD4^+$ concomitante ao aumento da carga viral que conduz ao estabelecimento da AIDS (COFFIN, 1995).

A contínua replicação viral estimula persistentemente o sistema imune desregulando a resposta efetora e a produção de citocinas contra os antígenos, o que em conjunto com a diminuição de linfócitos T $CD4^+$, resulta na redução da capacidade funcional da resposta imune induzindo um estado de imunossupressão grave (FAUCI et al., 1996; PANTALEO; FAUCI, 1996).

1.2 AIDS E DOENÇAS OPORTUNISTAS

1.2.1 Co-infecção entre HIV e *Leishmania*

A leishmaniose é uma doença causada por protozoários do gênero *Leishmania* que afeta várias espécies de mamíferos, incluindo o homem. As formas promastigotas flageladas da *Leishmania* são transmitidas pela fêmea de insetos flebotomíneos da família Psychodidae (*Phlebotomes* e *Lutzomyias*). A leishmaniose é endêmica em 98 países, sendo considerada uma das principais endemias em grande parte do continente americano, asiático, europeu e africano. Cerca de 12 milhões de pessoas são afetadas pela leishmaniose no mundo. Estima-se que, por ano, apareçam mais de 58.000 casos são de leishmaniose visceral (LV) e 220.000 casos de leishmaniose tegumentar (LT) (ALVAR et al., 2012). Recentes modificações nos fatores de risco de transmissão da leishmaniose contribuem para o aumento do número de casos, como por exemplo, a continua urbanização e a coinfecção com HIV-1 (DESJEUX, 2001; LYONS et al., 2003).

A *Leishmania* causa um amplo espectro de apresentações clínicas que incluem leishmaniose tegumentar (LT) e leishmaniose visceral (LV). Nas LT pode ocorrer desde

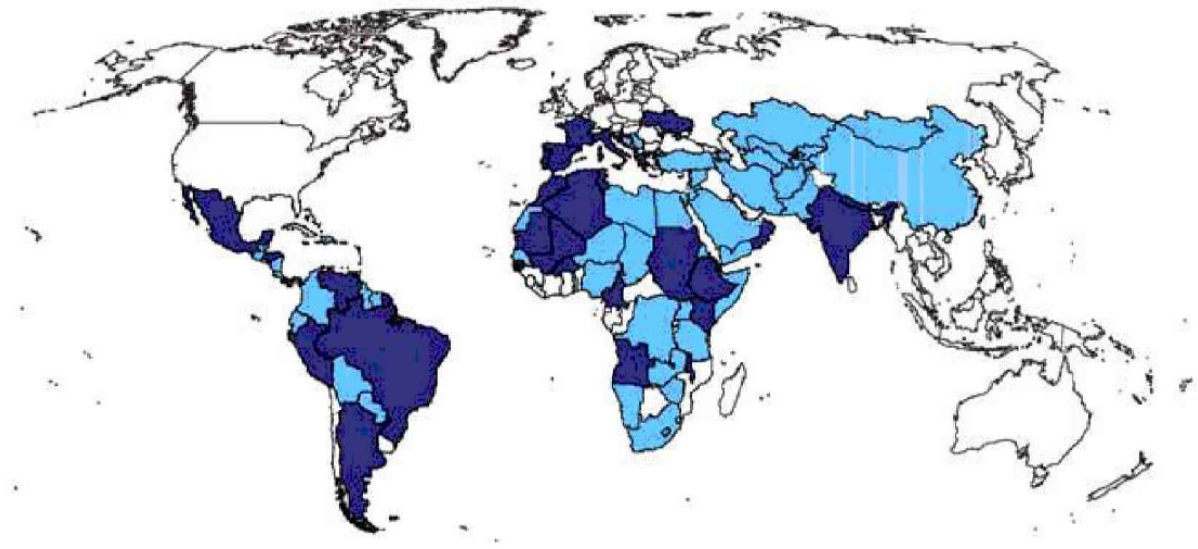
uma lesão cutânea que cura espontaneamente, lesões cutâneas localizadas ou disseminadas a lesões mucosas ou difusas. As apresentações clínicas de leishmaniose são resultado da associação entre as características do parasita e a resposta imune montada pelo hospedeiro. A imunidade celular e a produção de citocinas são os principais mecanismos de defesa contra a *Leishmania* (PEARSON; SOUSA, 1996). Uma resposta de linfócitos T *helper* 1 (Th1) produtora de interferon-gama (IFN-γ) é capaz de ativar macrófagos infectados. Nestes, as espécies reativas de oxigênio (H_2O_2) e os intermediários de nitrogênio (NO) são produzidos, o que resulta na morte intracelular da *Leishmania* (MURRAY et al., 1983).

As infecções subclínicas de *Leishmania*, que geralmente curam espontaneamente, estão associadas ao desenvolvimento de uma resposta imune celular tipo Th1 eficaz e bem regulada (BADARÓ et al., 1986). Pacientes com leishmaniose cutânea apresentam uma ou múltiplas lesões ulceradas e possuem uma resposta celular com produção elevada de IFN-γ e fator de necrose tumoral-alfa (TNF-α) e reduzida de interleucina-5 (IL-5) e IL-10 (BACELLAR et al., 2002). Pacientes com leishmaniose mucosa apresentam lesões na mucosa nasal e orofaríngea com intenso processo inflamatório que pode levar a destruição do septo nasal e aparecimento de lesões desfigurantes. Estes pacientes desenvolvem uma resposta Th1 exacerbada com baixas concentrações de IL-10 e fator de transformação e crescimento-beta (TGF-β) (BACELLAR et al., 2002; FARIA et al., 2005). As formas mais graves da infecção por *Leishmania*, como a cutânea difusa e a visceral, são associadas a um grande número de parasitas e a uma menor resposta Th1.

A LV é uma forma grave da doença, também conhecida como Kalazar, que se não tratada pode levar a morte. É caracterizada por hepatoesplenomegalia, febre, perda de peso e anemia. A LV é caracterizada pela anergia dos linfócitos, predominância de resposta Th2 e consequente incapacidade de ativar macrófagos (CARVALHO et al., 1988; ZWINGENBERGER et al., 1990; ATTA et al., 1998).

A coinfecção entre o HIV-1 e *Leishmania spp.* foi descrita em 35 países (DESJEUX; ALVAR, 2003). O número de casos de coinfecção aumentou a partir 1985 devido à sobreposição da distribuição geográfica dos casos de leishmaniose visceral e de AIDS (CRUZ et al., 2006). A coexistência das duas epidemias é resultado da disseminação da epidemia da AIDS para áreas rurais e da leishmaniose para áreas periurbanas (DESJEUX; ALVAR, 2003) (Figura 1).

Figura 1. Distribuição mundial da coinfecção por HIV/*Leishmania*



Distribuição mundial da leishmaniose (■) os países que reportaram coinfecção por HIV/Leishmania (■), 2001.

Fonte: Adaptado de Desjeux e Alvar, 2003.

Na Europa, 70 % da LV em adultos estão relacionadas com a infecção pelo HIV-1. Entre os casos europeus, 90 % são relatados na França, Itália, Portugal e Espanha. É provável que os números sejam ainda maiores em vários países da África e Ásia, que possuem falha no diagnóstico e deficiências no sistema de informação. Nas Américas, a maioria dos relatos de coinfecção tem origem no Brasil. Neste país, 37 % dos casos relatados de coinfecção por HIV-1/*Leishmania spp.* apresentaram a LV e 63 % a LT.A maioria das manifestações de LT é da forma mucocutânea ou mucosa (43 %) (RABELLO et al., 2003). A sobreposição da infecção por HIV-1 e pela *Leishmania spp.* ocorre em diversas áreas no Brasil. Nos últimos anos, as áreas de risco para a coinfecção por HIV-1/*Leishmania spp.* tem se ampliado devido às modificações no perfil epidemiológico da infecção pelo HIV, como o aumento da prevalência de AIDS em homens heterossexuais, em mulheres, na camada da população com menores níveis socioeconômicos e nas cidades de médio e pequeno porte, no interior do país.

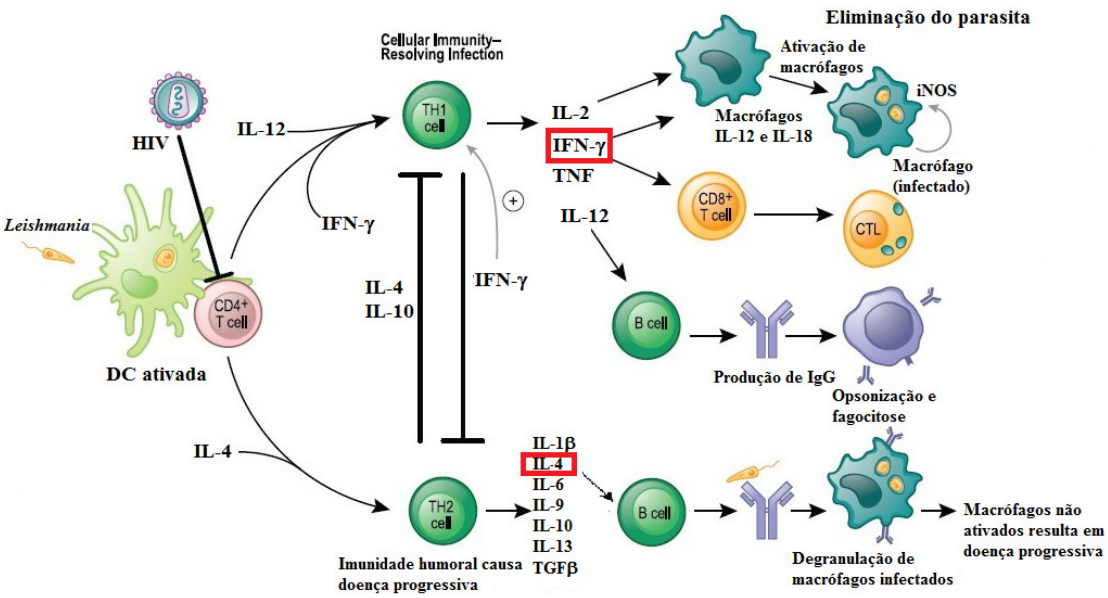
Em indivíduos coinfectados por HIV-1/*Leishmania spp.* é descrita a disseminação do parasita via sistema retículo endotelial, localizações atípicas como consequência da disseminação parasitária, deficiência na imunidade celular específica, elevadas taxas de recidiva e menor resposta terapêutica (CRUZ et al., 2006).

Os macrófagos têm sido reconhecidos como células importantes na patogênese da coinfecção por HIV-1/*Leishmania spp.*, pois são alvos primários do vírus e o local de infecção e multiplicação da *Leishmania spp.* (DALGLEISH et al., 1984; MELTZER et al., 1990; OLIVIER et al., 2003). A presença de ambos os microrganismos no mesmo tipo celular pode promover o descontrole infecção pela *Leishmania spp.* (TREMBLAY et al., 1996) e, por outro lado, induzir o aumento da replicação do HIV-1 (FOLKS et al., 1989; BERNIER et al., 1995).

A imunossupressão causada pelo HIV-1 prejudica a resposta celular específica. Nos pacientes coinfectados por HIV-1/*Leishmania spp.* que apresentam LV ocorre um aumento de citocinas Th2 (IL-4 e IL-10) quando comparado com pacientes com apenas LV (CACOPARDO et al., 1996; NIGRO et al., 1999). Estas alterações imunológicas podem estar correlacionadas à redução dos linfócitos T $CD4^+$ e a perda da capacidade dos linfócitos de reconhecer antígenos de *Leishmania spp.* e de estimular linfócitos B (MEDRANO et al., 1998; CRUZ et al., 2006). Possivelmente, a funcionalidade das células de memória também é afetada pela coinfecção por HIV-1/*Leishmania spp.*, pois tem sido relatada a perda da resposta positiva a reação de hipersensibilidade tardia (DTH) para antígenos de *Leishmania* em pacientes co-infectados com LT e LV (BADARÓ, 1997) (Figure 2).

Pacientes coinfectados por HIV-1/*Leishmania spp.* com LT apresentam quadros clássicos, manifestações atípicas, com o aumento de lesões cutâneas disseminadas e mucosas (LINDOSO et al., 2009). Nestes pacientes foi observada redução do número de linfócitos T de memória, ausência de linfoproliferação (DA-CRUZ et al., 1992; GOIS et al., 2014) e menores níveis de IFN-γ (RODRIGUES et al., 2011) em resposta aos antígenos de *Leishmania*. A inibição da resposta proliferativa e a ausência da produção do IFN-γ podem favorecer a disseminação do parasita para locais atípicos e contribuir apresentações clínicas disseminadas e de maior gravidade (WOLDAY et al., 1994; OLIVIER et al., 2003). Tendo em vista a redução na reposta Th1 presente em pacientes coinfectados por HIV-1/*Leishmania ssp.*, ainda não estão esclarecidos os mecanismos imunológicos envolvidos nas lesões de LT, principalmente os relacionados com dano mucoso.

Figura 2. Resposta imune na coinfecção por HIV/*Leishmania*



Fonte: Ezra e colaboradores, 2010

A introdução da HAART modificou a história natural da infecção e das doenças oportunistas (MOCROFT et al., 1998; KAPLAN et al., 2000). O número de casos de coinfecção por HIV-1/*Leishmania ssp.*nos países da Europa diminuiu (LOPEZ-VELEZ et al., 2001). Contudo, por causa do aumento da sobreposição das duas epidemias, a coinfecção passou a atingir os países que são os maiores focos endêmicos da leishmaniose. Os pacientes coinfectados por HIV-1/*Leishmania spp.* que recebem a terapia HAART apresentaram melhor taxa de sobrevivência e redução do risco de recidivas (CASADO et al., 2001; PINTADO et al., 2001). É provável que a restauração da resposta imune e a diminuição da carga viral possibilitem o melhor controle da infecção pela *Leishmania spp.*(LOPEZ-VELEZ et al., 2001). Entretanto, foi observado que entre 38 a 70 % dos pacientes coinfectados por HIV-1/*Leishmania spp.* recidivam em 24 meses após o tratamento anti-*Leishmania spp.* independente do status de HAART, da contagem de linfócitos T $CD4^+$ e da carga viral (CASADO et al., 2001; LOPEZ-VELEZ, 2003).

Os mecanismos imunopatogênicos pelos quais a *Leishmania spp.* e o HIV-1 interagem ainda não estão bem estabelecidos. Assim como, não está definido de que forma a infecção pelo HIV-1 compromete a resposta imune específica à *Leishmania ssp.*nos pacientes com LV. Desta forma, compreender as alterações imunológicas

encontradas nos pacientes coinfectados por HIV-1/*Leishmania ssp.*é importante para identificar estratégias de tratamento para reduzir a morbidade relacionada à leishmaniose nestes indivíduos.

1.2.2 Co-infecção HIV e *Mycobacterium tuberculosis*

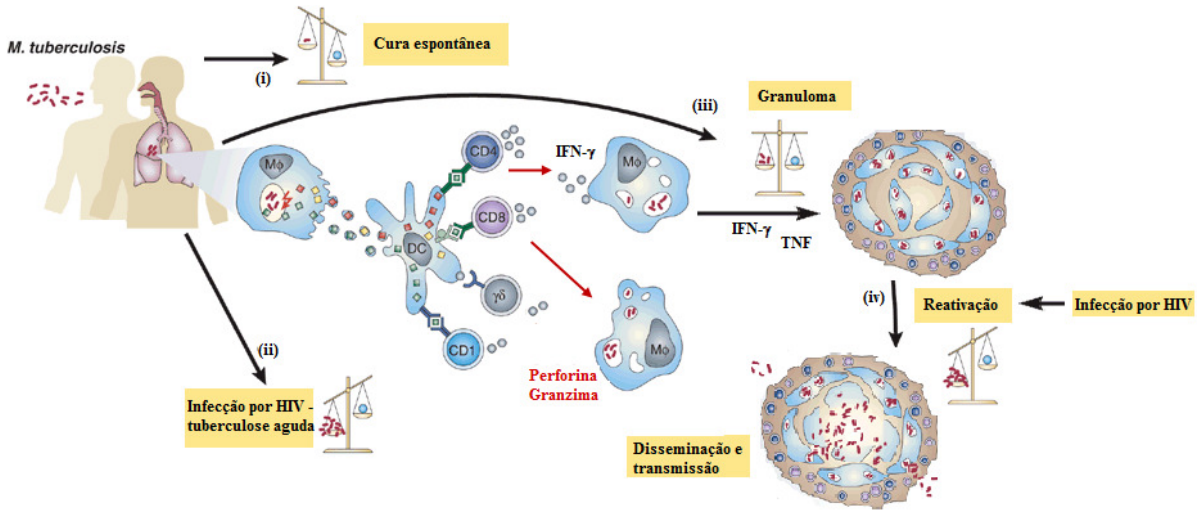
Segundo a OMS, a tuberculose (TB) é um grave problema da saúde pública. A cada ano surgem nove milhões de novos casos de TB e cerca de dois milhões de pessoas evoluem para o óbito (OMS, 2013). O Brasil encontra-se entre os 22 países que concentram 80 % dos casos de TB no mundo. Estes casos ocorrem principalmente em populações com baixa renda e escolaridade. Na Bahia, em 2012, foram diagnosticados 6.126 novos casos de TB. Especificamente, em Salvador, que é a quinta cidade com a maior incidência de TB no Brasil, foram confirmados 2.383 casos (BRASIL, 2014).

A infecção pelo *Mycobacterium tuberculosis* (Mtb) ocorre quando os bacilos são inalados pelo hospedeiro em aerossóis e conseguem alcançar os alvéolos pulmonares. Os macrófagos alveolares são as células-alvo principais para o Mtb. Os bacilos são reconhecidos pelas células da imunidade inata através de receptores *toll-like* (TLRs), os quais iniciam uma resposta inflamatória local,resultando no aumento do número de macrófagos e células dendríticas (DCs) no tecido pulmonar infectado. Após a ativação por citocinas e quimiocinas pró-inflamatórias, os macrófagos infectados tentam eliminar o Mtb pelos seus mecanismos bactericidas que envolvem a produção de espécies reativas de oxigênio ou nitrogênio (WALKER; LOWRIE, 1981; MACMICKING et al., 1997). As DCs endocitam as bactérias no tecido pulmonar e migram para os linfonodos drenantes a fim de iniciar a resposta imune adaptativa pela apresentação de antígenos aos linfócitos T virgens (CHACKERIAN et al., 2002).

A imunidade celular do tipo Th1 é essencial para o efetivo controle da infecção por Mtb. A imunidade celular resulta na formação do granuloma, que é essencial para conter o Mtb, pois funciona como uma barreira que delimita o sítio de infecção (TUFARIELLO et al., 2003). O granuloma é caracterizado pelo acúmulo local de macrófagos infectados ou não, células gigantes multinucleadas e células epiteliais, envoltas por um halo de linfócitos T $CD4^+$ e T $CD8^+$. Além destas células, o granuloma também é mantido pela presença do TNF-α. Em granulomas de pacientes com

tuberculose latente, observa-se uma expressão elevada de IFN-γ, TNF-α, IL-1β e baixa expressão de IL-4. Esta resposta é fundamental para a indução e manutenção da latência clínica e representa o equilíbrio entre a sobrevivência intracelular do Mtb e a resposta imune montada pelo hospedeiro (TUFARIELLO et al., 2003). A ausência do IFN-γ reduz a ativação dos macrófagos o que pode conduzir a reativação da TB (Figura 3).

Figura 3. Infecção por *Mycobacterim tuberculosis* e mecanismos imunológicos envolvidos



O *Mycobacterium tuberculosis* (Mtb) penetra no hospedeiro pela via inalatória. Três desfechos são possíveis para a infecção: (i) Eliminação imediata do Mtb pela resposta imune; (ii) Tuberculose (TB) primária, principalmente em indivíduos infectados por HIV; (iii) Contenção do Mtb no granuloma conduzindo a TB latente. Após o estabelecimento da latência, a infecção pode ser reativada levando ao rompimento do granuloma e a disseminação do Mtb (iv). Uma das causas da reativação pode ser a infecção pelo HIV.

Fonte: Adaptado de Kaufmann e McMuchael, 2005.

Tem sido demonstrado que os linfócitos T $CD8^+$ possuem um papel protetor na tuberculose. Os CTLs específicos, além de secretar IFN-γ, liberam grânulos citotóxicos (granzima e perforina) mediando à lise direta de macrófagos infectados (BROOKES et al., 2003; ANDERSSON et al., 2007; GREEN et al., 2012). As células natural killers (NK), células citotóxicas da imunidade inata, podem igualmente contribuir para controle da infecção pelo Mtb. Foi demonstrado *in vitro* que células NK são capazes de lisar macrófagos infectados e são fontes de IFN-γ (VANKAYALAPATI et al., 2005;

DHIMAN et al., 2009). Contudo, foi descrito que pacientes com TB apresentam menor frequência de células NK, menor expressão de receptores de ativação e menor produção de IFN-γ comparados a indivíduos saudáveis (BOZZANO et al., 2009).

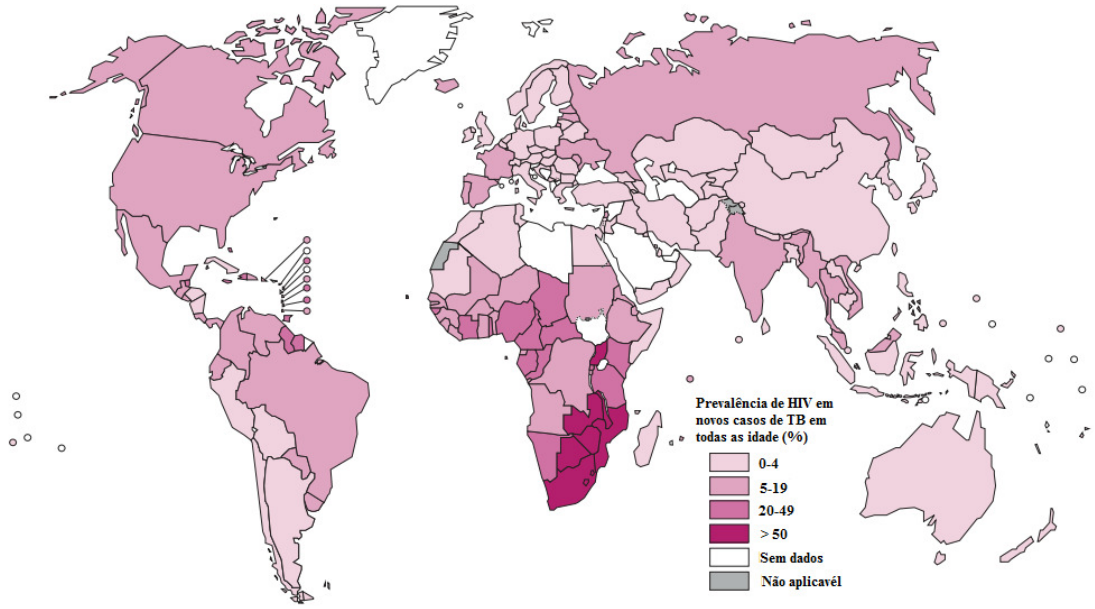
O desenvolvimento de TB ou da latência (manutenção de um número constante de bacilos em locais de infecção) pode depender de um equilíbrio entre linfócitos T $CD4^+$efetores e linfócitos T regulatórios (Treg). Tem sido reportado que a redução de linfócitos Treg e da produção de IL-10 pode exacerbar a resposta imune efetora e inflamatória contra o Mtb contribuindo para a patogênese da TB (REDFORD et al., 2011; URDAHL et al., 2011). Os linfócitos Treg são um subconjunto especializado de linfócitos T $CD4^+$ que suprimem a resposta das células T efetoras, incluindo linfócitos T $CD4^+$ e T $CD8^+$, *natural killer* (NK) e NK T, linfócitos B e células apresentadoras de antígenos (APCs) (SAKAGUCHI et al., 2010).

A TB e a infecção pelo HIV-1 são as principais doenças infecciosas em países em desenvolvimento. É estimado que cerca de 14 milhões de pessoas no mundo estão duplamente infectados por Mtb e HIV-1 (GETAHUN et al., 2010) (Figura 4). A coinfecção entre HIV-1 e Mtb acelera a deterioração clínica nos indivíduos coinfectados resultando em morte prematura. A TB é a maior causa de morte nos pacientes com AIDS, o que representa 26 % das mortes relacionadas com a AIDS, das quais 99 % ocorrem em países com recursos limitados (PAWLOWSKI et al., 2012). A TB é comumente mais grave em pacientes infectados por HIV-1, os quais geralmente apresentam manifestações atípicas de TB, como TB extra-pulmonar e TB disseminada (ACKAH et al., 1995; AARON et al., 2004).

Tendo em vista a importância da imunidade celular do tipo Th1 no controle da infecção pelo Mtb, as alterações causadas pelo HIV-1 na resposta imune específica conduzem a reativação da infecção por Mtb levando ao aumento da frequência de formas atípica, disseminada e extrapulmonar de TB (GILKS et al., 1990; LADO LADO et al., 1999; USTIANOWSKI; LOCKWOOD, 2003). Pacientes coinfectados por HIV-1/Mtb apresentam redução na resposta proliferativa aos antígenos do Mtb e na produção de IL-2, IFN-γ e TNF (ZHANG et al., 1994). A redução da expressão da citocinas do tipo Th1 em pacientes infectados por HIV-1 está associada ao aumento da suscetibilidade à TB. Desta forma, a alteração no equilíbrio entre as citocinas inflamatórias e anti-inflamatórias e a depleção de células efetoras contribuem para a disseminação de ambos os patógenos (BOCCHINO et al., 2000). Além disso, foi descrito um prejuízo na atividade citotóxica específicos para os antígenos de Mtb em

indivíduos coinfectados por HIV-1/Mtb (NIRMALA et al., 2001; KALOKHE et al., 2014), porém o mecanismo pelo qual a infecção pelo HIV-1 promove esta disfunção não foi claramente elucidado.

Figura 4. Prevalência estimada de HIV-1 em novos casos de tuberculose em 2012



Fonte: Global Tuberculosis Report, 2013

A HAART é capaz de restaurar a resposta de hipersensibilidade tardia ao PPD com redução do número de casos e da mortalidade associada a TB (LAWN et al., 2005b). Contudo, a incidência de TB continua elevada em indivíduos infectados por HIV, principalmente nas populações onde a doença é endêmica (LAWN et al., 2005a). O processo de restauração da resposta imune específica e funcional contra o Mtb após HAART não é bem compreendido, os dados sugerem que esta recuperação é apenas parcial (SHELBURNE et al., 2002; LAWN et al., 2005a). Além disso, o tratamento concomitante entre a HAART e o esquema de múltiplas drogas para tratamento de TB em pacientes coinfectados pelo HIV-1/Mtb possui uma elevada taxa de reações adversas com consequências fatais. Por isso, atualmente, tem sido evitado o tratamento com tuberculostáticos concomitante ao HAART, priorizando-se o início do tratamento para TB, contudo sem retardar por mais de oito semanas o início da HAART (LEMOS, 2008).

1.3 TERAPIA ANTIRRETROVIRAL ALTAMENTE ATIVA

A HAART conduz a supressão da replicação do HIV-1, que permite a reconstituição quantitativa e funcional do sistema imune (AUTRAN et al., 1997; CARCELAIN et al., 2001; LEDERMAN, 2001). O efeito de HAART na recuperação do número de linfócitos T $CD4^+$ e a restauração de subpopulações celulares ocorrem aparentemente em duas fases. Na primeira fase, que inicia até duas semanas após a introdução da HAART, ocorre um rápido aumento do número de linfócitos como consequência da redistribuição de linfócitos de memória ($CD4^+CD45RO^+$), que estavam sequestradas nos tecidos linfóides (AUTRAN et al., 1997; CARCELAIN et al., 2001). A segunda fase caracteriza-se por uma expansão lenta e gradual de células T *naives* timo-dependentes ($CD45RA^+CD62L^+$), a qual persiste por dois anos (AUTRAN et al., 1997). O aumento no número de células T $CD4^+$ é acompanhado da recuperação da proliferação linfocitária mediada pela IL-2, e da mudança do padrão de resposta celular do tipo Th2 para o tipo Th1 com produção de IL-2 e IFN-γ (IMAMI et al., 1999; WENDLAND et al., 1999; VALDEZ et al., 2000). Desta forma, o paciente torna-se capaz de montar uma resposta inflamatória ou de hipersensibilidade tardia contra antígenos de memória ou patógenos circulantes (WENDLAND et al., 1999; FRENCH et al., 2000).

Alguns pacientes sob HAART apresentam uma deterioração clínica como consequência da restauração da resposta imune. Nestes indivíduos observa-se a piora nos parâmetros clínicos e/ou laboratoriais apesar da elevação da contagem de linfócitos T $CD4^+$ e da redução da carga viral. A deterioração clínica concomitante à restauração do sistema imune promovida pela HAART resulta em uma intensa resposta inflamatória aos antígenos pré-existentes de uma infecção previamente tratada ou a antígenos latentes (SHELBURNE et al., 2002; MURDOCH et al., 2007). Este fenômeno foi denominado de Síndrome Inflamatória de Reconstituição Imune (IRIS) (SHELBURNE et al., 2002) e tem sido descrita em 16 % dos pacientes infectados por HIV que iniciam HAART (MULLER et al., 2010).

Vários patógenos são associados a IRIS. A grande maioria dos casos tem sido associada a infecções não parasitárias, que incluem: a) bactérias (Mtb, complexo *M. avium* e outras micobactérias não tuberculosas; b) vírus (citomegalovírus, vírus da varicela zoster, herpes vírus humano-8, vírus da hepatite B e C); e c) fungos

(*Pneumocystis jirovecii*, *Cryptococcus neoformans* e *Histoplasma spp.*). No entanto, o espectro de infecções associadas a IRIS continua a crescer, e já existem relatos associando a IRIS com algumas infecções parasitárias como leishmaniose, estrongiloidíase e esquistossomose (LAWN et al., 2005a; POSADA-VERGARA et al., 2005; GOIS et al., 2015).

A TB associada a IRIS (TB-IRIS) é uma das manifestações clínicas mais frequentemente relatada (SHELBURNE et al., 2002; FRENCH, 2009). Entre os indivíduos infectados por HIV com TB ativa, a incidência de IRIS varia, a depender da coorte, de 8 % a 43 % e a mortalidade está em torno de 3 % (NARITA et al., 1998; BREEN et al., 2004; MULLER et al., 2010). Em pacientes com TB-IRIS, frequentemente observa-se forma atípica, disseminada e ganglionar de TB, o aparecimento de abscessos múltiplos e sepses. É comum a presença de febre, linfadenopatia, aumento dos linfonodos e deterioração radiológica (BUCKINGHAM et al., 2004).

Duas formas de IRIS podem ser distinguidas: a forma "paradoxal" e a "revelada" (*unmasking*). A IRIS paradoxal ocorre após o início de HAART em pacientes previamente tratados. Nestes pacientes, a reação imunológica é direcionada contra patógenos não-viáveis ou antígenos residuais presentes no hospedeiro. Por outro lado, a IRIS revelada ocorre em pacientes que possuem infecção latente e, antes do início da HAART, não apresentaram manifestação clínica de doenças oportunistas. Estes pacientes só apresentam sintomas da doença associada a IRIS após o início da HAART devido a recuperação do sistema imune (LAWN et al., 2008; MEINTJES et al., 2008).

A imunopatogênese associada a IRIS é caracterizada pela expansão de células secretoras de IFN-γ específicas sugerindo que a restauração específica da resposta Th1 é a causa de IRIS (FRENCH, 1999; BOURGARIT et al., 2006; TAN et al., 2008; BOURGARIT et al., 2009). Contudo, outros estudos não observaram aumento da produção específica de INF-γem comparação aos pacientes que não desenvolvem IRIS (TAN et al., 2011; SKOLIMOWSKA et al., 2012; GOIS et al., 2015).

Uma elevada produção espontânea de citocinas e quimiocinas pró-inflamatórias derivadas de células da imunidade inata tem sido igualmente descrita nos pacientes com IRIS (STONE et al., 2002; BOURGARIT et al., 2006; OLIVER et al., 2010a; OLIVER et al., 2010b; TADOKERA et al., 2011). Além disso, é relatada a elevada expressão de marcadores de ativação nos linfócitos e a expansão de células NK (ALMEIDA et al.,

2002; HADDOW et al., 2010; SERETI et al., 2010; F et al., 2011). Estes dados em conjunto indicam que a resposta inflamatória exacerbada durante a IRIS pode ser causada pelos componentes da imunidade inata.

Recentemente, foi sugerido que a ausência de linfócitos T, tal como ocorre na AIDS, pode levar ao crescimento de patógenos intracelulares no interior de macrófagos inativados incapazes de eliminar o patógeno. Quando o número de linfócitos T $CD4^+$ é restaurado, após HAART, estes linfócitos podem estimular intensamente os macrófagos, bem como outras células do sistema imune inato, os quais produzem elevada quantidade de citocinas pró-inflamatórias, resultando em inflamação e destruição tecidual (BARBER et al., 2012). De fato, em recente estudo, observamos em um paciente infectado por HIV-1 com leishmaniose mucocutânea associada com IRIS níveis elevados de citocinas pró-inflamatórias e a ausência de uma resposta imune celular específica, a qual foi restaurada apenas após o tratamento da leishmaniose. Estes resultados podem indicar que a resposta inata não específica contribui igualmente para o desenvolvimento de IRIS (GOIS et al., 2015).

A imunopatogênese da TB-IRIS não está bem estabelecida. Particularmente, não está claro se a resposta inflamatória associada à doença resulta de uma resposta exacerbada específica aos antígenos de patógenos oportunistas, da produção citocinas pró-inflamatórias e quimiocinas pela resposta imune inata e/ou do desequilíbrio da resposta imune regulatória. A compreensão da imunopatogênese da IRIS pode auxiliar na identificação de biomarcadores associados ao desenvolvimento e ao prognóstico. Da mesma forma, pode contribuir para o esclarecimento de como ocorre a IRIS associada a patógenos específicos, como por exemplo: Mtb, *Mycobacterium avium, Leishmania*, *Cryptococcus neoformans*, citomegalovírus e *Histoplasma spp*.

2 OBJETIVOS

2.1 GERAL

Descrever o perfil da resposta imune celular aos antígenos de *Leishmania spp.* ou *Mycobacterium tuberculosis* em pacientes coinfectados com HIV.

2.2 ESPECÍFICOS

- Quantificar resposta proliferativa e as subpopulações de linfócitos T $CD4^+$ de memória específicas aos antígenos de *Leishmania* em pacientes infectados por HIV-1 com LV;
- Quantificar resposta proliferativa, as subpopulações de linfócitos T $CD4^+$e T $CD8^+$de memória e o perfil de citocinas produzida em resposta ao PPD em pacientes infectados por HIV-1 com TB;
- Avaliar se a HAART é capaz de restaurar a resposta polifuncional de linfócitos T no curso da TB;
- Avaliar a função citotóxica de linfócitos T $CD8^+$ e células NK em pacientes infectados por HIV com TB;
- Revisar sistematicamente a literatura sobre os casos de leishmaniose associada a IRIS;
- Revisar sistematicamente para descrever o perfil imunológico associado com o desenvolvimento de TB-IRIS em indivíduos infectados pelo HIV.

3 CAPÍTULO I

Para avaliar a resposta imune celular aos antígenos de *Leishmania spp.* de pacientes infectados por HIV-1 com LV, a frequência de linfócitos T $CD4^+$de memória central (MC) e efetora (ME) e a linfoproliferação em resposta ao antígeno solúvel de *Leishmania* (SLA) foi quantificada. Os resultados foram descritos no intitulado "*Cellular immune response in HIV-infected patients with visceral leishmaniais*", apresentado na seção 3.1 desta tese. Este manuscrito foi submetido para a revista Memórias do Instituto Oswaldo Cruz.

3.1 QUANTIFICAÇÃO DA RESPOSTA IMUNE CELULAR EM PACIENTES INFECTADOS POR HIV COM LEISHMANIOSE VISCERAL

Memórias do Instituto Oswaldo Cruz

MEMÓRIAS DO INSTITUTO OSWALDO CRUZ

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Keyword:	HIV, Visceral leishmaniasis, HIV/Leishmania co-infection, cellular immune response
Theme:	Leishmaniasis, Immunology

CELLULAR IMMUNE RESPONSE IN HIV-INFECTED PATIENTS WITH VISCERAL LEISHMANIAIS

Running Title: Cellular immune response in HIV/*Leishmania* co-infection

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Summary

Visceral leishmaniasis (VL) is an opportunistic disease frequent in HIV-infected individuals, particularly in regions where the both infections are endemic. The impairment in the adaptive immune responses caused by HIV-infection could be responsible for the severe presentation relapses commonly observed in individuals with HIV/VL co-infection. In this work, we quantified the specific memory T-lymphocyte subsets in response to stimulation by soluble *Leishmania* antigens in HIV-1-infected patients with VL. We found a decreased number and frequency of memory $CD4^+$ T-lymphocyte subsets in co-infected individuals that was restored following treatment for *Leishmania*.

Keywords: HIV; Visceral leishmaniasis; HIV/*Leishmania* co-infection; cellular immune response.

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Introduction

Approximately 34 million individuals worldwide are infected with Human immunodeficiency virus type 1 (HIV-1). The regions with the highest endemicity include Sub-Saharan Africa, the Caribbean, Eastern Europe and Central Asia (UNAIDS 2012). *Leishmania spp.* are known opportunistic pathogens in HIV-infected individuals (Badaro, 1997; Cruz et al., 2006). *Leishmania spp.* infection causes various clinical manifestations in humans that include tegumentary and visceral disease. Over the past decades, the epidemiology of *Leishmania spp.* and HIV-1 infections is increasingly overlapping in sub-tropical and tropical regions around the world (Karp& Auwaerter, 2007), resulting in greater numbers of co-infections. In the Mediterranean basin, northern India, Sudan and Ethiopia, visceral leishmaniasis (VL) has been considered an important public health problem in HIV-1infected patients (Mathur et al., 2006; ter Horst et al., 2008; Wolday et al., 2001). In the Americas, the majority of HIV-1/*Leishmania spp.* co-infection cases occur in Brazil (Orsini et al., 2012). From 2001 to 2005, 1.1 % of VL cases in Brazil were diagnosed in HIV-infected individuals (Brazil. 2011).

The clinical manifestations of leishmaniasis depend on the cellular immune response of the host. Interferon-gamma (IFN-γ) is essential for activation of macrophages and elimination of *Leishmania* (Liew et al., 1990). However, VL is characterized by predominance of Th2 cytokines and selective anergy of specific cell-mediated immunity, which allows the parasites to survive and replicate inside macrophages (Bacellar et al., 2000; Bourreau et al., 2003).

The immunosuppression caused by HIV-1 infection impairs the host immune response to *Leishmania* by interfering with the microbicidal activity of macrophages. In turn, *Leishmania* may induce HIV-1 replication, accelerating progression to acquired immune deficiency syndrome (AIDS) (Cacopardo et al., 1996). Patients co-infected with HIV-1/*Leishmania spp.* with VL present alteration in Th1/Th2 balance, resulting in increased Th2 cytokine response while the levels of Th1-associated cytokines (IL-12 and IL-18) are reduced (Cacopardo et al., 1996; Nigro et al., 1999; Wolday et al., 2000). This immune response profile can contribute to the development of more aggressive and relapsing VL, frequently observed in co-infected patients (Reus et al., 1999). Taken together, these findings suggest that HIV-1/*Leishmania spp.* co-infected patients may have a specific memory T-cells defect. Herein, we quantify the specific memory T-

lymphocyte subsets in response to stimulation by soluble *Leishmania* antigens of HIV-1-infected patients with VL.

Materials and Methods

Participants included in this study were enrolled at the Hospital Universitário Professor Edgard Santos, located in Salvador, Brazil. HIV-1/*Leishmania spp.* co-infected individuals newly diagnosed with VL and HIV-uninfected individuals with VL prior to leishmaniasis treatment were sequentially included. Co-infected individuals were evaluated before and immediately after treatment for leishmaniasis with pentavalent antimonials or amphotericin B, while VL patients were evaluated only prior to treatment. The diagnosis of VL was made with the presence of symptoms consistent with VL and histopathologic evidence of *Leishmania* in the spleen or bone marrow aspirates. Institutional review board approvals were obtained from Brazilian National Ethical Commission, Fundação Oswaldo Cruz and the University of California San Diego Human Research Protection Program.

Peripheral blood mononuclear cells (PBMCs) were separated by centrifugation over a Ficoll-Hypaque gradient(Sigma-Aldrich, St. Louis, USA), cryopreserved in 10 % dimethyl sulfoxide/fetal bovine serum (FBS) (LGC Biotecnologia, São Paulo, Brazil) and then stored in liquid nitrogen (-196 ºC). PBMCs were thawed and resuspended in RPMI medium (Sigma-Aldrich, St. Louis, USA) supplemented with 2mML-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) (Gibco, NY, USA) and 10 % FBS. Cells were incubated in the presence of anti-CD3 and anti-CD28 (1 μg/mL) monoclonal antibodies (BD bioscience, San Diego, CA, USA), and one of the following stimuli: soluble *Leishmania* antigens (SLA) (10 μg/mL) obtained from the *Leishmania chagasi* strain (MHOM/BR2000/Merivaldo2) (Baleeiro et al. 2006), phytohemagglutinin (PHA) (2 μg/ml) (Sigma-Aldrich, St. Louis, USA), or medium alone. Incubation was for 18 hours at 37 ºC in a humidified 5 % CO_2 atmosphere. After culture, PBMCs were washed with phosphate-buffered saline (PBS)/bovine serum albumin (BSA) and labeled with anti-CD3-allophycocyanin (APC), anti-CD4-fluorescein isothiocyanate (FITC), anti-CD45RA-phycoerythrin (PE), anti-CCR7-PECy7, anti-CD62L-PECy5 and isotype controls. *Naïve* cells were defined as $CD4^+CD45RA^+CD62L^+CCR7^+$, central memory (CM) cells were defined as $CD4^+CD45RA^-CD62L^+CCR7^+$ and effector memory (EM) cells were defined as $CD4^+CD45RA^-CD62L^-CCR7^-$.

To assess the proliferative response of $CD4^+$ and $CD8^+$ T-lymphocytes to SLA, $1x10^6$ PBMCs/mL were labeled with carboxyfluorescein succinimidyl ester (CFSE)

(Invitrogen, Eugene, OR, USA) as described by the manufacturer. PBMCs were incubated for five days at 37 ºC in a humidified 5 % CO_2 atmosphere with SLA (10 µg/mL), PHA (5 µg/mL) or medium alone. Then, PBMCs were washed with PBS/BSA and surface stained with anti-CD3-APC, anti-CD4-FITC, anti-CD8-PE and isotype controls. Cells were fixed with PBS-1% formaldehyde and acquired using a FACS Aria. Flow cytometry analysis was performed using FlowJo software version 7.6 (Tree Star Inc, Ashland, OR, USA), with at least 30,000 events analyzed per sample using forward- and side-scatter gating to define cell populations. The proliferation intensity was determined through the cell division index (DI). The threshold to define positive proliferative response was a DI above 0.06 for $CD4^+$ T-lymphocytes and above 0.09 for the $CD8^+$ T-lymphocytes as described previously (Pinto et al., 2011). As a comparison, the DI of HIV-1/*Leishmania spp.* co-infected individuals with VL were compared with DI of individuals with tegumentary leishmaniasis published previously (Gois et al., 2014).

Statistical analysis was performed using Prism software (GraphPad, San Diego, CA). The Kruskal-Wallis test was used to compare medians of study groups.

Results

Four HIV-1-infected individuals with confirmed diagnosis of VL were enrolled in this study. The median $CD4^+$ T-lymphocyte count was 200 cells/mm^3 (IQR 93-404 cells/mm^3) and viral load was 1.9 log/mL (IQR 1.9-6.8 log/mL). The demographic and clinical data of these co-infected individuals and mono-infected individuals are presented in Table 1.

Prior to leishmaniasis treatment, a lower frequency of CM and *naïve* $CD4^+$ T-lymphocytes in response to SLA was observed in HIV-1/*Leishmania spp.*co-infected individuals when compared to individuals with *Leishmania* mono-infection (Table 2). While the frequency of EM $CD4^+$ T-lymphocytes was similar between HIV-1/*Leishmania spp.* co-infected and *Leishmania* mono-infected individuals (Table 2), the absolute number of CM and EM $CD4^+$ T-lymphocytes following SLA stimulation were lower in the HIV-1/*Leishmania spp.* co-infected individuals. Following leishmaniasis treatment, the frequency of *naïve* (66.7 %), CM (38.9 %) and EM (32.7 %) $CD4^+$ T-lymphocyte subsets in response to SLA increased inHIV-1/*Leishmania spp.* co-infected individuals (Figure 1).

The lymphoproliferative response to SLA was undetectable in the individuals with VL, independent of HIV-1-infection. In contrast, in our previous work individuals with tegumentary leishmaniasis did have a lymphoproliferative response to SLA (Figure 2) (Gois et al., 2014). In one HIV-1/*Leishmania spp.* co-infected individual, restoration of lymphoproliferative response to SLA developed after the completion of treatment for leishmaniasis (data not shown).

Discussion

In this study, we quantified for the first time EM and CM $CD4^+$ T-lymphocyte subsets of HIV-1/*Leishmania spp.* co-infected individuals with VL in response to SLA. HIV-1/*Leishmania spp*. co-infected individuals showed an undetectable lymphoproliferative response to SLA, and reduced numbers of SLA-specific CM and EM $CD4^+$ T-lymphocyte subsets. The absence of specific lymphoproliferative response in both the HIV-1/*Leishmania spp*. co-infected and HIV-uninfected individuals with VL indicates an impairment of SLA-specific cellular immune responses. This is consistent with previous reports noting antigen-specific immunosuppression in subjects with VL (Carvalho et al., 1989). Interestingly, in all individuals with VL we observed Lymphoproliferation in response to PHA (data not shown) demonstrating that the immunodeficiency appears to be *Leishmania* antigen-specific (Gois et al., 2014). In addition to the impairment in proliferative response, previous studies have demonstrated a significant defect in IFN-γ production during HIV-1/*Leishmania spp*. co-infection (Cacopardo et al., 1996; Da-Cruz et al., 1992; Nigro et al., 1999). It is possible that the loss of this effector function of the cellular immune response may occur due to the reduced number of CM and EM $CD4^+$ T-lymphocyte subsets present in HIV-1-infected individuals. This reduced population of specific memory T-lymphocytes limits the development of an immune response able to kill *Leishmania,* consequently promoting the spread of the infection(Cruz et al., 2006).Another contributing factors may be the anergy of immune response in HIV-1/*Leishmania spp.* co-infected individuals from the systemic activation of the immune response and elevated apoptosis rate describe during HIV-infection. *Leishmania* infection may also contribute to this increased activation of immune system in HIV-infected individuals, including those receiving highly active antiretroviral therapy (HAART) (Santos-Oliveira et al., 2010).

Our results demonstrate that following leishmaniasis treatment, HIV-1/*Leishmania spp*. co-infected individuals restore SLA-specific memory $CD4^+$ T-lymphocytes. Indeed, the clinical resolution of VL is achieved after restoration of antigen-specific effector immune response (Carvalho et al., 1981; Sinha et al., 2006), and in rare cases a recovery of the lymphoproliferative response. However, despite this recovery (Sinha et al., 2006), a high relapse rate of VL in HIV-1-infected individuals is described (Alvar et al., 2008). It has been shown that *Leishmania* parasites may persist in the host after clinical resolution of VL, particularly in HIV-infected individuals

(Alvar et al., 1997; Ramirez& Guevara, 1997). The persistence of the viable parasites in the host and the inability of specific cellular immune response to control the parasite may explain the relapses seen in HIV-1-infected individuals (Moreno et al., 2000). Taken together, the results presented indicate that the specific treatment to leishmaniasis can contribute to the reconstitution of effector response against *Leishmania*. However, the frequent relapses observed in HIV/*Leishmania spp.*co-infected individuals suggest that the recovery of the number of cells and/or effector capacity of immune response may not be maintained over an extended period. These data needs to be confirmed, as well as the real impact of recovery and maintenance of specific effector immune response in preventing the relapses.

In summary, HIV-1/*Leishmania* co-infected individuals have a decrease inlymphoproliferative response to SLA that coincides with reduced number of specific CM and EM $CD4^+$ T-lymphocyte subsets. A recovery in the number and function of $CD4^+$ T-lymphocytes in response to SLA is observed following leishmaniasis treatment.

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Table 1.Clinical and epidemiological characteristics of HIV/*Leishmania* co-infected patients.

Patients	Age	Gender	Leishmaniasis treatment	CD4T-cells count(cell/mm^3)	Viral load (log/ml)	ART
Co-infected 02	34	M	NI	93	1.9	Yes
Co-infected 04	30	M	Amphotericin B	404	1.9	NI
Co-infected 07	28	M	Glucantime	NI	NI	No
Co-infected 10	29	F	Not treated	200	6.8	Yes
Mono-infected 01	39	M	Glucantime	NA	NA	NA
Mono-infected 02	19	F	Glucantime	NA	NA	NA
Mono-infected 03	29	M	Glucantime	NA	NA	NA

ART: Antiretroviral therapy; NI: not informed. NA: not applicable. M: man; F: female.

Table 2. Quantification of $CD4^+$ T-lymphocyte subsets in response to SLA from HIV/*Leishmania* co-infected patients and *Leishmania* mono-infected individuals.

$CD4^+$ T-lymphocyte subsets	Co-infected group (n=4)	Mono-infected group (n=3)
Naive		
Frequency (%)	11.9 (5.2-38.0)	60.7 (52.8-68-6)
Absolute count (cells/mm^3)	4 (1-19)	165 (92-237)
Central memory		
Frequency (%)	3.4 (0.2-9.3)	14.7 (10.9-26.2)
Absolute count (cells/mm^3)	2 (2-12)	164 (94-235)
Effector memory		
Frequency (%)	19.5 (12.5-22.9)	18.7 (16.6-37.6)
Absolute count (cells/mm^3)	3 (2-9)	243 (167-319)

Data represent median (interquartile range). SLA: Soluble *Leishmania* antigens.The $CD4^+$ T-lymphocyte subsets were quantified by flow cytometry. Naïve, central memory and effector memory cells were defined as $CD3^+CD4^+CD45RA^+CD62L^+CCR7^+$, $CD3^+CD4^+CD45RA^-CD62L^+CCR7^+$ and $CD3^+CD4^+CD45RA^-CD62L^-CCR7^-$, respectively.

Figures

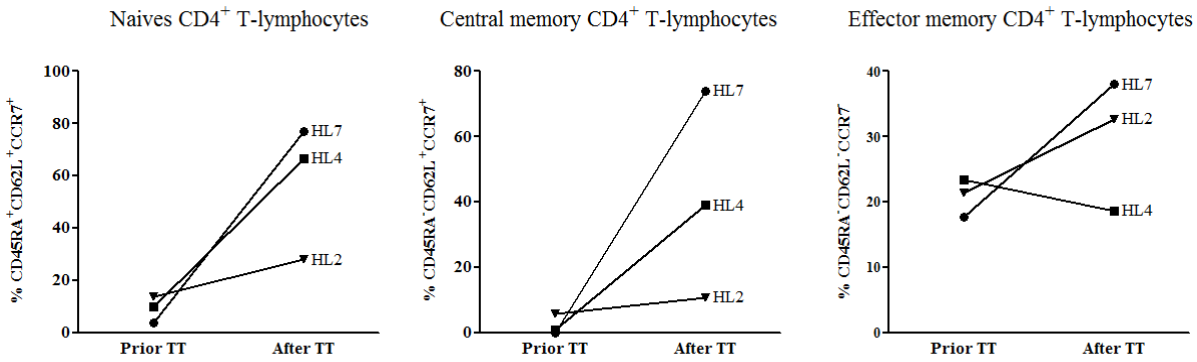

Figure 1. Frequency of $CD4^+$ T-lymphocyte subsets in response to soluble *Leishmania* antigens from HIV/*Leishmania* co-infected patients prior and after *Leishmania* treatment (TT). $CD4^+$ T-lymphocyte subsets specific to *Leishmania* antigens were quantified by flow cytometry.

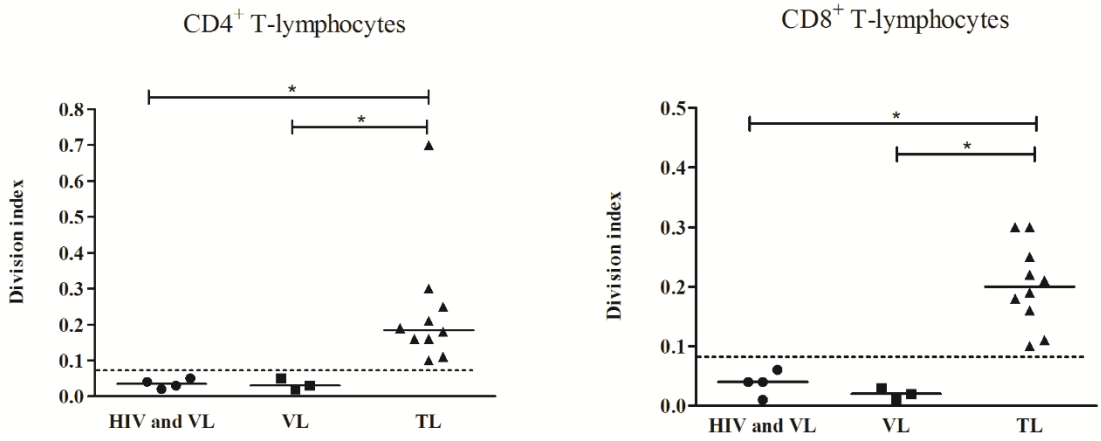

Figure 2. Proliferative response to soluble *Leishmania* antigens of $CD4^+$ and $CD8^+$ T-lymphocytes from HIV/*Leishmania* co-infected patients with visceral leishmaniasis (HIV/VL), *Leishmania* mono-infected patients with visceral leishmaniasis (VL) and *Leishmania* mono-infected patients with tegumentary leishmaniasis (LT). Medians are indicated by horizontal bars. The division index of $CD4^+$ and $CD8^+$ T-lymphocytes were calculated by software FlowJo. The dashed line represents the threshold to define positive proliferative response to $CD4^+$> 0.06 and $CD8^+$ T-cells> 0.09. P-value was calculated by Kruskal-Wallis test. *: $p<0.05$.

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4 CAPÍTULO II

Para descrever o perfil da resposta imune celular aos antígenos de Mtb em pacientes infectados com HIV com TB, realizamos a quantificação da resposta proliferativa, das subpopulações de linfócitos T $CD4^+$ e T $CD8^+$ de memória e do perfil de citocinas produzida em resposta ao PPD em pacientes infectados por HIV-1 com TB. Além disso, investigamos se a HAART é capaz de restaurar a resposta polifuncional de linfócitos T no curso da TB. Estes resultados são apresentados no manuscrito em preparação intitulado: "*Tratamento antiretroviral para HIV restaura parcialmente a resposta polifuncional de linfócitos T no curso da tuberculose*", na seção 4.1 desta tese.

A avaliação da função citotóxica de linfócitos T $CD8^+$ e células NK especifica aos antígenos de Mtb de pacientes infectados por HIV com TB foi realizada pela detecção da expressão de CD107A, IFN-γ e perforina em resposta ao PPD. Os resultados desta avaliação são apresentados no manuscrito em preparação intitulado: "*Functional activity of $CD8^+$ T-lymphocytes and NK cells of patients with HIV and tuberculosis"* na seção 4.2 desta tese.

4.1 TRATAMENTO ANTIRETROVIRAL PARA HIV RESTAURA PARCIALMENTE A RESPOSTA POLIFUNCIONAL DE LINFÓCITOS T NO CURSO DA TUBERCULOSE

TRATAMENTO ANTIRETROVIRAL PARA HIV RESTAURA PARCIALMENTE A RESPOSTA POLIFUNCIONAL DE LINFÓCITOS T NO CURSO DA TUBERCULOSE

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RESUMO

A terapia antiretroviral altamente ativa (HAART) reduziu a morbidade e a mortalidade relacionada à AIDS, contudo a incidência de tuberculose continua elevada em indivíduos infectados por HIV-1. O mecanismo de restauração da resposta imune celular efetora contra o *Mycobacterium tuberculosis* (Mtb) após HAART não é bem compreendida. O objetivo deste estudo foi avaliar se a HAART é capaz de restaurar a resposta polifuncional de linfócitos T no curso da tuberculose (TB). Foram avaliados pacientes com diagnóstico de TB infectados pelo HIV virgens de HAART (TB-HIV) ou em uso de HAART (TB-HIV-HAART), por um período inferior a seis meses. Um grupo com TB não infectado pelo HIV (TB) foi avaliado como controle. As células mononucleares do sangue periférico dos pacientes foram cultivadas na presença de PPD ou meio. Foram quantificadas a resposta linfoproliferativa, as frequências de linfócitos T $CD4^+$ e $CD8^+$ de memória central e efetora e o padrão de citocinas intracelulares (INF-γ e IL-2) por citometria de fluxo. Observou-se maior proliferação de linfócitos T $CD4^+$ e $CD8^+$ em resposta ao PPD no grupo TB-HIV-HAART comparado aos demais grupos. Não foram observadas diferenças nas frequências de linfócitos T $CD4^+$ com perfil efetor e de memória central entre os três grupos avaliados. Além disso, foi observada maior frequência de células polifuncionais (IFN-γ^+ e IL-2^+) nas subpopulações de T $CD4^+$ de memória central e T $CD4^+$ *naïves* e T $CD8^+$ *naïves* no grupo TB-HIV-HAART, comparado ao grupo TB-HIV (p=0,02, p=0,004 e p=0,03 respectivamente). Considerando-se todas as subpopulações de linfócitos T $CD4^+$, 41 % produzem IFN-γ no grupo TB-HIV-HAART, menos de 1 % no grupo TB-HIV e 65 % no grupo TB. Em relação a subpopulação T $CD8^+$, 58 % dos linfócitos do grupo TB-HIV-HAART produzem IFN-γ, menos de 1 % no grupo TB-HIV e 35 % no grupo TB. Os pacientes do grupo TB-HIV-HAART recuperam parcialmente a capacidade funcional frente ao PPD com aumento da produção de IFN-γ. Estudos futuros devem ser realizados para avaliar o efeito do tratamento tuberculostático sobre a resposta específica aos antígenos do Mtb. É possível que pacientes com melhor perfil funcional respondam mais rapidamente ao tratamento e tenham maior controle da infecção pelo Mtb.

Palavras-chave: HIV; Tuberculose; Coinfecção; HAART; Resposta imune celular.

INTRODUÇÃO

Segundo a Organização Mundial de Saúde (OMS), cerca de 37 milhões de pessoas estão infectadas por HIV no mundo, destas estima-se que 14 milhões estão infectados duplamente por HIV-1 e *Mycobacterium tuberculosis* (Mtb) (Getahun et al., 2010). A associação entre tuberculose (TB) e HIV-1 potencializa a morbidade e a mortalidade associada a ambos os patógenos, acelerando a deterioração clínica dos pacientes e resultando em morte prematura (Toossi et al., 2001; Pawlowski et al., 2012).

A introdução da terapia antiretroviral altamente ativa (HAART) em indivíduos com AIDS conduz a supressão da replicação do HIV-1 permitindo a reconstituição quantitativa e funcional do sistema imune (Autran et al., 1997; Carcelain et al., 2001; Lederman, 2001). O maior acesso à HAART reduziu a morbidade e a mortalidade relacionada à AIDS, contudo a incidência de TB continua elevada em indivíduos infectados por HIV-1, principalmente em populações onde as duas infecções são endêmicas (Lawn et al., 2005). A recuperação do número de linfócitos T $CD4^+$após HAART ocorre aparentemente em duas fases. Na primeira, que se inicia duas semanas após a introdução HAART, ocorre uma rápida redistribuição de linfócitos T $CD4^+$ de memória que estavam sequestradas nos tecidos linfóides (Autran et al., 1997; Carcelain et al., 2001). A segunda fase caracteriza-se por uma expansão gradual de células T naïves timo-dependentes (Autran et al., 1997). O processo de restauração da resposta imune específica e funcional contra o Mtb após HAART não é bem compreendida, os dados sugerem que esta recuperação é apenas parcial (Shelburne et al., 2002; Lawn et al., 2005).

A resposta imune protetora contra a infecção pelo Mtb depende de uma resposta celular de linfócitos T $CD4^+$ do tipo 1 (Th1) robusta, a qual é prejudicada devido a depleção dos linfócitos T $CD4^+$induzida pela infecção do HIV. Uma resposta polifuncional específica, caracterizada pela presença de linfócitos que produzem simultaneamente IL-2, IFN-γ e TNF, é fundamental para obter uma resposta capaz de sustentar sua própria expansão e garantir uma atividade efetora (Harari et al., 2005). Indivíduos infectados por HIV-1 com TB latente apresentam uma resposta de linfócitos T $CD4^+$polifuncionais preservada (Day et al., 2008). Porém a capacidade funcional das subpopulações de linfócitos T em responder ao Mtb nos pacientes infectados pelo HIV é pouco conhecida. Além disso, não está bem descrito se a HAART induz a recuperação da qualidade da atividade efetora de linfócitos T específica aos antígenos de Mtb. Este

estudo tem como objetivo avaliar se a HAART é capaz de restaurar a resposta de linfócitos T polifuncional no curso da tuberculose.

MATERIAL E MÉTODOS

Os indivíduos foram recrutados por busca ativa no Hospital Universitário Professor Edgar Santos, no Hospital Couto Maia e no Instituto Brasileiro para Investigação da Tuberculose (IBIT), todos localizados em Salvador, Brasil. Foram incluídos indivíduos infectados pelo HIV-1 com diagnóstico de TB que haviam iniciado HAART há menos de seis meses (grupo TB-HIV-HAART) ou virgens de HAART (grupo TB-HIV). Indivíduos não infectados pelo HIV-1 com diagnóstico de TB foram incluídos como controles (grupo TB). Foram excluídos pacientes que já haviam iniciado o tratamento para TB, indivíduos com outras causas de imunossupressão e gestantes. Os casos de TB foram definidos como paciente que apresentou sintomas respiratórios e teve uma baciloscopia positiva para Mtb. O diagnóstico de TB foi também considerado na presença de cultura positive ou história clínica de TB associada com testes complementares (Castelo Filho et al., 2004).

As células mononucleares do sangue periférico dos pacientes (PBMCs) foram isoladas a partir do sangue heparinizado por gradiente de Ficoll-Hypaque (Sigma-Aldrich, St. Louis, USA). Para avaliar a resposta proliferativa aos antígenos de Mtb, as PBMCs foram marcadas com *carboxyfluorescein succinimidyl ester* (CFSE, Molecular Probes, Eugene, OR, EUA) conforme as especificações do fabricante. Em seguida, as PBMCs marcadas foram incubadas em meio RPMI-1640 (Sigma-Aldrich, St. Louis, USA) suplementado com 2 mML-glutamina, penicilina (100 U/ml), estreptomicina (100 µg/ml) (Gibco, NY, USA) e 10 % de soro fetal bovino (LGC Biotecnologia, São Paulo, Brasil) e estimuladas com PPD (10 µg/mL) (Statens Serum Institute, Copenhagen, Denmark), PHA (5 µg/mL) (Sigma-Aldrich, St. Louis, USA) e meio por cinco dias a 37 °C e com 5 % de CO_2. Após a cultura, as PBMCs foram lavadas com PBS-BSA 0,2 % e marcadas com anticorpos monoclonais CD3-aloficocianina-cyanina 7 (APC-Cy7), CD4-ficoeritrina-cyanina 7 (PE-Cy7), CD8-APC por 20 minutos e fixadas com PBS-formaldeído 1 %. As células foram adquiridas no FACSFortesa (BD bioscience, San Diego, CA, USA) e analisadas utilizando o software Flowjo versão 7.6 (Tree Star Inc, Ashland, OR, USA). A intensidade de proliferação foi determinada através do índex de divisão (ID). Foi considerado o ID de 0,06 como ponto de corte para definir proliferação dos linfócitos T $CD4^+$ e 0,09 como ponto de corte para proliferação dos linfócitos (Pinto et al., 2011).

Para avaliar a frequência e função das subpopulações de linfócitos T $CD4^+$ e $CD8^+$ em resposta aos antígenos de Mtb, as PBMCs foram incubadas em meio RPMI-1640 suplementado com 2 mML-glutamina, penicillina (100 U/ml), estreptomicina (100 µg/ml) e 10 % de soro fetal bovino e estimuladas com PPD (10 µg/mL), PHA (5 µg/mL) e meio. As células foram cultivadas na presença de 1 µg/mL de anti-CD28, 1 µg/mL de anti-CD3 (BD bioscience, San Diego, CA, USA) por 18 horas à 37 ºC com 5 % de CO_2. Após duas horas de incubação, foi adicionado brefaldina A e mosensina (4 µg/mL). Após a cultura, as PBMCs foram incubadas com os seguintes anticorpos monoclonais: CD3-APC-Cy7, CD8-APC, CD4-ALEXA-700, CD45RA-PE-Cy5, CCR7-PE-Cy7 por 20 minutos e fixadas com PBS-formaldeído a 1 %. As PBMCs foram permeabilizadas com PBS-saponina 0,2 % e incubadas com anti-INF-γ-PE e anti-IL-2-FITC (BD bioscience, San Diego, CA, USA) por 30 minutos na temperatura ambiente. As PBMCs foram adquiridas no FACS Fortesa e analisadas usando o software Flowjo versão 7.6. Ao menos 30.000 eventos foram analisados por amostra.

As frequências de linfócitos e intensidade de proliferação foram apresentadas como medianas e intervalos interquartis. As diferenças entre os três grupos avaliados foram determinadas pelo teste Kruskal-wallis, seguido do pos-teste de Dunns. As diferenças foram consideradas estatisticamente significantes para valores de $p<0.05$. O software utilizado para análise foi o GraphPad Prisma.

Este estudo foi aprovado pelo comitê de ética em pesquisa da Fundação Oswaldo Cruz e todos os pacientes assinaram o termo de consentimento livre e esclarecido.

RESULTADOS

As principais características clínicas dos indivíduos incluídos no estudo são apresentadas na Tabela 1. A média da contagem de linfócitos T $CD4^+$ dos grupos HIV-TB e HIV-TB-HAART foi 23 células/mm^3 e 101 células/mm^3, respectivamente. A carga viral do grupo HIV-TB e HIV-TB-HAART foi 6,2 log/mm^3 e 6,0 log/mm^3, respectivamente (Tabela 1).

Observou-se uma diferença na resposta proliferativa dos linfócitos T $CD4^+$ e T $CD8^+$ frente ao PPD entre os grupos avaliados, com maiores índices de divisão no grupo HIV-TB comparados ao grupo HIV-TB-HAART (Figura 1).

Não foram observadas diferenças nas frequências de linfócitos T $CD4^+$ com perfil efetor e de memória central entre os três grupos avaliados. A maior razão entre as células T $CD4^+$ de memória efetora e memória central em resposta ao PPD foi observada no grupo HIV-TB-HAART (3,0), mas sem significância estatística quando comparada aos demais grupos. Em relação à subpopulação de linfócitos T $CD8^+$, observou-se uma tendência de menor frequência de linfócitos com perfil *naïve* no grupo TB comparado aos grupos TB-HIV e TB-HIV-HAART (p=0,08) (Tabela 2).

Quanto ao perfil de produção de citocinas em resposta ao PPD, foi observada menor frequência de células polifuncionais (IFN-γ^+ e IL-2^+) nas subpopulações de T $CD4^+$ de memória central e T $CD4^+$ *naïve*s no grupo TB-HIV, comparado aos grupos TB-HIV-HAART e TB (p=0,01 e p=0,004, respectivamente). Além disso, o grupo TB-HIV apresentou menores frequências de linfócitos monoprodutores de IFN-γ nas subpopulações de memória efetora e de memória central e *naïve* comparado ao grupo TB (p=0,03, p=0,002 e p=0,03, respectivamente) (Figura 2A). Em relação às subpopulações de linfócitos T $CD8^+$ foi observada uma tendência a menor frequência de linfócitos *naïves* polifuncionais e monofuncionais produtores de IFN-γ no grupo TB-HIV comparado ao grupo TB (p=0,08 e p=0,07, respectivamente). Além disso, foi observada menor frequência de linfócitos T $CD8^+$*naïves* monofuncionais produtores de IL-2 no grupo TB-HIV comparado aos demais grupos (Figura 3A). Considerando-se todas as subpopulações de linfócitos T $CD4^+$, 41 % das células do grupo TB-HIV-HAART produziram IFN-γ, enquanto essa proporção foi menor que 1 % no grupo TB-HIV e 65 % no grupo TB (Figure 2B). Em relação à subpopulação T $CD8^+$, 58 % dos

linfócitos do grupo TB-HIV-TARV produziram IFN-γ, enquanto essa proporção foi menor que 1 % no grupo TB-HIV e 35 % no grupo TB (Figura 3B).

DISCUSSÃO

Os resultados do presente estudo demonstram que a HAART induziu a restauração parcial da resposta específica ao PPD em indivíduos com TB ativa, mesmo quando administrada por um período inferior a seis meses. Pacientes infectados por HIV-1 com TB ativa, em uso de HAART, apresentaram uma resposta proliferativa das subpopulações de linfócitos T $CD4^+$ e T $CD8^+$mais robusta que a de indivíduos infectados com TB não tratados. Além disso, houve restauração de células polifuncionais, ou seja, duplo produtoras de IL-2 e de INF-γ e de células monoprodutores de IFN-γ.

É bem conhecido o papel dos linfócitos T $CD4^+$na resposta imune protetora contra o Mtb, principalmente pela produção de IFN-γ. Esta citocina é essencial para a ativação dos mecanismos microbicidas dos macrófagos com produção de espécies reativas de oxigênio (H_2O_2) e os intermediários de nitrogênio (NO) que destoem o bacilo (Goldsack; Kirman, 2007). Porém, apenas a capacidade de produzir IFN-γ não assegura a proteção imunológica. Recentemente, foi proposto que a associação entre o estabelecimento da proteção imunológica e capacidade funcional dos linfócitos T em responder a antígenos é multifatorial (Lalvani; Millington, 2008). Além dos linfócitos T $CD4^+$, os linfócitos T $CD8^+$ tem igualmente um papel na defesa contra o Mtb. Linfócitos T $CD8^+$ são capazes de secretar IFN-γ e de exercer uma ação citotóxica sobre macrófagos infectados (Woodworth; Behar, 2006). Igualmente, foi demonstrado que o IFN-γ produzido pelos linfócitos T $CD4^+$ é fundamental para a sobrevivência e função dos linfócitos T $CD8^+$ contra Mtb (Green et al., 2012).

Dados da literatura indicam um predomínio de linfócitos T $CD4^+$ e $CD8^+$de memória efetora polifuncionais circulantes em pacientes infectados por HIV-1 com TB latente. Estes resultados sugerem que a resposta polifuncional é importante para promover o controle da infecção, inclusive em indivíduos infectados pelo HIV-1. Respostas polifuncionais são associadas a um melhor controle do patógeno e geralmente estão presentes em infecções com baixa carga antigênica, a exemplo de infecções latentes. Por outro lado, uma resposta monofuncional, unicamente com produção de IFN-γ predomina em infecções agudas ou com carga antigênica elevada, a exemplo da TB ativa (Harari et al., 2005). De fato, no presente estudo foi observado que a maior parte dos linfócitos T $CD4^+$ dos pacientes com TB ativa, não infectados pelo HIV-1,

produziam apenas IFN-γ, enquanto em pacientes com TB ativa infectados pelo HIV-1, virgens de tratamento, a maior parte dos linfócitos T $CD4^+$ e T $CD8^+$produziam apenas IL-2. Uma correlação inversa entre frequência de linfócitos T $CD4^+$ específicos para antígenos de Mtb produtores de IL-2 e carga viral foi observada em pacientes infectados pelo HIV-1 com TB latente, sugerindo que essas subpopulação tem um papel protetor na manutenção da forma latente da infecção (Day et al., 2008). É provável que níveis insuficientes de produção de IFN-γ contribuíram para o estabelecimento de formas mais graves e disseminadas de TB. Estes resultados sugerem que o controle da disseminação do bacilo depende da presença de subpopulações capazes de produzir IFN-γ e IL-2.

A maioria dos pacientes do grupo TB-HIV-HAART estavam sob HAART por cerca de um mês. Os resultados demonstram que HAART promove uma recuperação rápida da produção de IFN-γ em resposta estimulação antigênica induzindo tanto células monofuncionais como polifuncionais. Apesar do aumento da capacidade funcional induzida por HAART, esta recuperação não foi suficiente para o estabelecimento de uma resposta imune capaz de conter a infecção causada pelo bacilo, tendo em vista que os muitos pacientes estudados apresentavam formas disseminadas e atípicas de TB. Para estabelecer uma resposta imune protetora contra a infecção pelo Mtb é necessária uma recuperação funcional completa dos linfócitos que envolva a capacidade de produzir múltiplas citocinas (IL-2, IFN-γ e TNF-α).

Para que ocorra a restauração de uma resposta celular capaz de conter patógenos oportunistas é necessário não apenas a redução da carga viral como um incremento de ao menos 60 linfócitos T $CD4^+/mm^3$ após o início de HAART (Li et al., 1998). É possível no grupo TB-HIV-HAART, o número absoluto destas células não tenha suficiente para promover uma resposta protetora. De fato, os pacientes do grupo TB-HIV-HAART ainda apresentavam uma importante imunossupressão, com baixa contagem de linfócitos T $CD4^+$ e carga viral elevada.

Além do perfil de produção de citocinas, características fenotípicas dos linfócitos T $CD4^+$ e $CD8^+$polifuncionais parecem implicar na capacidade de controlar a infecção pelo Mtb. Os linfócitos polifuncionais nos pacientes TB-HIV-HAART eram predominantemente de células com fenótipo de memória central e *naïves*. Linfócitos de memória são divididos em duas subpopulações de acordo com a expressão de CCR7 e CD45RA (Sallusto et al., 1999). Linfócitos de memória central migram preferencialmente para os tecidos linfóides, enquanto linfócitos de memória efetora se

direcionam para o sítio da infecção. Durante a exposição ao antígeno, linfócitos T de memória efetora tem um maior potencial para produzir citocinas imediatamente após a ativação. Por outro lado, linfócitos T de memória central., após exposição antigênica se tornam efetores e, em seguida, podem agir mediando à resposta imune (Zaph et al., 2004). Linfócitos *naïves* geralmente circulam em direção aos órgãos linfóides secundários e tem uma resposta menos efetiva a estimulação antigênica e produz baixos níveis de citocinas (Sallusto et al., 1999).

Tem sido relatado que linfócitos T regulatórios e a produção IL-10 limitam a resposta imune contra o Mtb contribuindo para a patogênese da TB (Redford et al., 2011; Urdahl et al., 2011). No presente estudo a frequência de células T regulatórias produtoras de IL-10 foi semelhante entre todos os grupos avaliados, inclusive os não infectados pelo HIV (dados não mostrados).

Em conclusão, os resultados aqui apresentados indicam que os pacientes infectados pelo HIV e diagnóstico de TB em uso de HAART recuperam parcialmente a capacidade funcional frente ao PPD com aumento da produção de IFN-γ. Estudos futuros devem ser realizados para avaliar o efeito do tratamento tuberculostático sobre a resposta específica aos antígenos do Mtb. É possível que pacientes com melhor perfil funcional respondam mais rapidamente ao tratamento e tenham maior controle da infecção pelo Mtb.

TABELAS

Tabela 1. Características clínicas de indivíduos incluídos no estudo

Identificação	Gênero	Idade	Contagem de linfócitos T $CD4^+$ (células/mm^3)	Carga viral do HIV (log/mm^3)	Forma clínica de TB
Grupo TB-HIV(n=07)					
TB-HIV 1	M	ND	ND	ND	Disseminado
TB-HIV 2	M	ND	ND	ND	Disseminado
TB-HIV 3	M	ND	ND	ND	Disseminado
TB-HIV 4	M	32	22	5,5	Pulmonare ganglionar
TB-HIV 5	F	27	19	6,5	Disseminado
TB-HIV 6	M	27	47	4,5	Ganglionar
TB-HIV 7	M	40	3	6,4	Pulmonar
Grupo TB-HIV-HAART (n=06)					
TB-HIV-HAART 1	M	45	184	5,5	Pulmonar
TB-HIV-HAART 2	M	ND	11	5,7	Ganglionar
TB-HIV-HAART 3	F	32	146	4,9	Pleural
TB-HIV-HAART 4	F	35	77	6,0	Meningite
TB-HIV-HAART 5	F	34	24	5,7	Disseminado
TB-HIV-HAART 6	M	28	166	6,7	Pulmonar
Group TB (n=08)					
TB 1	F	41	771	NA	Pulmonar
TB 2	F	59	640	NA	Pulmonar
TB 3	M	ND	ND	NA	Pulmonar
TB 4	M	ND	ND	NA	Pulmonar
TB 5	M	ND	ND	NA	Pulmonar
TB 6	F	ND	ND	NA	Pulmonar
TB 7	M	ND	ND	NA	Pulmonar
TB 8	M	ND	ND	NA	Pulmonar
TB 9	M	ND	ND	NA	Pulmonar

HAART: Terapia antiretroviral de altamente ativa; M: masculino; F: feminino; NA: Não aplicável; ND: Não determinado.

Tabela 2. Frequência de subpopulações de linfócitos T $CD4^+$ e $CD8^+$

Subpopulação (%)	TB-HIV (n=7)		p-valor	TB-HIV-HAART (n=6)		p-valor	TB (n=9)		p-valor
T $CD4^+$	Meio	PPD		Meio	PPD		Meio	PPD	
Naïve	16,5 (6,5-44,6)	7,5 (0,0-11,9)	0,47	9,6 (3,8-11,1)	3,5 (1,3-7,2)	0,25	3,2 (0,3-7,6)	2,0 (1,4-15,0)	0,1
ME	56,2 (25,0-64,4)	49,7 (14,8-74,1)	0,58	32,1 (23,4-63,8)	39,6 (17,8-62,4)	0,87	57,4 (28,8-82,7)	30.9 (27,6-81,2)	0,91
MC	7,2 (5,4-23,9)	10,2 (5,6-27,0)	0,06	35,3 (11,1-51,9	14,0 (6,7-52,8)	0,62	8,3 (2,9-33,2)	18,5 (6,3-43,0)	0,73
Razão ME/MC	4,6 (0,4-8,1)	2,2 (0,2-3,9)	0,31	1 (0,4-7,1)	3,0 (0,9-4,9)	0,9	10,3 (1,7-21,9)	1,7 (0,8-13,2)	0,06
T $CD8^+$	Meio	PPD		Meio	PPD		Meio	PPD	
Naïve	28,3 (6,0-49,9)	14,3 (6,1-51,0)	0,30	11,2 (2,6-31,6)	15,1 (8,2-32,2)	0,62	3,2 (0,3-12,9)	4,4 (0,3-15,1)	0,84
ME	48,1 (11,4-66,3)	47,3 (14,7-69,3)	0,93	47,3 (24,3-58,4)	47,3 (27,0-53,6)	0,62	47,8 (24,5-75,2)	39,1 (23,2-78,7)	0,74
MC	8,1 (5,9-16,3)	7,7 (4,3-14,3)	0,81	7,5 (2,6-8,8)	14,2 (11,7-17,5)	0,12	7,8 (1,0-18,1)	11,2 (3,6-13,7)	0,38
Razão ME/MC	1,7 (0,2-11,0)	3,3 (0,3-11,4)	0,37	5,3 (1,6-14,0)	2,9 (1,1-6,7)	0,37	6,1 (4,1-19,4)	6,1 (2,8-13,7)	0,69

Os dados foram apresentados em mediana (intervalo interquartil). Teste Wilcoxon ($p<0,05$). Células *naïve*: $CD45RA^+$ $CCR7^+$; Células de memória central (MC): $CD45RA^-$ $CCR7^+$ e Células de memória efetora (ME): $CD45RA^-CCR7^-$. A razão entre ME/MC foi calculada dividindo a frequência de ME pela frequência de MC em cada paciente.

FIGURAS

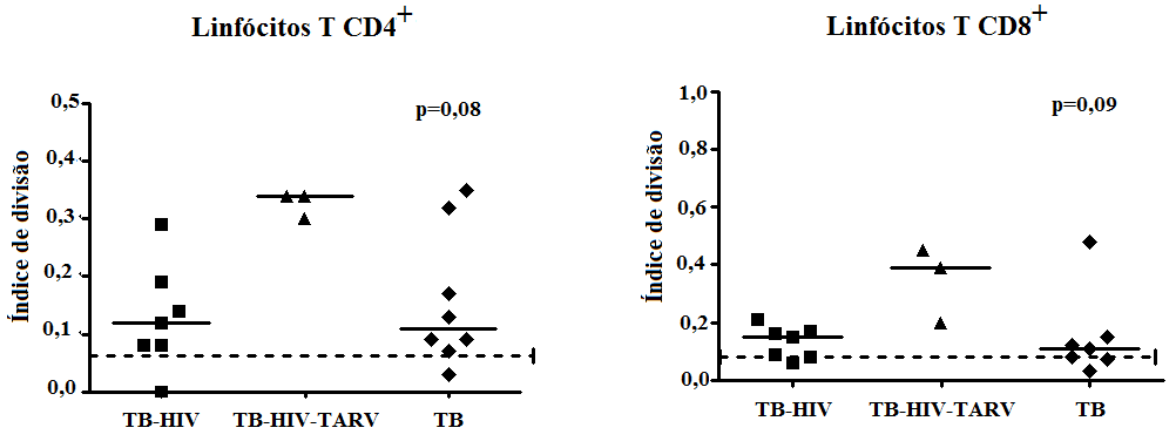

Figura 1. Resposta proliferativa ao PPD de linfócitos T $CD4^+$e $CD8^+$ de pacientes infectados por HIV com tuberculose virgens de HAART (TB-HIV), pacientes infectados por HIV com tuberculose sob HAART (TB-HIV-HAART) e pacientes não infectados por HIV com tuberculose (TB). As barras horizontais representam as medianas. A resposta proliferativa foi avaliada calculando o índice de divisão (DI) usando o software FlowJo. A linha pontilhada representa o limite de detecção que define resposta proliferativa positiva para os linfócitos T $CD4^+$é DI>0,06 e para T $CD8^+$ DI> 0,09. O p-valor foi calculado pelo teste Kruskal-Wallis. *: $p<0,05$.

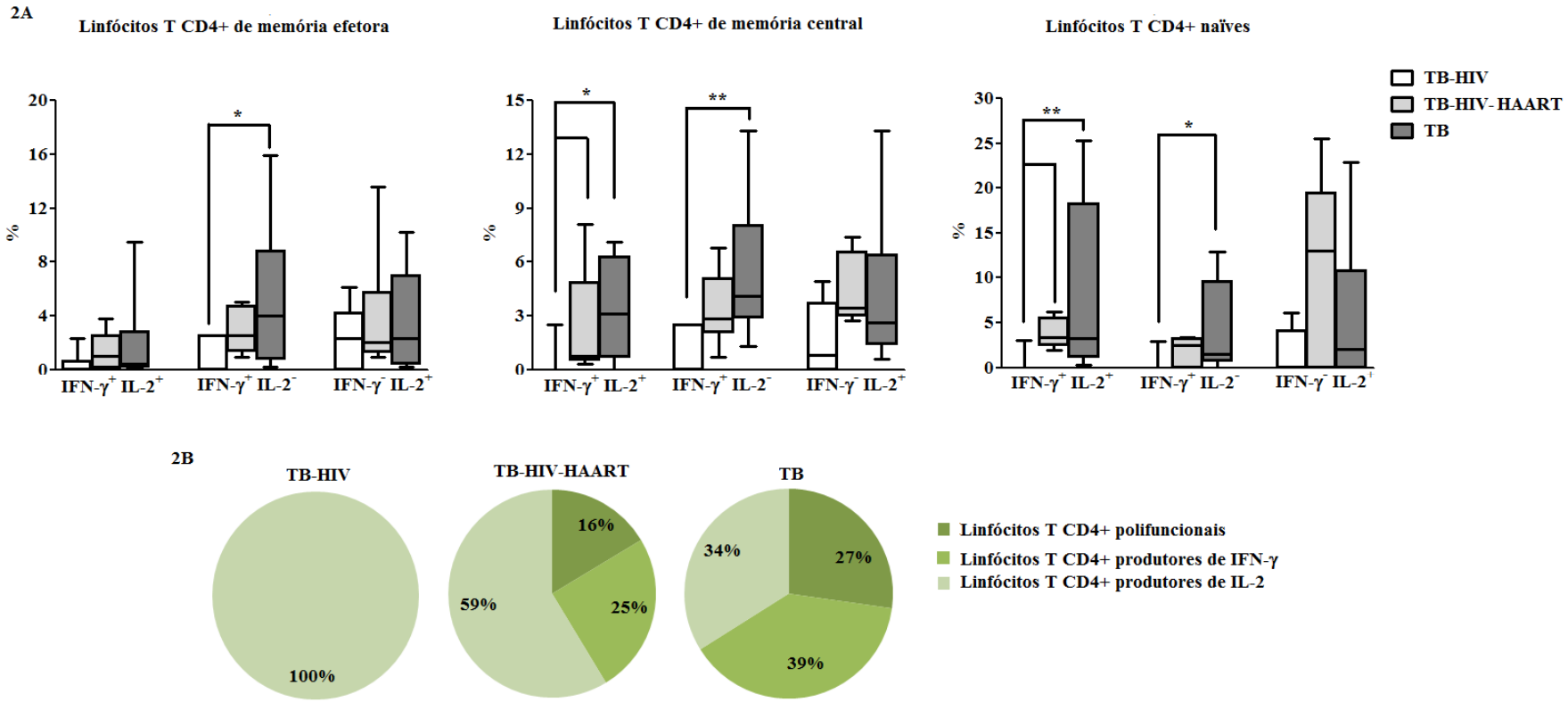

Figura 2. Perfil de citocinas (IL-2 e IFN-γ) produzidas pelas subpopulações de linfócitos T $CD4^+$ em resposta ao PPD de pacientes infectados por HIV com tuberculose virgens de HAART (TB-HIV), pacientes infectados por HIV com tuberculose sob HAART (TB-HIV-HAART) e pacientes não infectados por HIV com tuberculose (TB). Células *naïve*: $CD45RA^+$ $CCR7^+$; Células de memória central (MC): $CD45RA^-CCR7^+$ e Células de memória efetora (ME): $CD45RA^-CCR7$. O p-valor foi calculado pelo teste Kruskal-Wallis. *: p<0,05; **<0,001. A) Frequência de linfócitos T $CD4^+$ produtores de citocinas B) O circulo representa as proporções de todas as subpopulações de linfócitos T $CD4^+$ produtoras de citocinas. Linfócitos T $CD4^+$ polifuncional são células produtoras de IFN-γ e IL-2.

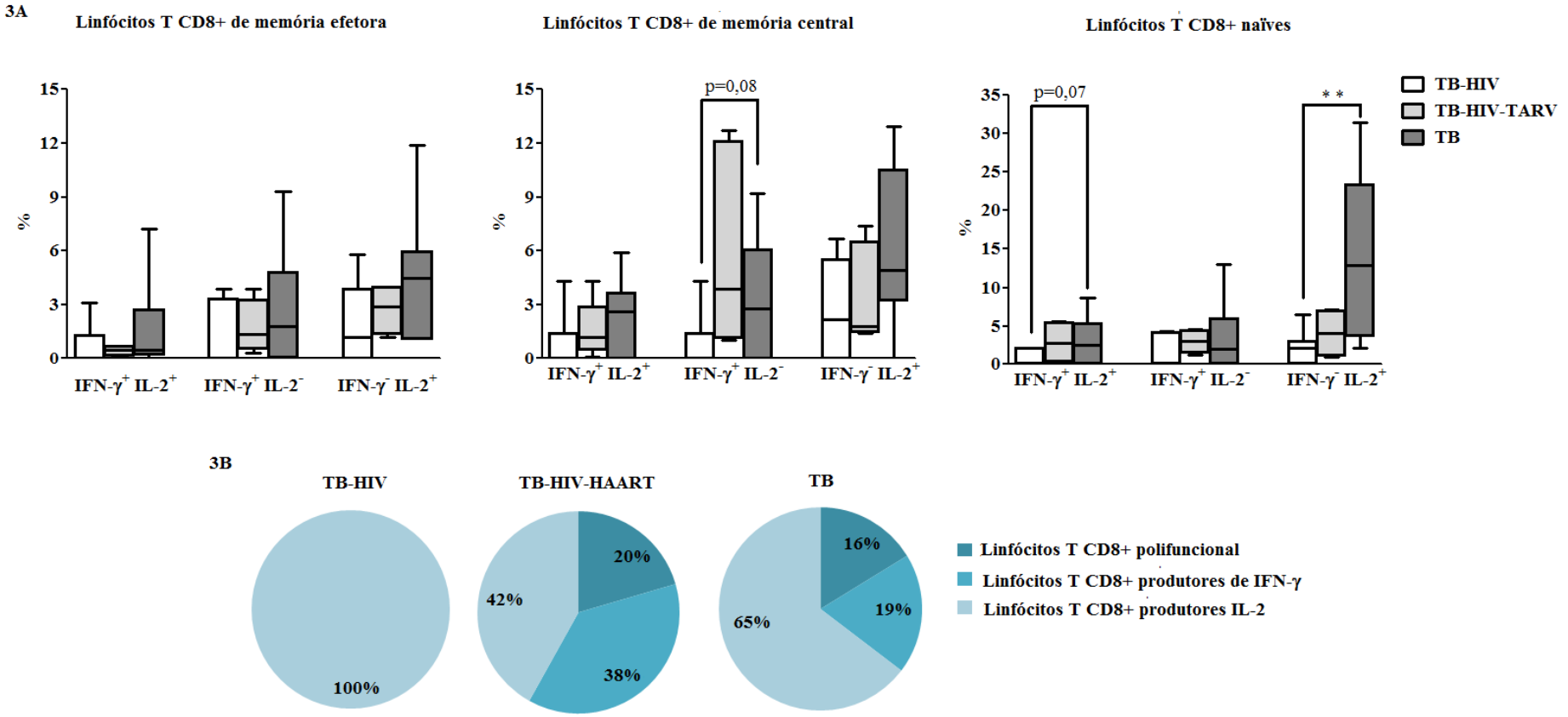

Figure 3. Perfil de citocinas (IL-2 e IFN-γ) produzidas pelas subpopulações de linfócitos T CD8$^+$ em resposta ao PPD de pacientes infectados por HIV com tuberculose virgens de HAART (TB-HIV), pacientes infectados por HIV com tuberculose sob HAART (TB-HIV-HAART) e pacientes não infectados por HIV com tuberculose (TB). Células *naïve*: CD45RA$^+$ CCR7$^+$; Células de memória central (MC): CD45RA$^-$CCR7$^+$ e Células de memória efetora (ME): CD45RA$^-$CCR7. O p-valor foi calculado pelo teste Kruskal-Wallis. *: p<0,05; A) Frequência de linfócitos T CD8$^+$ produtores de citocinas B) O circulo representa as proporções de todas as subpopulações de linfócitos T CD8$^+$ produtoras de citocinas. Linfócitos T CD8$^+$ polifuncional são células produtoras de IFN-γ e IL-2.

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4.2 ATIVIDADE FUNCIONAL DE LINFÓCITOS T CD8$^+$ E CÉLULAS NK DE PACIENTES COINFECTADOS POR HIV E TUBERCULOSE

FUNCTIONAL ACTIVITY OF CD8$^+$ T-LYMPHOCYTES AND NK CELLS OF PATIENTS WITH HIV AND TUBERCULOSIS

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ABSTRACT

Tuberculosis is one of the main opportunistic disease in HIV-1-infected patients. Recently, it was described a protective role of $CD8^+$ T-lymphocytes and NK cells in the control of *Mycobacterium infection* (Mtb). This study evaluated the cytotoxic function of $CD8^+$ T-lymphocytes and NK cells from HIV-1-infected individuals during active tuberculosis. Twelve HIV-1-infected patients (HIV-TB) and three HIV-1-uninfected patients (TB) with active tuberculosis were included in the study. The frequency of $CD8^+$ T-lymphocytes and NK cells in peripheral blood was determined by flow cytometry. Peripheral blood mononuclear cells were cultured with PPD or medium, in the presence of CD107a, for 18h. The cytotoxic activity of $CD8^+$ T-lymphocytes and NK cells was evaluated by CD107a degranulation assay and intracellular detection of perforin and IFN-γ using flow cytometry. A reduced frequency of CD107a $^+$NK cells in response to PPD was observed in HIV/TB patients when compared to TB individuals. The results suggest that HIV-infection promotes an impairment of cytotoxic function, particularly in the degranulation activity of NK cell. This might be an additional mechanism to promote the survival and spread of Mtb and hence the development of active TB.

KEY-WORDS: HIV; tuberculosis; CD8 T lymphocytes; NK cells; cytotoxicity; degranulation; IFN-gama.

INTRODUCTION

Almost 14 million of individuals are co-infected with *Mycobacterium tuberculosis* (Mtb) and HIV-1 worldwide (Getahun et al., 2010). Tuberculosis (TB) is responsible for roughly 26 % of AIDS-related deaths, especially in developing countries that concentrate 99 % of these deaths (Pawlowski et al., 2012). The immunosuppression induced by HIV-1 increases the occurrence of TB by both increasing the susceptibility to primary infection and reactivating a latent tuberculosis infection. Moreover, Mtb infection induces an increased viral replication and high progression to AIDS (Toossi et al., 2001). HIV-1-infected patients often develop atypical manifestations of TB as extrapulmonary and disseminated TB (Ackah et al., 1995; Aaron et al., 2004).

Protective response against Mtb depends on the action of interferon-gamma (IFN-γ) produced mainly by T helper 1 lymphocyte (Th1). IFN-γ activates macrophages inducing the production of nitric oxide synthase (NOS2), which is essential for elimination of Mtb (Saito; Nakano, 1996). The $CD8^+$ T-lymphocytes also have a protective role in the control of Mtb growth. These cells also secrete IFN-γ and have a cytotoxic effect on infected macrophages by driving their degranulation activity (Brookes et al., 2003; Andersson et al., 2007; Green et al., 2012).The natural killer (NK) cells, cytotoxic cells of innate immunity, can also contribute to Mtb control.

Previous studies have demonstrated that NK cells mediate *in vitro* direct cytotoxicity via perforin and granulysin against infected macrophages and are source of IFN-γ (Vankayalapati et al., 2005; Dhiman et al., 2009). However, other study describes that in patients with TB are observed low the frequency of NK cells, low expression of activation receptors (NKp30, and NKp46) and low IFN-γ production (Bozzano et al., 2009). The activation and subsequent degranulation of NK cells depends on complex interactions between receptors present on their surface and those on the target cell (Cheent; Khakoo, 2009).

Few studies have addressed the impact of HIV-1/Mtb co-infection on the cytotoxic activity of $CD8^+$ T lymphocytes and NK cells to macrophages infected with Mtb (Kalokhe et al., 2014). Recently, it was reported that HIV-1-infected individuals with latent Mtb infection have an impairment in the Mtb-specific $CD8^+$ T lymphocytes cytotoxic activity (Kalokhe et al., 2014). In addition, a reduction on the functional activity of NK cells was also found (Nirmala et al., 2001; Rao et al., 2008). The mechanism by which HIV-1-infection promotes the impairment of this Mtb-specific

cytotoxic activity remains unclear. The hypothesis of this study is that alterations of the activity of cytotoxic cells might be due to a decreased degranulation capacity or lower production of cytotoxic granules. Thus, herein we evaluated the cytotoxic function of $CD8^{+}$T-lymphocytes and NK cells in HIV-1/Mtb co-infected patients.

METHODS

Patients included in the present study had active TB with HIV-1 infection (group TB-HIV) or only TB (group TB). They were selected at Hospital Universitário Professor Edgar Santos, Hospital Couto Maia and Instituto Brasileiro para Investigação da Tuberculose (IBIT), all located in Salvador, Brazil. Healthy individuals recruited among students and laboratory staff were included as uninfected controls. Individuals who had started treatment for TB, with other causes of immunosuppression and pregnant were excluded. Case of TB was definite as a patient who experienced respiratory symptoms and had a smear-positive test for Mtb. TB diagnosis was also considered in presence of a positive culture or clinical history of TB associated with complementary tests (Castelo Filho, Kritski et al., 2004). Clinical data of individuals included in the study are shown in Table 1.

To determine the frequency of $CD8^+$ T-lymphocytes and NK cells, whole blood were incubated in the presence of the following monoclonal antibodies: CD3-allophycocyanin-cyanina 7 (APC-Cy7), CD8-APC and CD56-phycoerythrin-cyanina 5 (PE-Cy5) (BD Bioscience, San Diego, CA, USA) for 20 minutes at room temperature. Then, erythrocytes were lysed using lysing solution (Becton-Dickinson, San Jose, CA, USA) and cells were fixed with PBS-1 % formaldehyde. Cells were acquired on FACS Fortesa (BD Bioscience, San Diego, CA, USA) and analyzed using FlowJo software version 7.6 (Tree Star Inc., Ashland, OR, USA).

Cytotoxic activity of $CD8^+$ T-lymphocytes and NK cells was evaluated by CD107a degranulation assay and intracellular detection of perforin and IFN-γ. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-Hypaque (Sigma-Aldrich, St. Louis, USA). PBMCs were incubated in RPMI-1640 (Sigma-Aldrich, St. Louis, USA) supplemented with 2 MML-glutamine, penicillin (100 U/ml), streptomycin (100 mg/ml) (Gibco, NY, USA) and 10 % fetal bovine serum (LGC Biotechnology, São Paulo, Brazil) and stimulated with PPD (10 μg/mL) (Statens serum Institute, Copenhagen, Denmark), PHA (5 μg/mL) (Sigma-Aldrich, St. Louis, USA) or medium. Cells were cultured in the presence of anti-CD28 (1 μg/ml), anti-CD3 (1 μg/ml) (BD Bioscience, San Diego, CA, USA) and anti-CD107a-FITC (5 μL) (BD bioscience, San Diego, CA, USA) for 18 hours at 37 °C in a humidified 5 % CO_2 atmosphere. Brefaldina A and mosensin (4 μg/ml) were added after two hours of

incubation. Then, PBMCs were incubated with the following monoclonal antibodies: CD3-APC-Cy7, CD8-APC, CD56-PE-Cy5 for 20 minutes and fixed with 1 %. PBS-formaldehyde. PBMCs were permeabilized with PBS-0.2 % saponin and incubated with anti-IFN-γ-PE and anti-perforin-PE (BD Bioscience, San Diego, CA, USA) for 30 minutes at room temperature. Finally, PBMCs were acquired in FACS Fortesa and analyzed using the FlowJo software version 7.6 (Figure 1).

Statistical analyzes were performed using GraphPad Prism and statistical differences was determined by Mann-Whitney test or Kruskal-Wallis test. Considered statistically significant $p<0.05$.

This study was approved by the Instutional research board of Fundação Oswaldo Cruz and all patients signed an informed consent.

RESULTS

Regarding the frequencies of $CD8^+$ T-lymphocytes and NK cells, TB/HIV group had higher frequency of $CD8^+$ T-lymphocytes (median 22.4 %, IQR 3.6-46.6 %) and lower frequency of NK cells(median 0.9 %, IQR 0.6-1.6 %), compared with TB group (16.1 % IQR 13.4-19.4 % and 1.3 %, IQR 1.0-3.3 %, respectively).

To evaluate the activation profile of $CD8^+$ T-lymphocytes and NK subsets, the frequencies of cells expressing CD107, perforin or IFN-γwere quantified in the absence of stimulus in TB-HIV and TB groups as well as in uninfected controls (Table 2). Higher frequency of IFN-γ^+CD8^+T-lymphocytes was found in TB group and TB/HIV group compared to uninfected controls (p=0.004). Moreover, frequency of IFN-γ^+ NK cells was higher in TB-HIV group compared to both TB group and uninfected controls, yet this difference was not statistically significant.

Next, the frequencies of $CD8^+$ T-lymphocytes and NK cells and the expression of CD107, perforin or IFN-γfollowing PPD stimulation was measured in both cell subsets, in TB-HIV and TB groups. The frequencies of PPD-specific $CD8^+$ T-lymphocytes expression of CD107 or perforin were similar between groups (Table 2). Regarding NK cells, lower frequency of $CD107a^+$ was found in TB-HIV group (16.2 % IQR 7.0-29.4 %) compared to TB group (35.5 % IQR 19.0-57.3 %) (p=0.04). An increase in the frequencies of $CD8^+$ T-lymphocytes expressing $perforin^+$ or $CD107A^+$ in response to PPD, compared with unstimulated cells was only observed in TB-HIV group ($p<0.05$). The frequencies of PPD-specific $CD8^+$ T-lymphocytes and NK cells expressing IFN-γ^+ were similar to those observed in cells cultured without stimulus, for all evaluated groups (Table 2).

DISCUSSION

The results obtained in the present study demonstrated that individuals infected with HIV-1 with active TB have a lower frequency of NK cells in addition to a decreased degranulation activity in response to PPD antigens compared to individuals with TB alone. This indicates a dysfunction in the specific cytotoxic activity of NK cells. LAMP-1 or CD107a molecule is a marker of $CD8^+$ T-lymphocytes degranulation. CD107ais expressed on cell surface during the release of cytotoxic granules in the virological synapse between cytotoxic cells and infected target cells (Alter et al., 2004).Previous studies, using others methods to evaluate cytotoxicity (chromium release assay and dioctadecyloxa carbocyanine dye-based flow cytometry) have also reported the reduction of NK cells cytotoxic activity in TB patients regardless their HIV-1 serological status (Nirmala et al., 2001; Rao et al., 2008).

The decreased NK cells degranulation capacity might be due to a failure in activation of these cells or in the recognition of target cells. In fact, it was reported that NK cells from individuals with latent Mtb-infection express low levels of activation receptors, which might contribute to the survival and spreading of bacillus (Bozzano et al., 2009). Moreover, HIV-1itself also contributes to the impairment of NK cells since it induces a reduction in the frequency of these cells, in the cytokines production (TNF-α and IFN-γ) and in the cytotoxic activity (Scott-Algara et al., 1992; Mavilio et al., 2003). Furthermore, the strong activation of the immune system driven by HIV-1 infection promote a spontaneous degranulation of NK cells, which might reflect in low detection *in vitro* of degranulation activity and/or reduced of pathogen-specific degranulation activity. According, high frequency of IFN-γ^+ $CD8^+$ T-lymphocytes was found in TB-HIV group in absence of PPD stimulation. Despite the defective degranulation activity of NK cells in TB-HIV individuals found herein, the intracellular level of perforinin those was similar to that of TB patients.

Regarding the $CD8^+$ T-lymphocytes, frequency of cells expressing perforin or $CD107a^+$ was similar in both groups. Taken together, these results suggest that $CD8^+$ T-lymphocytes of HIV-TB patients have a degranulation activity preserved. These results contrast with recent results indicating a reduced degranulation ability of $CD8^+$ T-lymphocytes from HIV-1/Mtb co-infected individuals (Kalokhe et al., 2014). A low expression of both perforin and antimicrobial granulosyn of $CD8^+$ T-lymphocytes in

pulmonary tissue from patients with chronic pulmonary TB was also described (Andersson et al., 2007). However, patients included in the present study had an acute TB disease and were evaluated before starting TB treatment. Moreover, we analyzed peripheral blood T-lymphocytes.

In this study, no difference in the cytotoxic function of both CD8-T lymphocytes and NK cells was found when TB-HIV patients were stratified according to antiretroviral therapy (ART) (data not shown). However, patients on ART were still immunosuppressed, with low $CD4^+$ T-cell counts and high viral load.

In conclusion, the results obtained herein suggest that HIV-1-infection promotes impairment of cytotoxic response, particularly in the degranulation activity of innate NK cell. This might be an additional mechanism to promote the survival and spreading of Mtb and hence the development of active TB.

Table 1. Clinical characteristics of HIV/TB and TB groups

Identification	Gender	Age	$CD4^+$ T-lymphocytes count (cells/mm^3)	HIV-1 viral load (log/mm^3)	ART use	TB clinic form
TB-HIV group (n=12)						
TB-HIV 1	M	ND	ND	ND	No	Disseminated
TB-HIV 2	M	ND	ND	ND	No	Disseminated
TB-HIV 3	M	32	22	5.5	No	Pulmonary and lymph node
TB-HIV 4	F	27	19	6.5	No	Disseminated
TB-HIV 5	M	27	47	4.5	No	Lymph node
TB-HIV 6	M	40	3	6.4	No	Pulmonary
TB-HIV 7	M	45	184	5.5	Yes	Pulmonary
TB-HIV 8	M	ND	11	5.7	Yes	Lymph node
TB-HIV 9	F	32	146	4.9	Yes	Pleural
TB-HIV 10	F	35	77	6.0	Yes	Meningitis
TB-HIV 11	F	34	24	5.7	Yes	Disseminated
TB-HIV 12	M	28	166	6.7	Yes	Pulmonary
TB group (n=06)						
TB 1	F	41	771	NA	NA	Pulmonary
TB 2	F	59	640	NA	NA	Pulmonary
TB 3	M	ND	ND	NA	NA	Pulmonary
TB 7	M	ND	ND	NA	NA	Pulmonary
TB 8	M	ND	ND	NA	NA	Pulmonary
TB 9	M	ND	ND	NA	NA	Pulmonary

ART: Antiretroviral therapy; M: male; F: female; NA: Not applicable; ND: Not determined.

Table 2. Expression of IFN-γ, perforin or CD107A on $CD8^+$ T-lymphocytes and NK cells

Subsets (%)	Medium				PPD		
	HIV/TB co-infected (n=12)	TB mono-infected (n=6)	Uninfected (n=10)	p-value	HIV/TB co-infected (n=12)	Mtb mono-infected (n=6)	p-value
IFN-γ^+ $CD8^+$ T lymphocytes	0.9 (0.0-1.7)	2.7 (1.4-5.6)	0.05 (0.0-0.2)	0.004	2.4 (0.0-4.9)	5.5 (1.1-10.1)	0.21
Perforin$^+$ $CD8^+$ T lymphocytes	3.8 (2.0-5.8)	6.2 (1.1-23.3)	1.6 (0.7-9.8)	0.5	5.0 # (3.7-9.0)	4.5 (2.8-23.5)	0.9
$CD107A^+$ $CD8^+$ T lymphocytes	1.6 (0.4-2.6)	4.4 (2.0-10.5)	1.3 (0.4-7.2)	0.24	2.7 # (1.1-9.6)	4.1 (1.2-10.7)	0.52
IFN-γ^+ NK cells	12.2 (4.0-22.0)	13.4 (5.8-18.3)	1.3 (0.0-10.8)	0.09	14.3 (4.2-17.8)	9.7 (5.9-19.5)	0.96
Perforin$^+$ NK cells	15.6 (10.3-30.4)	20.4 (10.5-29.2)	35.2 (16.5-72.1)	0.1	17.2 (12.7-39.8)	16.3 (3.6-23.0)	0.45
$CD107A^+$ NK cells	7.7 (4.0-22.0)	15.0 (1.4-18.9)	8.2 (5.1-14.1)	0.97	16.2 (7.0-29.4)	35.5 (19.0-57.3)	0.04

The data are presented as median (interquartile range).NK cell was determined on CD3-$CD56^+$ cell subset. P-value was calculated using Kruskal-Wallis test or Mann Whitney test to compare HIV/TB group and TB group. Wilcoxon test was used to compare cells cultured with medium and PPD for each group (#: $p<0.05$).

FIGURES

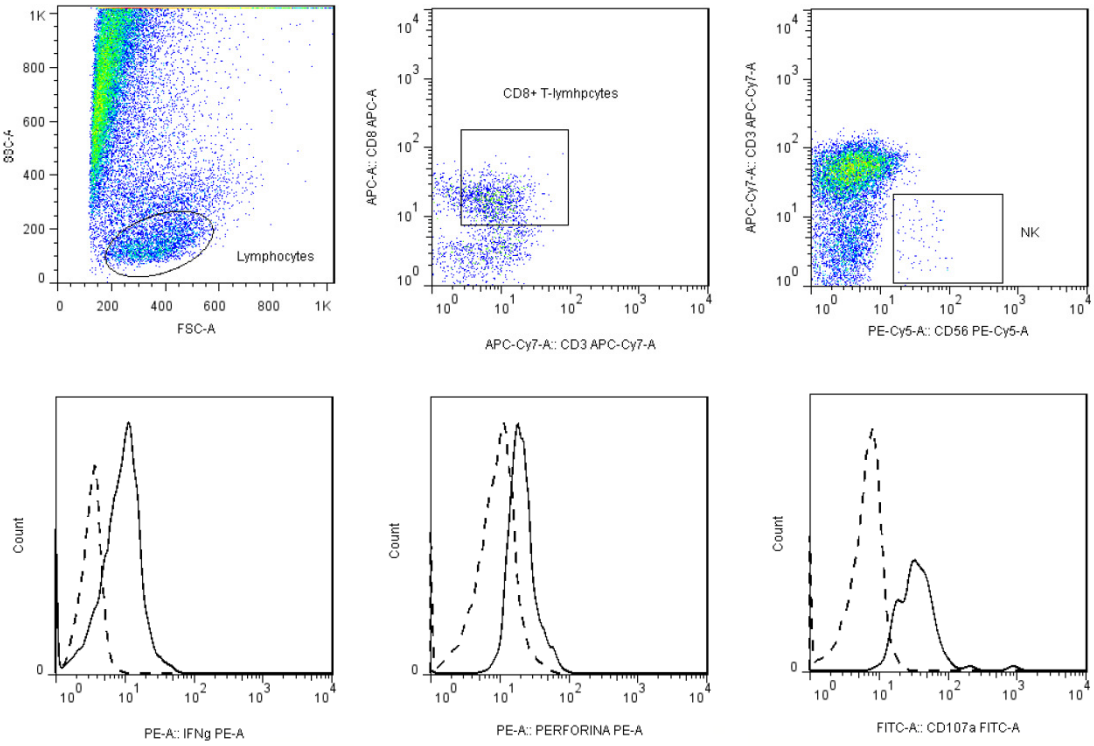

Figure 1. Representative flow cytometry analysis on $CD8^+$ T-lymphocytes ($CD3^+CD8^+$) and NK cells ($CD3^-CD56^+$) of HIV/TB patients and TB individuals. In the flow cytometry analysis the forward- and side-scatter gating were used to define lymphocyte populations. IFN-γ^+, perforin$^+$ and $CD107A^+$ cells were determined by overleaping histograms from each cell subset ($CD8^+$T-lymphocytes or NK cells) and isotype control.

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5 CAPÍTULO III

Durante a busca de pacientes co-infectados por HIV e *Leishmania* foi identificado um caso de leishmaniose mucocutânea associado à IRIS. Este caso e a avaliação da resposta imune celular aos antígenos de *Leishmania* envolvidas na manifestação de IRIS foram publicados (Gois et al., 2015). Durante este relato, observamos que existem poucos casos de leishmaniose associada a IRIS descritos na literatura. Desta forma, uma revisão sistemática de literatura sobre os casos de leishmaniose associada a IRIS foi realizada e publicada na revista *JIAPAC*, com o título: *"Leishmaniasis as a manifestation of immune reconstitution inflammatory syndrome (IRIS) in HIV-infected patients: a literature review"*. Esta revisão é apresentada na seção 5.1 desta tese.

A TB é uma das apresentações mais frequentemente relatados de IRIS. Recentemente, tem sido discutido quais as alterações imunológicas estão presentes em indivíduos coinfectados que podem causar TB-IRIS após a introdução de HAART. Para esclarecer qual a resposta imune está envolvida na TB-IRIS, foi realizada uma revisão sistemática da literatura intitulada: *"Immunological profile of HIV-infected patients with tuberculosis associated-immune reconstitution inflammatory syndrome: a systematic review"*. Esta revisão foi publicada recentemente na revista *Clinical & Cellular Immunology* e é apresentada na seção 5.2 desta tese.

5.1 LEISHMANIOSE COMO MANIFESTAÇÃO DA IRISEM PACIENTES INFECTADOS POR HIV: UMA REVISÃO DE LITERATURA

State-of-the-Art Review

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Leishmaniasis as a Manifestation of Immune Reconstitution Inflammatory Syndrome (IRIS) in HIV-Infected Patients: A Literature Review

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Abstract

Introduction: After the onset of highly active antiretroviral therapy (HAART), some HIV-infected patients present a severe inflammation in response to a latent or a previously treated opportunistic pathogen termed immune reconstitution inflammatory syndrome (IRIS). Few reports of tegumentary and visceral leishmaniasis have been described in association with IRIS. **Methods:** A systematic literature review of IRIS in association with leishmaniasis identified 34 reported cases. **Results and Discussion:** The majority of these occurred in males 4 months following the onset of HAART. The mean CD4 count before HAART was 94 $\pm$ 77 cells/mm^3, increasing to 5 times the initial value between the onset of HAART and IRIS presentation. Visceral leishmaniasis and post–kala-azar dermal leishmaniasis were the most commonly reported clinical manifestations, followed by tegumentary leishmaniasis and uveitis. **Conclusions:** Commonly found characteristics included cutaneous involvement, regardless of *Leishmania* species; appearance of lesions unrelated to time of probable *Leishmania* infection; rapid recovery of CD4 count following HAART; and rapid progression.

Keywords

HIV/AIDS, immune reconstitution inflammatory syndrome, IRIS, leishmaniasis, co-infection HIV/*Leishmania*

Introduction

Highly active antiretroviral therapy (HAART) has dramatically changed the natural course of HIV infection by decreasing the occurrence of opportunistic infections and, consequently, the mortality associated with AIDS. However, after the onset of HAART, some patients experience clinical deterioration following an increase in CD4 count and a decrease in HIV viral load. This worsening is usually due to the clinical manifestation of a latent or a previously treated opportunistic pathogen that paradoxically presents as a severe clinical manifestation. The immune response against these types of pathogens results in severe inflammation as a consequence of the restored immune response termed as immune reconstitution inflammatory syndrome (IRIS).[1–3]

The majority of IRIS cases are associated with nonparasitic infections, including (*a*) bacteria (*Mycobacterium tuberculosis*, the *Mycobacterium avium* complex, and other nontuberculous mycobacteria); (*b*) viruses (cytomegalovirus, varicella zoster virus, herpes simplex virus, human herpes virus 8, JC virus, and hepatitides B and C); (*c*) fungi (*Pneumocystis jirovecii*, *Cryptococcus neoformans*, and *Histoplasma* spp).[4,5] However, other parasitic infections associated with IRIS, such as *Strongyloides stercoralis* and *Schistosoma mansoni*, have also been previously described.[5,6] The risk of IRIS is mainly associated with severe immunosuppression at the start of HAART.[5]

To date, very few reports of tegumentary and visceral leishmaniasis (PL), as well as post–kala-azar dermal leishmaniasis (PKDL), have been described in association with IRIS in HIV-infected patients from several countries.[7–17] Furthermore,

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Table 1. Reported Characteristics of HIV-Infected Patients with Leishmaniasis in Association with IRIS in the Literature.

Author, year	Country	N	Age/ Gender	Leishmaniasis IRIS	Primary Manifestation	*Leishmania* Species	Treatment
Ridolfo et al, 2000[17]	Italy	1	36/F	PKDL	VL	*L infantum*	LAmphB
Gilad et al, 2001[15]	Ethiopia	1	32/M	PKDL	VL	*L* sp	SSG
Blanche et al, 2002[18]	Burkina Faso	1	34/M	Uveitis	DCL and ganglionic	*L major*	Corticosteroid, LAmphB, INF-γ, Enucleation, Sbv
Bittencourt et al, 2003[19]	Brazil	1	28/M	PKDL	VL	NR	Sbv
Berry et al, 2004[20]	France	2	45/F 36/M	LV	Asymptomatic Asymptomatic	*L infantum, L infantum*	AmphB AmphB
Meenken et al, 2004[21]	Netherlands	1	NR / M	Uveitis	VL	*L donovani*	Corticosteroid, SSG, Flu, Enucleation
Posada-Vergara et al, 2005[14]	Brazil	2	52/M 46/M	MCL MCL	Asymptomatic DCL	*L* sp, *L brasilienses*	AmphB, Sbv
Alsina-Gibert et al, 2006[22]	Spain	1	48/M	PKDL	VL	*L* sp	LAmphB, Sbv, allopurinol
Kerob et al, 2006[11]	Senegal	1	55/M	CL	CL	*L major*	Flu
Rihl et al, 2006[26]	Germany/ Spain	1	42/M	PKDL and VL	VL	*L* sp	SSG, miltefosine
Stark et al, 2006[24]	Australia/ Greece	1	45/M	PKDL	VL	*L infantum*	LAmphB
Antinori et al, 2007[13]	Italy	1	46/M	PKDL and uveitis	VL	*L infantum*	Pentamidine, miltefosine
Guffanti et al, 2008[25]	Italy	1	40/F	PKDL	VL	*L* sp	Miltefosine
Sinha et al, 2008[10]	Nicaragua	1	39/M	DCL	Asymptomatic	*L chagasi*	LAmphB
ter Horst et al, 2008[8]	Ethiopia	13	33[a]/NR	VL	VL	*L* sp	NR
Chrusciak-Talhari et al, 2009[16]	Brazil	1	32/M	DCL	CL	*L guyanensis*	Sbv, prednisone
Patel et al, 2009[12]	India/Kuwait	1	35/M	VL	Asymptomatic	*L* sp	AmphB, antibiotic
Tadesse et al, 2009[23]	Ethiopia	1	25/M	PKDL	VL	NR	SSG
Auyeung et al, 2010[9]	Australia/ Cyprus	1	60/M	VL	Asymptomatic	*L donovani*	Corticosteroid, LAmphB
Gelanew et al, 2010[7]	Ethiopia	1	38/M	PKDL and VL	Asymptomatic	*L donovani*	Sbv

Abbreviations: PKDL, post–kala-azar dermal leishmaniasis; VL, visceral leishmaniasis, MCL, mucocutaneous leishmaniasis, CL, cutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis; INF-γ, interferon gamma; F, female; M, male; NR, not reported; Flu, fluconazol; SSG, sodium stibogluconate; Sbv, pentavalent antimonial; LAmphB, lipisomal amphotericin B; AmphB, amphotericin B; IRIS, immune reconstitution inflammatory syndrome.
[a]Mean age.

many cases may remain underreported due to the difficulty in diagnosing leishmaniasis in association with IRIS because of the absence of universal criteria. The present study conducts a review of the international literature pertaining to cases of leishmaniasis in association with IRIS.

Methods

The literature considering the cases of leishmaniasis as a manifestation of IRIS in HIV-infected individuals was analyzed. The search was performed in MEDLINE and BIREME, the Brazilian regional library of medicine, using the following key words: immune reconstitution inflammatory syndrome, cutaneous leishmaniasis, mucocutaneous leishmaniasis (MCL), PKDL, VL, and ocular leishmaniasis. No restrictions were placed with regard to the time of publication or language of publication. Articles were selected if all of the following criteria were met: the patient serology for HIV was positive, there was evidence of a decrease in viral load and an increase in the CD4 count following the onset of HAART, leishmaniasis presented with an inflammatory or atypical manifestation, and *Leishmania* parasites were detected in lesions. All other literature reviews were excluded.

Literature Review

To date, 34 cases of leishmaniasis as a manifestation of IRIS have been described worldwide (Table 1). Males (77%) predominated among the cases described. The mean age of patients was 39 (ranging from 28 to 60 years). The most frequent clinical presentation was VL (19 cases),[7–9,12,20,26] followed by PKDL (10 cases).[7,13,15,17,19,22–26] In 2 cases, VL and PKDL were diagnosed simultaneously.[7,26] Tegumentary leishmaniasis was reported in 5 cases[10,11,14,16], and MCL was reported in 2 of the 5 cases,[14] while diffuse cutaneous leishmaniasis (DCL) was reported in 2 others.[10,16] Uveitis as a consequence of leishmaniasis was reported in 3 cases,[13,18,21] wherein 1 was found to be associated with PKDL.[13] The mean time between the onset of HAART and the occurrence of IRIS manifestations was 4 months (range: 6 days-111 months). Mean CD4 counts were 94 ± 77 cells/mm^3, ranging from 4 to 256 cells/mm^3 (Table 2). Sixteen patients had an increase in CD4 count following

Table 2. CD4 Counts and Viral Loads before and after HAART.

Author, year	CD4 Count prior to HAART	CD4 Count after HAART	% Increase in CD4 Count	Viral Load prior to HAART	Viral Load after HAART	Leishmaniasis IRIS	Time of HAART, Months
Ridolfo et al, 2000[17]	35	157	348	NR	<50	PKDL	8
Blanche et al, 2002[18]	4	91	2175	381 000	<50	Uveitis	4
Berry et al, 2004[20]	186	226	21	1 700 000	NR	VL	10 days
Berry et al, 2004[20]	15	66	340	354 000	NR	VL	6 days
Meenken et al, 2004[21]	60	740	1133	NR	NR	Uveitis	26
Posada-Vergara et al, 2005[14]	38	65	71	750 000	NR	MCL	1
Posada-Vergara et al, 2005[14]	24	283	1179	NR	400	MCL	1
Alsina-Gibert et al, 2006[22]	69	158	128	578	<200	PKDL	11
Rihl et al, 2006[26]	28	116	314	<50	NR	PKDL and VL	22
Stark et al, 2006[24]	176	374	112	NR	NR	PKDL	84
Antinori et al, 2007[13]	71	321	352	73 000	<50	PKDL and uveitis	111
Guffanti et al, 2008[25]	200	584	192	NR	<50	PKDL	2
Sinha et al, 2008[10]	115	200	74	700	<400	DCL	6
Chrusciak-Talhari et al, 2009[16]	16	184	1050	NR	NR	CL	10
Auyeung et al, 2010[9]	55	75	36	>100 000	562	VL	5

Abbreviations: HAART, highly active antiretroviral therapy; PKDL, post–kala-azar dermal leishmaniasis; VL, visceral leishmaniasis; MCL, mucocutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis; NR, not related; IRIS, immune reconstitution inflammatory syndrome.

HAART, with an average gain of 235 ± 198 cells/mm^3 (57-40 cells/mm^3), corresponding to a 5-fold rise. The elapsed time between onset of HAART and manifestation of IRIS was not related to the degree of CD4 boost and viral load before therapy.

In the 2 cases reported by Berry et al,[20] the diagnosis of VL in association with IRIS was inconclusive, as manifestations appeared shortly after the onset of HAART (6-10 days). In addition, both patients had leukopenia and thrombocytopenia prior to the onset of HAART, which may be due to the natural course of VL instead of IRIS. The largest series of cases of VL in association with IRIS was reported in Ethiopia; however, scarce clinical data were presented regarding these patients. In these cases, IRIS was diagnosed 3 months after the onset of HAART, based solely on positive serology for *Leishmania*, or due to the presence of *Leishmania* spp in splenic aspirate. All these patients previously had negative serology for *Leishmania*.[8] It is known[27] that 50% of HIV/*Leishmania* co-infected individuals have negative serology for *Leishmania*, especially those with CD4 counts of less than 100 cells/mm^3. Therefore, perhaps VL as a manifestation of IRIS was an uncertain diagnosis in this series of cases reported in Ethiopia. Moreover, in a prospective study conducted by de la Rosa et al,[28] VL in association with IRIS was not observed in 11 patients with the subclinical form of the disease, who were treated with HAART during 29 months of follow-up. Four patients with active VL who started HAART during the clinical manifestation of VL presented a regression of symptoms.

A total of 10 cases of PKDL were reported in association with IRIS.[7,13,15,17,19,22–26] Post–kala-azar dermal leishmaniasis was diagnosed between 1 and 111 months after the onset of HAART. In only 1 patient, the previous diagnosis of VL was unconfirmed. Following the onset of antiretroviral treatment (ART), CD4 counts increased in all 10 cases. Furthermore, skin lesions appeared only after HAART was initiated, characterizing a new event related to a preexisting *Leishmania* infection. Taken together, these findings suggest a stronger association between PKDL and IRIS compared to VL in association with IRIS.

Regarding the 5 reported cases of cutaneous leishmaniasis associated with IRIS, *Leishmania* infection was commonly disseminated. In 1 case in Brazil, the dissemination of lesions occurred during CD4 count recovery,[16] whereas in the case described by Kerob et al,[11] several nodules containing *Leishmania* appeared following combined ART and amphotericin B treatment.

Sinha et al[10] reported a case of DCL in association with IRIS in 1 HIV-positive individual on ART. In contrast to classical DCL, which is an anergic form of leishmaniasis characterized by disseminated non ulcerating skin lesions, this patient had fever, weight loss, diarrhea and splenomegaly. In addition, *Leishmania chagasi* was isolated from this patient's lesions, which had not been previously reported in cases with DCL. In this case, PKDL should have been considered as a possible diagnosis due to the patient's clinical presentation.

Two cases of cutaneous leishmaniasis with mucosal involvement have been previously described.[14] In both patients, mucosal damage occurred shortly after immune recovery following HAART in contrast to the classical course observed in uninfected individuals wherein mucosal damage occurs

later. Moreover, the species isolated in these 2 cases was *Leishmania braziliensis*, which is commonly associated with the mucosal form of leishmaniasis. Treatment with pentavalent antimony and amphotericin B resulted in the resolution of lesions in the 2 cases, although the authors did not specify the use of corticosteroids.

Uveitis as a manifestation of IRIS, resulting from *Leishmania* infection, was reported in 3 HIV-infected patients undergoing HAART. One patient had uveitis concomitantly with PKDL and was treated successfully with miltefosine.[13] In 2 patients, uveitis resulted in blindness in the affected eye, despite treatment with high doses of corticosteroids.[18,21] In these cases, as well as in the patient reported herein, the isolated species was *L braziliensis*, which is commonly associated with the mucosal form of leishmaniasis.

Discussion

The present study reviewed cases of severe leishmaniasis in HIV-infected individuals as a manifestation of IRIS. Common characteristics found in these patients were cutaneous involvement regardless of the *Leishmania* species isolated, onset of disease regardless of when the patients were infected with *Leishmania*, as well as a rapid progression to severe forms of the disease in association with a rapid CD4 count recovery following ART. The median CD4 count before the onset of HAART was over 50 cells/mm^3 in almost all cases, in comparison with lower CD4 counts found in patients with other infectious diseases in association with IRIS.[5,29] In the majority of the reviewed cases, the length of time between the onset of HAART and occurrence of IRIS was 6 months, similar to what was observed in other infectious diseases associated with IRIS. The only exception was a patient who developed PKDL and uveitis as a manifestation of IRIS 9 years after the onset of HAART. However, this patient was unsuccessfully treated[13] during this period, and IRIS occurred following rescue therapy, when the CD4 count rose from 71 to 321 cells/mm^3. This finding suggests that leishmaniasis as a manifestation of IRIS occurs largely as a result of immune response recovery, despite the length of the recovery period or the initial CD4 count. Gelanew et al[7] reported on 3 HIV-infected patients with PKDL, in which a definitive IRIS diagnosis was impossible due to the absence of standardized clinical and laboratory criteria.[7] However, PKDL in association with IRIS could be considered in one of these cases, since the onset of symptoms was concomitant with an increase in CD4 (from 40 to 93 cell/mm^3) following HAART. A diagnosis of IRIS would be conceivable in several notable cases detailed in the literature, involving an aggressive co-infection with HIV/*Leishmania*.[30,31] Yet, in most of these reports, the length of time between the initiation of HAART and the occurrence of leishmaniasis is incomplete or absent, and relevant information regarding CD4 counts and viral load, before and after HAART, is missing.

Two cases of patients infected with HIV and MCL as a manifestation of IRIS have been described.[14] Common finding are dissemination of lesions, frequently found on the arms, lower limbs, and feet. In addition, lesions are also observed in the nasal, oropharyngeal, and genital mucosa in these patients. Moreover, in the 2 patients reported by Posada-Vergara et al,[14] the onset of leishmaniasis, in one case, and the worsening of leishmaniasis, in the other, occurred 1 month after the onset of HAART. It has been well established that mucosal damage in individuals with leishmaniasis who are not HIV positive is usually associated with an exacerbation of the cellular immune response and elevated production of proinflammatory cytokines. In this form of leishmaniasis, the Montenegro skin test is positive, indicating a positive delayed-type hypersensitivity response to *Leishmania* antigens.[32,33]

In several reports, patients experienced rapid healing of lesions in response to combined amphotericin B and corticosteroid treatments.[9,12] From an immunological point of view, a parallel could be established between IRIS and type 2 leprosy reactions. Leprosy is the prototype of granulomatous disease in which the immune response can result in a paradoxical exacerbation of a patient's lesions, which are typically characterized as erythema nodosum leprosum.[34] Treatment with anti-inflammatory immunomodulatory drugs, such as thalidomide, is required to control the exacerbation of leprosy.[35,36] Further studies should be conducted to evaluate the effectiveness of combined ART and corticosteroid therapy during the first 6 months of treatment in HIV-infected individuals with severe immunosuppression, who are at potential risk for life-threatening immune restoration disease.

ART results in a decreased incidence of opportunistic infections and longer patient survival.[1] Viral loads markedly decrease in tandem with an increase in CD4 counts, resulting in the restoration of immune function in more than 70% of the affected individuals.[1,6] Restoration of CD4 counts seems to take place in 2 phases: the first phase occurs 2 weeks after treatment and lasts for 3 months. There is a relatively rapid redistribution of sequestered memory T lymphocytes (CD4+CD45RO+) from the lymphoid tissues into the bloodstream following a decrease in viral load.[37,38] The second phase starts 6 months after viremia control and is characterized by less profuse and more gradual expansion of naive T cells (CD45RA+CD62L+), persisting for 1 to 2 years.[38] The swift restoration of the T-cell repertoire may contribute to the development of IRIS, especially in individuals with high amounts of antigens caused by either the presence of killed microorganisms during previous infections or prior subclinical opportunistic infections.[6] The immunological mechanisms involved in IRIS development remain unclarified. Elevated production of proinflammatory cytokines is frequently found during the course of IRIS, most notably high levels of interleukin 6, interferon gamma, and tumor necrosis factor-α.[39–41] The recovery of pathogen-specific T cells may play a role in the intensity of the inflammatory response, as evidenced by several reports which found an increase in T cells specific to *M tuberculosis*, *M avium*, and *Cryptococcus neoformans* in patients who developed IRIS in association with these diseases.[42,43] However, the importance of specific T cells warrants further investigation, as

other studies found no association between the recovery of *M tuberculosis*–specific T cells and tuberculosis in association with IRIS.[44,45] To date, the literature contains no reports linking the recovery of *Leishmania*-specific T cells with IRIS-associated leishmaniasis. It is also possible that other mechanisms could contribute to the intensity of inflammatory responses during IRIS, such as the excessive activation of innate immune cells[3] or the impairment of a regulatory T-cell response.[44,46]

In conclusion, leishmaniasis as a manifestation of IRIS may present as a new disease or as the progression of latent disease following the introduction of HAART and consequent restoration of immunity. Further studies should be conducted to clarify the immunological aspects involved in IRIS development as well as to establish relevant criteria to aid in the definitive and prompt diagnosis of IRIS.

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5.2 PERFIL IMUNOLÓGICO DE PACIENTES INFECTADOS POR HIV COM TUBERCULOSE ASSOCIADA À IRIS: UMAREVISÃO SISTEMATICA

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Research Article Open Access

Immunological Profile of HIV-Infected Patients with Tuberculosis Associated-Immune Reconstitution Inflammatory Syndrome: A Systematic Review

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Abstract

Objective: This study systematically reviews the literature that describes the immunological profile associated with the development of tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) in HIV-infected individuals.

Methods: Between the primary and secondary searches, a total of 20 articles were selected for the final analysis.

Results: The results obtained herein indicated that TB-IRIS was associated with the recovery of Mtb-specific immune response, demonstrated by an increased frequency of specific IFN-g-producing cells and specific multifunctional T-lymphocytes (TNF and IFN-γ-producing). In addition, an increased production of inflammatory cytokines and chemokines was found in TB-IRIS patients compared to non-IRIS individuals.

Conclusion: These data suggest that expansion of Mtb-specific cells may not be the main factor for the occurrence of IRIS. Further studies are needed to better evaluate the dynamic of restoration of Mtb-specific memory cells and to clarify the role of innate immune responses in immunopathogenesis of TB-IRIS patients

Keywords: HIV, AIDS, Tuberculosis, Immune reconstitution inflammatory syndrome, Antiretroviral therapy, HAART, Mycobacterium tuberculosis antigens, Cytokines, Specific immune

Introduction

Highly active antiretroviral therapy (HAART) had a major impact in reducing mortality and morbidity associated with AIDS and a significant improvement in patients' quality of life [1,2]. Although HAART is effective at controlling viral replication and inducing partial restoration of CD4+ T-lymphocyte repertoires, around 16% of treated patients experience a clinical deterioration [3,4]. They present an overwhelming inflammatory response against pre-existing antigens that is named inflammatory immune reconstitution syndrome (IRIS) [5,6]. This syndrome results from the immune system's restored ability to mount a potent inflammatory response after HAART. IRIS can manifest as a paradoxical response, in which clinical worsening occurs when patients start on pathogen-specific therapy and HAART simultaneously. Alternatively, it can occur as an unmasking IRIS, in which a latent opportunistic infection is identified following HAART initiation [7,8].

Mycobacterium tuberculosis (Mtb) infection is one of the pathogens most commonly associated with IRIS, especially in endemic areas for tuberculosis [6-10]. Patients with tuberculosis-associated IRIS (TB-IRIS) frequently present fever, tachycardia, exacerbation of tuberculosis symptoms, peripheral and mediastinal lymphadenopathy, neurological symptoms, and abdominal manifestations that include hepatosplenomegaly and cavitary masses [11-13].

The immune reconstitution following HAART is characterized by an increase in the number of CD4+ T-lymphocytes, restoration of lymph oproliferative response to memory antigens, and a shift from a T helper (Th) type 2 to a type 1 cytokine profile, with an increase in IL-2 and IFN-g levels [14-17]. Although the immunological mechanism involved in TB-IRIS remains partially unclear, it has been suggested that the intense inflammatory response results from an exaggerated antigen-specific response [18,19]. Moreover, the production of spontaneous pro-inflammatory cytokines and chemokines [19] and/or an imbalance in the immune regulatory response [20] are found in the course of TB-IRIS. Studies assessing the immune pathogenesis of TB-IRIS are scarce, include small samples of patients and describe different aspects of the immune response. The present study aims to systematically review the pertinent literature to describe the immunological profile associated with the development of TB-IRIS in HIV-infected individuals.

Methods

The systematic review was performed in the Medline, Scielo, Lilacs and Web of Science virtual databases by two independent researchers. Languages were restricted to Portuguese, English, Spanish and French,

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and there was no limit for the year of publication. The following keywords were used: "Immune Reconstitution Inflammatory Syndrome", "Immune reconstitution disease", "Immune restoration syndrome", "Immune restoration disease", "*Mycobacterium tuberculosis*", "M. tuberculosis", "Mycobacterium tuberculosis antigens", "tuberculin", "PPD", "Tuberculosis", "Treg cell", "T-lymphocytes", "CD4-positive T-lymphocytes", "CD8 T-lymphocytes", "FOXP3", "Th1 cell", "type 1 helper T cells", "Th1 response", "IL-6", "tumor necrosis factor alpha", "TNF-alpha", "IFN-gamma", "Interferon-gamma Release Tests", "cytokines", "Chemokines", "C-Reactive Protein", "antibodies", "specific immune", "HIV", "AIDS", "antiretroviral therapy" and "HAART". All keywords, except "type 1 helper T cells", were found in the Mesh database.

Studies were selected according to the following criteria: original articles and articles whose results presented the assessment of markers of the Mtb-specific immune response in HIV-infected patients with TB-IRIS. The exclusion criteria were: letters to the journals and animal model studies. These criteria were first applied to titles and abstracts and then to full text articles. The final article selection was defined by consensus between the two researchers. Secondary search was additionally performed from references included in the original articles.

The following information was systematically extracted from each article: (1) basic information (title, year, authors, objectives, and keywords), (2) study design, (3) methods used for evaluation of the immune system (innate and antigen-specific response), (4) subjects (setting, sample, data collection, procedures and tools), and (5) results obtained. The systematic literature review was structured according to the PRISMA checklist.

$CD4^+$ T-cell count, HIV viral load and the number of Mtb-specific IFN-g-producing cells evaluated by enzyme-linked immunospot assay (ELISPOT) of TB-IRIS patients and non-IRIS were extracted from all articles where information was available. The data of TB-IRIS patients and non-IRIS were compared using U Mann-Whitney test ($p<0.05$).

Results

From the primary search 97 articles were selected of which 80 were excluded. Among the articles excluded, 30 were duplicates and 49 were ineligibles and one was unable to obtain full text. (Figure 1). The secondary search added three articles, totaling 20 studies. When considering study designs, prospective studies were the most frequent (15), followed by case reports (3), case-control (1) and cross-sectional study (1). The majority of patients were from Sub-Saharan Africa [18, 20-29] and Asia (Malaysia, Cambodia, and Thailand) [19,30-36]. Only one study included European patients [37]. Three studies evaluated patients from the same African cohort [18,21, 22], two from the same Malaysian cohort [30,35] and four from the same Cambodian cohort [19,31-33-36]. Patients classified as HAART-associated tuberculosis (TB-ART) [19,31-33-36] were considered herein as unmasking TB-IRIS since TB occurred after reconstitution of the immune system and the control of viral replication. A total of 237 patients with TB-IRIS were reported, and those evaluated by more than one study were only counted once (Table 1). Ninety percent of the studies (18/20) compared the immune response of patients with TB-IRIS to the HIV-infected individuals without IRIS (non-IRIS, n=417). IRIS occurred on average 28 days (SD 24 days) after HAART initiation. The increase in $CD4^+$ T-lymphocyte counts after HAART was significant in both groups with and without IRIS, while a significant reduction in viral load was observed only in patients with IRIS (Figure 2).

Study design	Origin of patients	TB-IRIS group N	Control group (non-IRIS) N	Time between HAART onset and IRIS (days)	$CD4^+$ T-lymphocyte (at pre-HAART) (cell/ mm3)	$CD4^+$ T-lymphocyte (at IRIS) (cell/ mm3)	References
Case report	England	1	2	35	150	294	[37]
Prospective	Sub-Saharan Africa*	7 (pIRIS)	12	23	32	107	[18]
Prospective	Malaysia †	3 (pIRIS)	8	Patient 1: 21 Patient 2: 84 Patient 3: 98	NI	NI	[30]
Prospective	Sub-Saharan Africa*	11 (pIRIS)	13	23	26	86	[21]
Cross-sectional	South Africa	35 (pIRIS)	19	14	51	181	[20]
Prospective		10 (pIRIS)	41	15	195	NI	
Prospective	Sub-Saharan Africa*	11 (pIRIS)	13	26	37	108	[22]
Prospective	Cambodia §	15 (pIRIS)	55	10	45	NR	[31]
		11 (uIRIS)	206	10			
Prospective	Thailand	22 (pIRIS)	104	14	35	144	[32]

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Prospective	Cambodia §	15 (pIRIS)	30	10	45	NI	[33]
Prospective	Cambodia §	15 (pIRIS)	30	10	45	NI	[19]
Case report	Thailand	1 (uIRIS)	4	60	46	155	[34]
Case report	South Africa	22 (pIRIS)	22	14	NI	NI	[23]
Prospective	Malaysia †	3 (pIRIS)	9	49	15	147	[35]
Case-control	South Africa	18 (uIRIS)	58 (HIV) 51 (HIV-TB)	55	115	154	[24]
Prospective	South Africa	1 (uIRIS) 5(pIRIS)	NI	28	4 42	41	[25]
Prospective	South Africa	8 (TB-MDR) 3 (TB-RM)	25 (TB-FS)	14	50 55	NI	[26]
Prospective	Cambodia §	15 (pIRIS)	30	10	45	NI	[36]
Prospective	South Africa	16	18	14	93	158	[27]
Prospective	Gambia	20 (pIRIS)	16	21	60	100	[20]
Prospective	Uganda	18 (pIRIS)	18	14	19	NI	[29]

pIRIS: tuberculosis associated with paradoxical IRIS; uIRIS: tuberculosis associated with unmasking IRIS; NI: Not informed; TB-MDR: tuberculosis multi-drug resistant; TB-RM: tuberculosis mono-resistant to rifampicin; TB-FS: tuberculosis full sensitive;*, †, §:patients evaluated in the same studies.

Table 1: Characteristics of included studies.

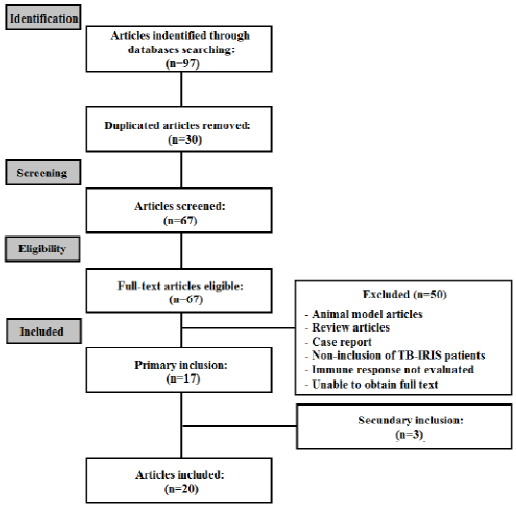

Figure 1: Flow diagram for selecting studies for systematic review.

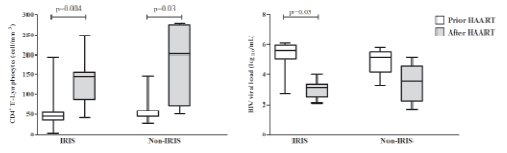

Figure 2: $CD4^+$ T-cell counts and HIV viral load (VL) of TB-IRIS patients and non-IRIS individuals evaluated. Data were extracted from all studies where information was available [18-28-31-37]. The data are presented as median (interquartile range), U Mann-Whitney test ($p<0.05$).

The specific response to Mtb antigens was analyzed in 15 studies (Table 2). An increase in the number of Mtb-specific IFN-g-producing cells was observed in TB-IRIS patients following HAART [18,22-30-35] or when compared to non-IRIS individuals [18-20-22-35]. The number of Mtb-specific IFN-g-producing cells evaluated using ELISPOT was seven times higher in TB-IRIS patients compared to non-IRIS individuals (p=0.03) (Figure 3) [18-20-22-30-35]. In two studies, no increase was observed during TB-IRIS [25,29]. Moreover, a higher frequency of monofunctional ($CD4^+$IFN-g+, $CD8^+$IFN-g+ and $CD8^+TNF^+$) [28] and multifunctional ($CD4^+$IFN-g+TNF+IL-2-) T-lymphocytes [22,28] was found in TB-IRIS patients compared to non-IRIS individuals. Increased production of chemokines and Th1 cytokines (CXCL-9, CXCL10, IL-1b, IL-6, IL-8, TNF, IL-2, IL-12, and IFN-g) was found in

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TB-IRIS patients compared to non-IRIS individuals in four studies [18-23-35,36]. The IFN-g level evaluated by IFN-g release assay (IGRA) was found to be similar in both groups [31-33]. Only one study observed no difference in IL-2 and IL-12 production in TB-IRIS and non-IRIS individuals [32]. Regarding humoral immune responses, two out of three patients with TB-IRIS evaluated by Tan et al (2008) had an increase of anti-PPD IgG compared to TB patients not infected with HIV [30]. Anti-PGL-Tb1 was found only in non-IRIS individuals [21].

Evaluated antigen	Immunological assay	Main results	References
PPD, ESAT-6, 85B	IFN-γ-producing cells (ELISPOT); cytokines (chemiluminescence)	Increased number of PPD-specific IFN-γ-producing cells and higher IL-2, IL-12, IFN-γ, CXCL9 and CXCL10 levels in TB-IRIS patients compared to baseline and to non-IRIS individuals	[18]
PPD, ESAT-6	IFN-γ-producing cells (ELISPOT)	Increased number of PPD-specific IFN-γ-producing cells during IRIS (two out of three patients), compared to baseline. No IFN-γ response to ESAT-6 antigen	[30]
PGL-Tb1, ESAT-6, CFP10	Antibodies (ELISA)	Similar anti-ESAT-6/CFP10 and anti-ManLAM antibody levels in TB-IRIS and non-IRIS individuals	[21]
PPD, ESAT-6, 38kD, Acr 1 e 2	IFN-γ-producing cells (ELISPOT)	Increased number of ESAT-6 and PPD-specific IFN-γ-producing cells in TB-IRIS patients compared to non-IRIS individuals	[20]
PPD	IFN-γ-producing cells (ELISPOT), (ICC, flow cytometry)	Increased number of PPD-specific IFN-γ-producing cells in TB-IRIS patients compared to non-IRIS. Multifunctional (IFN-γ+TNF-a+IL-2-) $CD4^+$ T-cells	[22]
RD1, PPD	IFN-γ level (IGRA)	Similar IFN-γ levels between pTB-IRIS and non-IRIS patients. Increased IFN-γ levels in uTB-IRIS patients compared to controls	[31]
RD1 e PPD	IFN-γ level (IGRA); IL-2 and IL-12 (ELISA)	Increased IFN-γ levels to PPD during IRIS compared to baseline. Similar IFN-γ, IL-2 and IL-12 levels in TB-IRIS patients compared to non-IRIS individuals	[32]
PPD	IFN-γ level (IGRA); IL-5 (ELISA)	Similar IFN-γ and IL-5 levels in TB-IRIS patients and non-IRIS individuals	[33]
Mtb H37Rv, PPD	Cytokines (RT-PCR and ELISA)	Increase quantity of cytokines transcripts (IL1-β, IL-5, IL-6, IL-10, IL-13, IL-17a, IFN-γ, GM-CSF and TNF) and of IL-12p40, IL-1β, GM-CSF, TNF, IL-10, IL-6, IL-2 and IL-8 levels in TB-IRIS patients compared to non-IRIS individuals	[23]
PPD e Lipomannan	IFN-γ-producing cells (ELISPOT); cytokines (ELISA)	Increase number of PPD-specific IFN-γ-producing cells and of TFN levels in response to lipomannan Mtb antigen in TB-IRIS patients compared to non-IRIS individuals	[35]
PPD	IFN-γ-producing cells (ICC, flow cytometry)	Increased frequency of PPD-specific IL-2, IL-10 and TNF-producing $CD4^+$ T-cells following resolution of IRIS	[25]
ESAT-6, 38 kD, Acr 1 e 2, PPD e MtbH37Rv	IFN-γ-producing cells (ELISPOT); cytokines (flow cytometry)	Similar frequency of specific IFN-γ-producing cells among TB-IRIS groups (TB-MDR, TB-RM and TB-FS). Higher IFN-γ/IL-10 and L-2/IL-10 ratios in TB-IRIS FS compared to TB-IRIS MDR and TB-IRIS RM	[26]
PPD e RD1	Cytokines (flow cytometry)	Increased of CXCL10 levels in TB-IRIS patients compared to no-IRIS individuals	[36]
PPD, ESAT-6, CFP10	IFN-γ-producing cells (ICC, flow cytometry)	Higher frequency of PPD-specific $IFN\text{-}\gamma^+CD4^+$, $IFN\text{-}\gamma^+TNF^+CD4^+$, $IFN\text{-}\gamma^+CD8^+$ and TNF^+CD8^+ T-cells in TB-IRIS patients compared to non-IRIS individuals	[28]

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PPD, ESAT-6. CFP10	IFN-γ-producing cells (ELISPOT); cytokines (flow cytometry)	Similar number of specific IFN-γ-producing cells among TB-IRIS patients and non-IRIS individuals	[29]

pIRIS: tuberculosis associated with paradoxical IRIS; uIRIS: tuberculosis associated with unmasking IRIS; non-IRIS: patients without IRIS; ELISPOT: enzyme linked immunospot assay; PPD: purified protein derivative; ESAT-6: early secretory antigenic target-6; RD1: region of difference 1; PGL-Tb1: glycolipid antigen of Mtb; ELISA: enzyme-linked immunosorbent assay; IGRA: interferon-gamma release assay; ICC: intracellular cytokines; RT-PCR: reverse transcription polymerase chain reaction. TB-MDR: tuberculosis multi-drug resistant; TB-RM: tuberculosis rifampicin mono-resistant; TB-FS: tuberculosis fully sensitive.

Table 2: Evaluation of the immune response to Mycobacterium tuberculosis antigens in patients with TB-IRIS.

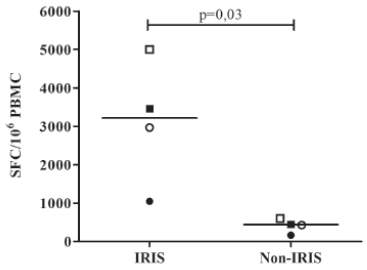

Figure 3: Quantification of Mycobacterium tuberculosis (Mtb)-specific IFN-g-producing cells of TB-IRIS patients and non-IRIS individuals using ELISPOT. Data were extracted as median from all articles that used ELISPOT to quantify IFN-g-producing cells in response to Mtb antigens (PPD, ESAT-6, 85B, 38KD, Acr 1 and 2 and lipomannan) [**18- 20- 22- 30- 35**]. TB-IRIS group: median: 3,216 SFC/106 PBMC, IQR 1,528-4,616 SFC/106 PBMC; non-IRIS group: (441.5 SFC/106 PBMC, IQR 233.5-563.3 SFC/106 PBMC). Mann-Whitney test ($p<0.05$).

The Table 3 summarizes the phenotypic profile of different cell subsets and the ex vivo cytokine levels in patients with TB-IRIS. The spontaneous production of Th1-type (IFN-γ, IL-2, IL-12) and innate (TNF, IL-6, IL-1β, IL-18, CXCL10, EGF and HGF) cytokines were higher in patients with TB-IRIS compared to non-IRIS individuals [18,19-23,24-27-34-37]. Moreover, a three-fold increase in C-reactive protein (CRP) plasmatic levels was found in TB-IRIS patients [20-24]. Six studies performed phenotypic analyses of T-lymphocyte subsets, natural killer (NK) cells, monocytes and dendritic cells [18-20-22-25-30-35]. Activation of $CD4^+$ T-lymphocytes, macrophages and NK cells were observed by several authors [18-24-30-35]. The expansions of KIR-TCRγδ+Vδ2+ T-cells as well as the reduction of Myeloid Dendritic Cells (MDC) were unchanged during TB-IRIS, when these alterations were preexistent before HAART [22]. Tan et al found an increased expression of TLR2 in monocytes and MDCs of TB-IRIS patients. Increased expression of TLR2 was associated with high levels of TNF and IL-12p40 and low levels of IL-10 [35].

Immunological assay	Main results	Reference
Plasma cytokines (ELISA)	Higher IL-6 level in TB-IRIS patients compared to non-IRIS individuals	[37]
Cell phenotyping (WB, flow cytometry)	Increased TNF, IL-6, IL-1β, IL-10, RANTES, and MCP-1 levels and high frequency of activated $CD4^+$ T-cells in TB-IRIS patients	[18]
Cell phenotyping (WB, flow cytometry)	Similar frequencies of activated $CD4^+$ and $CD8^+$ T-cells and $CD4^+$ Treg cells among TB-IRIS and non-IRIS individuals	[20]
Cell phenotyping (PBMC, flow cytometry)	Higher frequency of activated $CD4^+$ T-cells and $CD4^+$ Treg cells (CD25+CD127low and CTLA-4+) in TB-IRIS patients compared to healthy individuals.	[30]
Cell phenotyping (WB, flow cytometry)	Lower frequency of TCRγδ and Vδ2+ T cells expressing CD94/NKG2 and CD158ah,b in TB-IRIS patients compared to non-IRIS individuals.	[22]
Plasma cytokines (flow cytometry and ELISA)	Increased IL-18 and CXCL10 levels and decreased CCL2 in TB-IRIS patients compared to non-IRIS individuals.	[19]
Plasma cytokines (ELISA)	At baseline, higher TNF and IL-10 levels in TB-IRIS patient compared to non-IRIS individuals. Higher IFN-γ levels in TB-IRIS patients after HAART compared to baseline.	[34]

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Plasma cytokines (Luminex technology)	Higher frequency of activated NK cell and C-reactive protein, IL-8, EGF, and HGF levels in TB-IRIS patients compared to non-IRIS patients.	[24]
Cell phenotyping (whole blood, flow cytometry)	Higher expression of TLR2 on mDC and monocytes of TB-IRIS patients compared to non-IRIS individuals	[35]
Cytokines (RT-PCR)	Increase quantity of IL-2, IL-5, IL10, IL-12p40, IL-13, IL-15, IL-17A, TGF-β, and TNF transcripts in TB-IRIS patients compared to non-IRIS individuals	[23]
Cell phenotyping (WB, flow cytometry)	Expansion of central memory CD4+ T-cells following HAART.	[25]
Cytokines levels (CSF, flow cytometry)	Higher TNF, IFN-γ and IL-6 levels in TB-IRIS patients compared to non-IRIS individuals	[27]

Table 3: Cytokine and phenotypic profile of patients with TB-IRIS evaluated ex vivo.

Discussion

The results of this systematic review show that the presence of TB-IRIS was concomitant with the restoration of the Mtb-specific immune response. In the majority of the evaluated studies (11/15), a higher number of Mtb specific-IFN-γ producing cells [18-20-22-35] and of specific multifunctional T-lymphocytes (IFN-γ and TNF-producing) [22-28] as well as higher production of Th1 cytokines [18-23-35,36] were found in TB-IRIS patients compared to non-IRIS individuals. However, the peak in the number of antigen-specific IFN-γ-producing cells did not always coincide with the onset of IRIS, occasionally occurring after IRIS resolution [22-25]. In only four out of 15 studies, three using IGRA and one using ELISPOT, differences in IFN-γ production in response to Mtb antigens were not reported between groups [29-31-33]. Interestingly, the aforementioned study using ELISPOT also found a low production of IFN-γ in response to cytomegalovirus and influenza antigens in TB-IRIS patients, suggesting that those patients were immunosuppressed [29]. None of studies using IGRA found any difference in the production of IFN-g between TB-IRIS and non-IRIS groups. This could indicate that ELISPOT sensitivity to Mtb antigens may be higher than IGRA in patients with advanced HIV infection [38,39].

The low IGRA sensitivity observed could also be explained by the antigenic components of this test, ESAT-6, CFP-10 and TB 7.7, which are derived from region of difference 1 (RD1) in the Mtb genome. In fact, two studies that evaluated ESAT-6 response in patients with paradoxal TB-IRIS using ELISPOT detected low number of spot forming cells (SFC), whereas the number of SFC in response to protein purified derivate to Mtb (PPD) was high [18, 30]. Conversely, higher IFN-γ-production in response to both PPD and RD1 antigens, including ESAT-6, was observed in patients with unmasking TB-IRIS compared to non-IRIS individuals [31]. As RD1 antigens are solely derived from Mtb, as opposed to PPD, it has been proposed that the inflammatory response in unmasking TB-IRIS would be triggered by antigens from live bacteria, while in paradoxal TB-IRIS that response is mainly triggered by antigens from dead bacteria [30]. Thus, IGRA could be useful distinguishing unmasking and paradoxal TB-IRIS.

This systemic review also found a low frequency of polyfunctional (IFN-g+IL-2+TNF+) $CD4^+$ and $CD8^+$ T-lymphocytes in response to PPD in TB-IRIS patients. The majority of patients had a specific multifunctional T-lymphocytes secreting IFN-γ and TNF, but not IL-2 in response to Mtb antigens stimulation [22-28]. These findings suggest that the quality of the specific immune response to Mtb antigens recovery is limited [28]. Mono-functional T-lymphocytes ($CD4^+$IFN-g+) response is mainly found during persistent infection with high antigen load, as occurs during an infection associated with IRIS. Maintaining a high level of antigens impairs the establishment of a polyfunctional response capable of sustaining its own expansion and effector activity [40].

In addition to a high secretion of Th1 cytokines (IFN-γ, IL-2, IL-12) in response to Mtb antigens, several studies found higher production of cytokines and chemokines released from innate immune cells (TNF, IL-6, IL -1β, IL-10, IL-18, CCL-5, CCL-2, CXCL10) [18,19-23,24-34,35-37] in patients with TB-IRIS compared to non-IRIS individuals. Non-specific release of proinflammatory cytokines and chemokines may induce the systemic inflammatory reaction present in IRIS. It has been proposed that once HAART controls the viral load and promotes the reconstitution of T-lymphocyte repertoires, specific lymphocytes could produce cytokines that stimulate macrophages which were previously infected by intracellular pathogens during the period of immunosuppression (AIDS). In turn, these macrophages and other cells of the innate immune system would secrete high levels of proinflammatory cytokines and chemokines, which would result in the inflammatory manifestations of IRIS [41].

Moreover, inflammatory response during TB-IRIS could also be caused by a dysfunction of regulatory T-lymphocytes. Two studies found similar frequencies of $Foxp3^+CD4^+$ T-cells in patients with TB-IRIS and non-IRIS individuals [20,42]. Tan et al studying three patients with TB-IRIS, observed an increase in the proportion of T-cells with regulatory profile ($CD4^+CD25^+CD127^{lo}$ and $CD4^+$CTLA-4+) in comparison to healthy controls, but these findings have not been compared to patients who did not develop IRIS [30]. This review was unable to find studies evaluating the regulatory T-cell function in TB-IRIS patients. This review has some limitation we did not assess the evidence strength of results presented in the articles included and also the risk of bias in these articles.

In conclusion, the findings presented in this review suggest that during TB-IRIS an increase in the specific response to Mtb antigens occurs, as evidenced by a higher number of IFN-γ producing cells and by higher levels of cytokines. The potential role of innate immune response, with increased production of proinflammatory cytokines and chemokines, as well as activated NK cells and macrophages was also observed during TB-IRIS. Taken together, these data suggest that expansion of Mtb specific cells may not be the determining factor for the occurrence of IRIS. Further studies are needed to better evaluate

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the dynamic of restoration of Mtb-specific memory cells and to clarify the role of innate immune responses in immunopathogenesis of TB-IRIS. Modulating the proinflammatory cytokine storm observed during IRIS may be beneficial to patients by decreasing morbidity and mortality of TB-IRIS patients.

Declaration of Conflicting Interests

There is no conflicting interest.

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6 DISCUSSÃO

A leishmaniose e a tuberculose são duas infecções oportunistas frequentes em pacientes infectados pelo HIV-1. Nesta tese, avaliamos em um primeiro momento a resposta de memória central e efetora no curso da leishmaniose visceral e da tuberculose ativa em indivíduos infectados pelo HIV-1. Pacientes co-infectados por HIV-1/*Leishmania spp.* demonstraram ausência de reposta proliferativa dos linfócitos T $CD4^+$ e $CD8^+$ em resposta ao SLA e redução na frequência de subpopulações de linfócitos T $CD4^+$, a qual foi restaurada após o tratamento para LV. Nos pacientes com TB-HIV foi igualmente observado ausência de resposta proliferativa de linfócitos T $CD4^+$ e $CD8^+$ e de células produtoras de IFN-γ em resposta ao PPD. Além disso, foi observado que o HIV-1 promove a redução da atividade degranulativa de células NK o que pode contribuir para o descontrole da infecção e desenvolvimento de TB ativa. Nos pacientes com TB-HIV, HAART foi capaz de induzir uma recuperação parcial de células específicas produtoras de IFN-γ, bem como da proliferação em resposta ao PPD.

Os pacientes coinfectados por HIV-1/*Leishmania spp.* apresentaram uma resposta proliferativa indetectável ao SLA e o número reduzido de linfócitos T $CD4^+$ de MC e ME específicos para o SLA. Além disso, observou-se a restauração quantitativa da subpopulações de linfócitos T $CD4^+$ após o tratamento para a leishmaniose visceral. Estes resultados indicam em prejuízo na resposta imune celular específica. Estudos anteriores também demonstraram alterações funcionais durante a co-infecção pelo HIV-1/*Leishmania spp.*, como a redução na produção de IFN-γ (DA-CRUZ et al., 1992; CACOPARDO et al., 1996; NIGRO et al., 1999).

O prejuízo da função efetora pode estar correlacionado ao número reduzido de linfócitos T $CD4^+$ de memória, bem como a incapacidade funcional (qualidade da resposta) dos linfócitos em responder a antígenos de memória. A incapacidade funcional de responder aos antígenos pode ser devido às alterações do sistema imune causadas pela infecção do HIV-1 como, por exemplo, a ativação generalizada e a elevada taxa de apoptose.

Embora os resultados tenham demonstrado a recuperação da resposta imune após tratamento da leishmaniose, elevadas taxas de recidivas são comuns em indivíduos coinfectados. Foi descrito que apesar da resolução clinica de leishmaniose após o tratamento, é frequente a persistência de parasitas em indivíduos infectados por HIV-1 (ALVAR et al., 1997; RAMIREZ; GUEVARA,1997). A sobrevivência de parasitas no hospedeiro em

conjunto com as alterações na resposta imune celular causada pelo HIV-1 favorece o aparecimento das recidivas.

Em relação à coinfecção HIV-1/Mtb, foi também observada ausência da resposta proliferativa ao PPD dos linfócitos T $CD4^+$ e $CD8^+$ e de linfócitos T $CD4^+$ e $CD8^+$ produtoras de IFN-γ em resposta ao PPD nos pacientes com TB-HIV virgens de HAART. Interessantemente, observamos que pacientes com TB-HIV após HAART (grupo TB-HIV-HAART) apresentaram proliferação dos linfócitos T $CD4^+$ e $CD8^+$ e expansão de células polifuncionais produtoras de IL-2 e de INF-γ e células monofuncionais produtoras de IFN-γ em resposta ao PPD.

O IFN-γ é essencial para o controle de microrganismos que infectam macrófagos, como o Mtb e a *Leishmania*. O IFN-γ é produzido durante a resposta celular Th1 e atua ativando a atividade microbicida dos macrófagos. A ausência de IFN-γ é associado ao escape do patógeno e ao estabelecimento de doença ativa. Porém, apenas a capacidade de produzir IFN-γ não indica que os linfócitos T possuem uma atividade efetora capaz de eliminar patógenos intracelulares. Tem sido demonstrada a importância do estabelecimento de uma resposta de linfócitos T $CD4^+$ e $CD8^+$polifuncionais específicas caracterizadas pela produção simultânea de IL-2, IFN-γ e TNF. Linfócitos T polifuncionais são importantes para obter uma resposta capaz de sustentar sua própria expansão e garantir uma atividade efetora. Respostas polifuncionais geralmente estão presentes em infecções com baixa carga antigênica, a exemplo de infecções latentes. Por outro lado, respostas monofuncionais produtoras de IFN-γ são predominantes em infecções agudas ou com carga antigênica elevada, como ocorre na TB ativa (HARARI et al., 2005).

Os pacientes com TB ativa, não infectados por HIV, tem um predomínio de linfócitos T $CD4^+$monofuncionais produtores de IFN-γ, enquanto os linfócitos T $CD4^+$ monofuncionais produtores de IL-2 são mais frequentes em pacientes infectados por HIV com diagnóstico de TB ativa. Dados da literatura indicam que em pacientes infectados por HIV com tuberculose latente existe um predomínio de linfócitos T $CD4^+$ e T $CD8^+$de memória efetora polifuncionais circulantes (KALOKHE et al., 2014). Estes resultados sugerem que a resposta polifuncional é realmente mais eficaz para promover o controle da infecção pelo Mtb, inclusive em indivíduos infectados pelo HIV-1.

Apesar do aumento da capacidade funcional após HAART, esta recuperação não foi suficiente para o estabelecimento de uma resposta imune capaz de conter a infecção causada pelo bacilo em pacientes infectados por HIV-1.

A maioria dos pacientes coinfectados por HIV/Mtb que faziam uso de HAART estavam a cerca de um mês em tratamento. Os resultados demonstram que HAART promove uma recuperação rápida da produção de IFN-γ em resposta a estimulação antigênica induzindo tanto células monofuncionais como polifuncionais. Tal recuperação é semelhante ao que ocorre em pacientes com TB-IRIS. A caracterização fenotípica e funcional das células produtoras de IFN-γ durante a IRIS foi realizada em poucos estudos. Os autores observaram em resposta ao PPD uma baixa resposta polifuncional e a predominância de resposta monofuncionais produtoras de IFN-γ ou TNF-α, mas não de IL-2 (BOURGARIT et al., 2009; WILSON et al., 2013). O que coincide que o perfil observado em pacientes com TB ativa infectados ou não pelo HIV.

Além do perfil de produção de citocinas, características fenotípicas dos linfócitos T $CD4^+$ e $CD8^+$ polifuncionais parecem implicar na capacidade de promover o controle de infecções. Linfócitos de memória são divididos em duas subpopulações de acordo com a expressão de CCR7 e CD45RA, moléculas associadas à migração linfocitárias (SALLUSTO et al., 1999). Após a exposição secundária ao antígeno, linfócitos T ME tem maior potencial para produzir citocinas imediatamente após a ativação, enquanto linfócitos T MC são estimulados a proliferar e diferenciar-se em células efetores para, em seguida, agir contra o antígeno (ZAPH et al., 2004). Linfócitos *naïve*s tem uma resposta menos efetiva a estimulação antigênica e produz baixos níveis de citocinas(SALLUSTO et al., 1999). Nos pacientes co-infectados por HIV-1/*Leishmania* ambas as populações de memória estavam reduzidas em comparação aos indivíduos mono-infectados. Enquanto a freqüência destas populações foram semelhantes entre os pacientes co-infectados por HIV-1/Mtb e os pacientes com TB não infectados pelo HIV. Em relação à capacidade funcional destas subpopulações, linfócitos polifuncionais nos pacientes TB-HIV-HAART eram predominantemente de células com fenótipo de memória central e naïves. No entanto, a mesma avaliação não foi realizada em pacientes co-infectados por HIV-1/*Leishmania.*

Recentemente, foi proposto que os linfócitos T $CD8^+$ tem igualmente um papel na defesa contra o Mtb. Linfócitos T $CD8^+$ e células NK são capazes de secretar IFN-γ e de direcionar suas ações citotóxicas contra macrófagos infectados (VANKAYALAPATI et al., 2005; WOODWORTH; BEHAR, 2006; DHIMAN et al., 2009). Nós avaliamos a função citotóxica de linfócitos T $CD8^+$ e de células NK em pacientes coinfectados por HIV-1/Mtb. Observamos uma alteração na atividade citotóxica das células NK em resposta ao PPD. Os resultados indicaram que a infecção pelo HIV-1 promove redução na capacidade de

degranulação das células NK contribuindo para o descontrole da infecção e desenvolvimento de TB ativa.

Outro aspecto avaliado na presente tese, foi a ocorrência de doenças oportunistas associadas à IRIS, especialmente Leishmaniose e Tuberculose. A IRIS é comumente relatada em indivíduos infectados por HIV-1 com imunossupressão grave (<50 linfócitos T $CD4^+$/μL) e elevada carga antigênica de um patógeno oportunista (French, 2012). Desta forma, a persistência de parasitas viáveis em indivíduos infectados por HIV-1, como observados na coinfecção HIV-1/*Leishmania,* pode contribuir para o surgimento da leishmaniose ou da tuberculose como manifestação de IRIS.

Recentemente, relatamos um caso de leishmaniose tegumentar associado à IRIS descrevendo as alterações imunes observadas antes e após tratamento com anfotericina B e corticosteróides (GOIS et al., 2015). Em nossa revisão de literatura identificamos 34 casos relatados de leishmaniose associada a IRIS no mundo. A maioria dos casos ocorreu, em média, quatro meses após introdução da HAART. Foram identificados como fatores associados ao desenvolvimento de IRIS a contagem de linfócitos T $CD4^+$ menor que 100 células/mm^3 antes de HAART e um aumento de cinco vezes no número inicial de linfócitos T $CD4^+$. Apresentação clínica mais frequente foi de LV, enquanto somente cinco casos de LT associados a IRIS foram descritos. É provável que o número de casos de leishmaniose associada a IRIS é subestimado devido à ausência de critérios universais para o diagnóstico. De fato, vários casos reportados na literatura não possuíam dados suficientes para confirmar o diagnóstico de IRIS.

A TB-IRIS, por outro lado, foi bastante descrita e caracterizada, pois é uma das manifestações de IRIS mais frequentes. É conhecido que a IRIS ocorre após a reconstituição quantitativa dos linfócitos T $CD4^+$ e o controle da carga viral após HAART. Vários autores descreveram uma maior expansão de células produtoras de IFN-γ em resposta ao PPD comparada a pacientes sem IRIS indicando que a recuperação da resposta imune especifica ao Mtb causa da IRIS. Além disso, foi também documentado a produção exacerbada e inespecífica de citocinas pró-inflamatórias, bem como a elevada ativação de células NK e de macrófagos. Em conjunto os dados sugerem que, a expansão de células específicas produtoras de IFN-γ não explica o desenvolvimento da IRIS. Nossa revisão de literatura evidencia o papel da imunidade inata no surgimento da IRIS.

7 CONSIDERAÇÕES FINAIS

Os resultados apresentados na presente tese indicam que a infecção pelo HIV-1 induz alterações na resposta celular de memória central e efetora aos patógenos avaliados. Essas alterações são parcialmente restauradas no curso da HAART. Estudos futuros devem ser conduzidos para melhor avaliar a dinâmica de restauração quantitativa e qualitativa dos linfócitos T de memória central e efetora específicos e no curso da HAART.

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