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TESE DE DOUTORADO

LINFÓCITOS T CD4^{+} E AS DISTINTAS APRESENTAÇÕES CLÍNICAS DA TUBERCULOSE

PAULO SÉRGIO DE MORAIS DA SILVEIRA MATTOS

Salvador – Bahia
2022

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Tese apresentada ao Curso de Pós-Graduação em Patologia Humana para a obtenção do grau de Doutor.

Orientador: Dr. Bruno Bezerril Andrade

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Paulo Sérgio de Morais da Silveira Mattos

FOLHA DE APROVAÇÃO

Salvador, 29 de abril de 2022.

COMISSÃO EXAMINADORA

Dra. Tonya Azevedo Duarte
Professora
UFBA

Dra. Iukary Oliveira Takenami
Professora
UNIVASF

Dr. Ricardo Riccio Oliveira
Pesquisador
IGM/ FIOCRUZ

Dr. Jorge Clarêncio Souza Andrade
Pesquisador
IGM/ FIOCRUZ

Dr. Bruno de Bezerril Andrade
Pesquisador
IGM/FIOCRUZ

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Aos meus tios e primos
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Aos meus sogros

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“A tarefa não é tanto ver aquilo que ninguém
viu, mas pensar o que ninguém ainda
pensou sobre aquilo que todo mundo vê.”

(**Arthur Schopenhauer**)

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RESUMO

INTRODUÇÃO: O diagnóstico da tuberculose (TB) pode ser realizado por detecção de *M. tuberculosis* (MTB) em testes microbiológicos, como baciloscopia e cultura ou ensaios de biologia molecular. A intepretação desses exames é limitada, pois não trazem informações sobre a repercussão sistêmica,celular, inflamatória e apresentam baixa sensibilidade em indivíduos paucibacilar. **OBJETIVO:** Avaliar a ativação celular e as alterações fenotípicas em linfócitos T CD4$^+$ em indivíduos com TB, coinfectados ou não com HIV. **MÉTODOS:** Foram desenvolvidos estudos e análise em dois momentos e universos. O primeiro foi um estudo de caso controle com pacientes infectados com TB residentes no Brasil. Marcadores de ativação celular (CD38, HLA-DR e/ou Ki-67) foram validados em células T CD4$^+$ específicas para MTB em discriminar TB pulmonar (TBP), TB extrapulmonar (TBEP) e TB latente (ILTB). O efeito da coinfecção pelo HIV no desempenho do diagnóstico das distintas formas de TB foi avaliado. O segundo estudo de cooorte foi desenvolvido com pacientes de TB-HIV, residentes no sul da Índia, antes e após o início da terapia antiretroviral (TARV). Nesse estudo, foram analisadas alterações fenotípicas em células T CD4$^+$, durante a ocorrência da síndrome inflamatória de reconstituição imune (SIRI) e sua relação com a inflamação sistêmica. Adicionalmente, subpopulações de linfócitos T CD4$^+$ e biomarcadores no sangue periférico foram analisados. **RESULTADOS:** Os pacientes com TBP e TBEP apresentaram uma maior frequência de células T CD4$^+$ IFN-γ^+, com expressão de CD38, HLA-DR em comparação com ILTB. As frequências de células HLA-DR+ ou Ki-67+ distinguiram com precisão TBP de TBPE. A infecção pelo HIV não afetou a capacidade desses marcadores em distinguir TB ativa de ILTB ou TBPde TBPE. No segundo estudo, a frequência de células T CD4 *naive* (CD27$^+$CD45RO$^-$), bem como de memória efetora (CD27$^-$CD45RO$^+$) distinguiram os indivíduos que desenvolveram a SIRI dos que não apresentaram, entre a 2° e6° semana após o início da TARV. Análises posteriores revelaram que TARV pode reconstituir diferentes quantidades de subconjuntos de linfócitos T CD4$^+$ com expansão preferencial decélulas CXCR3$^+$ CCR6$^-$ em pacientes com TB-SIRI. Além disso, houve expansão e restauração funcional de linfócitos T CD4$^+$ CXCR3$^-$CCR6$^+$, com expressão de marcadores de memória central (CD27$^+$CD45RO$^+$) e citocinas correspondentes, com redução nas células CXCR3$^-$CCR6$^+$ após TARV apenas naqueles que desenvolveram TB-SIRI. **CONCLUSÃO:** Os marcadores de ativação celular em células T CD4$^+$ específicas para MTB distinguiram TB ativa de ILTB e TBP de TBPE, independentemente do *status* de infecção pelo HIV. Os subconjuntos de células T CD4$^+$ estão fortemente associadas com SIRI.

Palavras-chave: Tuberculose. Células T CD4$^+$. HIV. Biomarcadores.

MATTOS, Paulo Sérgio de Morais da Silveira. **Between CD4$^+$ T lymphocytes and the different clinical presentations of tuberculosis**. 2022. 86f. 2022. 86f. Tese (Doutorado em Patologia) – Universidade Federal da Bahia, Faculdade de Medicina, Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, 2022.

ABSTRACT

INTRODUCTION: The diagnosis of tuberculosis (TB) is performed by means of the detection of *M. tuberculosis* (MTB*)* in microbiological tests, such as smear and culture and molecular biology assays. The interpretation of these tests is limited, as they do not provide information on the systemic, cellular, and inflammatory repercussions and they have low sensitivity in paucibacillary individuals. **OBJECTIVE:** To evaluate cell activation and phenotypic changes in CD4$^+$ T lymphocytes in individuals with tuberculosis, with or without HIV. **METHODS:** Initially, in a cohort from Brazil, the use of CD38, HLA-DR and/or Ki-67 cell activation markers on MTB-specific CD4$^+$ T cells to discriminate pulmonary tuberculosis (PTB) from extrapulmonary tuberculosis (EPTB) from latent tuberculosis infection (LTBI), as well as EPTB from PTB, was validated. We also tested the effect of HIV coinfection on diagnostic performance. Added to this thesis, another manuscript covering South Indian patients with TB-HIV before and after initiation of ART, determining the phenotypic changes in CD4$^+$ T cells during Tuberculosis-immune reconstitution inflammatory (SIRI) and their relationship with systemic inflammation. In this study, we analyzed subpopulations of T lymphocytes and biomarkers in peripheral blood. **RESULTS**: EPTB and PTB patients had a higher frequency of T CD4$^+$ IFN-γ^+ cells expressing CD38, HLA-DR compared to LTBI. Furthermore, the frequencies of HLA-DR$^+$ or Ki-67$^+$ cells accurately distinguished EPTB from PTB. HIV infection did not affect the ability of these markers to distinguish active tuberculosis from LTBI or EPTB from PTB. In the second study, the frequency of naive CD4 T cells (CD27$^+$CD45RO$^-$), as well as of effector memory (CD27$^-$CD45RO$^+$) distinguished individuals who developed the inflammatory reconstitution syndrome from those who did not, in the 2nd-6th week after starting ART. Further analysis revealed that ART reconstituted different amounts of subsets of CD4$^+$ T lymphocytes with preferential expansion of CXCR3$^+$ CCR6$^-$ cells in TB-SIRI patients. In addition, there was an expansion and functional restoration of CXCR3$^-$CCR6 CD4$^+$ lymphocytes expressing central memory markers (CD27$^+$ CD45RO$^+$) and corresponding cytokines, with a reduction in CXCR3$^-$CCR6$^+$ cells after ART only in those who developed TB-SIRI. **CONCLUSION**: Cell activation markers on CD4$^+$ T cells specific for MTB distinguished active TB from LTBI and EPTB from PTB, regardless of HIV infection status. CD4$^+$ T cell subsets are strongly associated with SIRI.

Keywords: Tuberculosis. CD4$^+$ T cells. HIV. Biomarker.

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LISTA DE ABREVIATURAS E SIGLAS

AIDS – Síndrome da Imunodeficiência Adquirida

BAAR – Bacilo Álcool-Ácido Resistente

BCG – Bacilo de Calmette-Guérin

CCR – Receptor de quimiocina

CD – Cluster de diferenciação

CR – Receptor de complemento

CXCR – Receptores de quimiocinas

FT – Fator tecidual

HIV - vírus da imunodeficiência humana

HLA – Antígeno leucocitário humano

IFN – Interferon

IL – Interleucina

ILTB – Tuberculose latente

Ki-67 – Marcador de Proliferação celular antígeno Ki-67

MAs – Macrófagos alveolares

MHC – Complexo Principal de Histocompatibilidade

MTB – *Mycobacterium tuberculosis*

NK – Natural Killer

PCR – Reação de polimerase em cadeia

PCR – Proteína C reativa

PPD – Derivado proteico purificado

RM – Receptor de manose

SIRI – Síndrome inflamatória de reconstituição imunológica

SNC – Sistema Nervoso Central

TARV – Terapia antirretroviral

TB – Tuberculose

TBA – Tuberculose ativa

TBEP – Tuberculose extrapulmonar

TBP – Tuberculose pulmonar

TGF – Fator de crescimento transformador

Th – T helpers

TNF – Fator de necrose tumoral

Treg – T reguladora

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1 **INTRODUÇÃO**

1.1 EPIDEMIOLOGIA

A tuberculose (TB), é um problema de saúde pública que apresenta distribuição global, com maiores registros de casos na Índia, Indonésia, China, Filipinas, Paquistão, Nigéria, Bangladesh e África do Sul (Figura 1) (ORGANIZAÇÃO MUNDIAL DA SAÚDE, 2021). Em 2020, foi responsável por infectar aproximadamente 9,9 milhões de pessoas e causar 1,5 milhões de óbitos no mundo, representando uma das principais causas de óbito por doença infecciosas, superando o HIV e perdendo apenas para a doença do coronavírus (COVID-19). Durante a pandemia da COVID-19 houve redução das taxas de casos notificados de TB em países com alta carga da doença, o que indica uma provável subnotificação do número total de casos e óbitos descritos para o período. Além disso, a pandemia prejudicou os programas de tratamento e prevenção para a erradicação da TB, anteriormente instituídos pela Organização das Nações Unidas (ONU), devido a interrupção do acesso aos serviços de saúde, redução dos recursos e o remanejamento de profissionais de saúde e equipamentos para o combate à pandemia (GLAZIOU, 2020; KANCHAR; SWAMINATHAN, 2019).

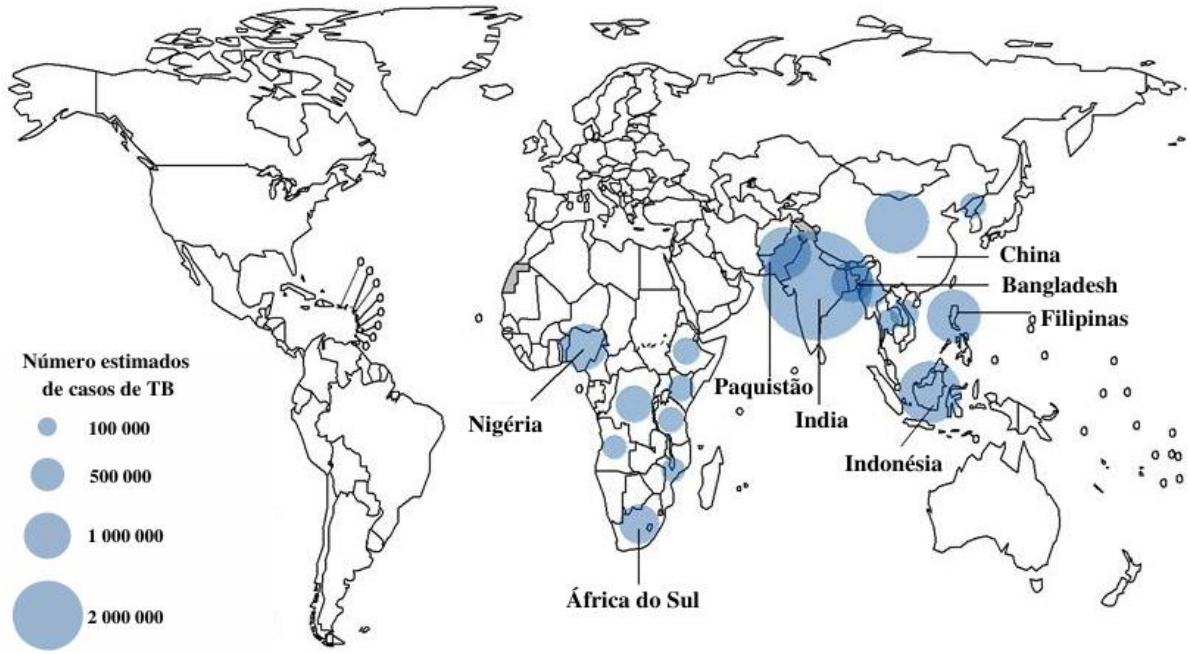

Figura 1 - Incidência estimada de tuberculose em 2020, para países com pelo menos 100.000 casos incidentes.
Fonte: Adaptado de (WHO, 2021)

Assim como outros países, o Brasil tem apresentado declínio nas taxas de incidência e mortalidade nas últimas décadas, principalmente associado à melhoria das condições de vida da população e à realização de programas de controle da TB (PELISSARI *et al.*, 2018). Entretanto, o Brasil continua entre os 30 países de alta carga para a TB, sendo, portanto, considerado prioritário para o controle da doença no mundo (ORGANIZAÇÃO MUNDIAL DA SAÚDE, 2019). Em 2020, o Brasil registrou 66.819 casos novos de TB, com um coeficiente de incidência de 31,6 casos por 100 mil habitantes. Em 2019, foram notificados cerca de 4,5 mil óbitos pela doença, com um coeficiente de mortalidade de 2,2 óbitos por 100 mil habitante. Os estados com maiores números de casos notificados de TB foi São Paulo seguido pelo Rio de Janeiro, Rio Grande do Sul, Bahia e Amazonas. As cidades que apresentaram maiores número de casos de TB nesse período foram o Rio de Janeiro, São Paulo, Manaus, Salvador, Fortaleza (ARRIAGA *et al.*, 2021).

1.2 O AGENTE ETIOLÓGICO

A TB é causada por *Mycobacterium tuberculosis* (MTB) ou *bacilo de Koch*, assim chamado por ter sido identificado pelo cientista alemão *Robert Koch*, em 1882. O MTB é uma bactéria pertencente ao complexo *Mycobacterium tuberculosis*, do qual também fazem parte as espécies *M. bovis*, *M. bovis – BCG*, *M. africanum*, *M. microti*, *M. caprae* e *M. pinnipedii* (RIOJAS *et al.*, 2018).

O MTB é um bacilo irregular de 0,3 a 0,5μm de diâmetro e de comprimento variável. Trata-se de uma bactéria intracelular facultativa, aeróbia estrita, não encapsulada, não formadora de esporos, imóvel e de crescimento lento, as colônias são visíveis em meio sólido 10 a 28 dias, o que é extremamente lenta em comparação com outras bactérias (CAMPOS, 2006).

O envoltório celular do MTB é uma estrutura multiforme. A parede celular, gerada por um complexo formado entre as moléculas de arabinogalactano, peptídeoglicanocano e ácidos micólicos (ácidos graxos complexos de 70-80 carbonos), é responsável pela forma característica da bactéria, além de conferir importantes efeitos biológicos, como a indução da formação de granuloma e atuar como barreira efetiva contra agentes antimicrobianos convencionais (ABRAHAMS; BESRA, 2016). Nela estão presentes também moléculas de glicolipídios que, em conjunto com certas proteínas, dão as características antigênicas da bactéria; e por ser o componente mais externo da estrutura bacteriana, confere proteção contra diversos fatores externos e agentes imunológicos de defesa do hospedeiro (Figura 2) (SHAKU; EALAND; KANA, 2020).

A capacidade tintorial do bacilo álcool-ácido resistente, presente em do MTB deve-se à alta composição lipídica da parede celular (aproximadamente 40-60% de seu peso) conferida pela presença dos ácidos micólicos, que garantem hidrofobicidade – que dificulta a penetração de corantes aquosos – e resistência ao processo de descoloração por álcool-ácido, à ação de agentes químicos e antibióticos. Por isso, o MTB é também chamado de Bacilo Álcool-Ácido Resistente (BAAR) (HIGHSMITH; SULLIVAN, 2019).

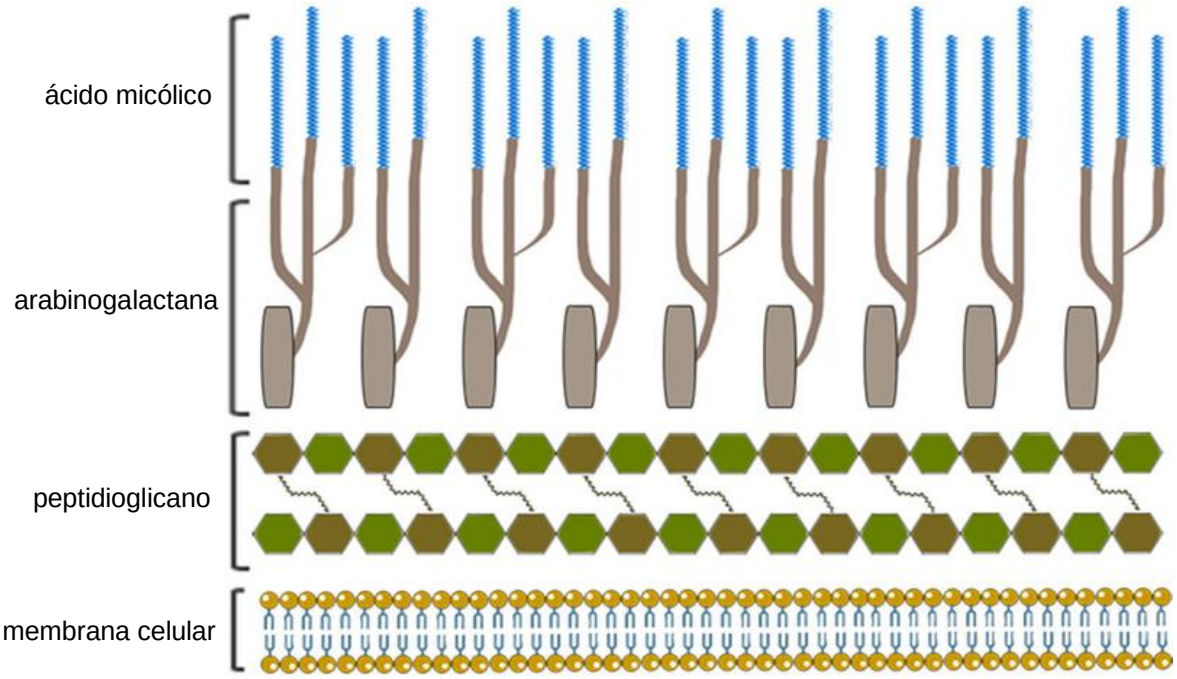

Figura 2 - Representação esquemática da estrutura da parede celular do *Mycobacterium tuberculosis.*
Fonte: Adaptado de (SENICAR et al., 2020)

1.3 TRANSMISSÃO E INFECTIVIDADE

A transmissão do MTB decorre da eliminação de gotículas de saliva contaminada, de forma direta de um doente com TB ativa pulmonar pela tosse, fala ou espirro, inaladas por outro indivíduo (Figura 3). No entanto, o contato com o bacilo, pode ou não resultar no desenvolvimento da doença (AHMAD, 2011). A chance de uma evolução favorável é determinada por fatores que podem comprometer o sistema imune como, idade avançada, desnutrição, tabagismo, infecção pelo HIV, neoplasias e outras doenças, além de outras características do agente agressor como virulência da cepa, carga bacilífera e indução de hipersensibilidade (NARASIMHAN *et al.*, 2013). As pessoas em contato próximo com casos infecciosos (familiares, profissionais de saúde, detentos, dentre outros) possuem risco elevado de infecção (MARTINEZ *et al.*, 2017).

Possíveis determinantes que levam a casos mais graves ou fatais da TB estão relacionados aos antígenos secretados, componentes de superfície celular, enzimas envolvidas no metabolismo celular e reguladores transcricionais (CHAI; ZHANG; LIU, 2018; JAIN *et al.*, 2007; SAKAMOTO, 2012).

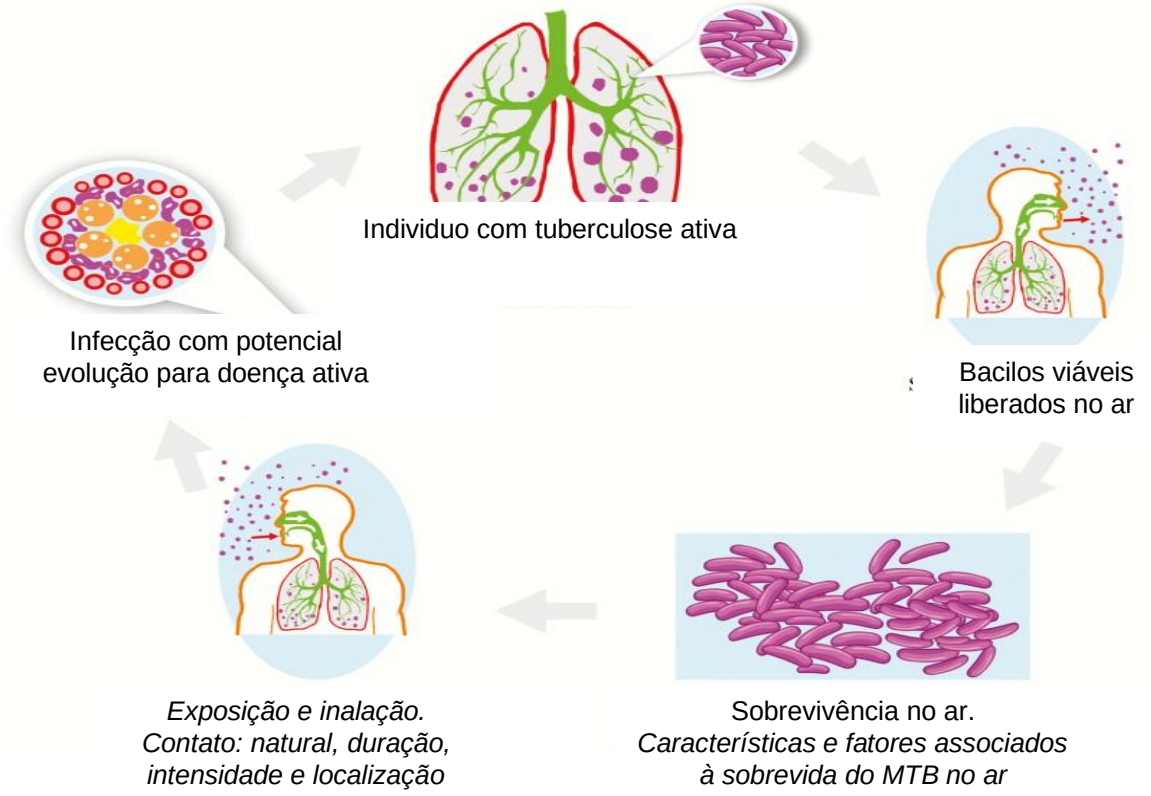

Figura 3 - Cascata de transmissão da tuberculose.
Fonte: Adaptado de (CHURCHYARD et al., 2017)

1.4 ASPECTOS CLÍNICOS

A TB primária ou primo-infecção é uma forma da doença que acomete principalmente os pulmões e linfonodos de pessoas sem imunidade específica, geralmente crianças saudáveis e adultos jovens que não foram previamente expostos ao MTB (HUNTER, 2018). A evolução ocorre de forma insidiosa e lenta nos primeiros anos após a infecção. Em alguns casos, em decorrência da disseminação dos bacilos, via hematogênica, o foco primário pode progredir e consequentemente levar ao desenvolvimento de lesões granulomatosas pequenas e difusas (TB miliar). Na TB primária também podem ser atingidas regiões extrapulmonares: meningoencefalite, pleural e pericárdio (MILBURN, 2001).

A TB pós primaria, diferentemente da TB primária, apresenta-se com maior ocorrência em adultos. Dois principais fatores estão envolvidos com o aparecimento dessa forma: 1)

reativação de uma infecção primária: o indivíduo apresenta competência imunológica e consegue deter a infecção inicial, mas a doença desenvolve-se posteriormente a partir de um foco latente; 2) reinfecção exógena: há uma nova infecção e o sistema imune não é capaz de conter a sua progressão (HUNTER, 2018). Na TB pós primária os indivíduos exibem uma infecção geralmente de curso crônico, com sintomas generalizados, como febre, adinamia, anorexia, emagrecimento e sudorese noturna, bem como sintomas específicos do local acometido (SAKAMOTO, 2012).

A maioria dos indivíduos infectados pelo MTB apresentará a infecção latente (ILTB) e apenas 5-10% desses, desenvolverá a TB ativa (TBA) (REICHLER *et al.*, 2018). Dentre os pacientes que desenvolvem TBA, 84% têm a forma pulmonar (TBP) e 16% extrapulmonar (TBEP) (ORGANIZAÇÃO MUNDIAL DA SAÚDE, 2020).

A TBP acomete principalmente os adultos, mas também são descritos casos em crianças. Os principais sintomas são tosse e expectoração, com a evolução da doença, o paciente também poderá apresentar: dor torácica, dispneia, anorexia, febre, emagrecimento, hemoptise, astenia e sudorese noturna (ALCAÏS *et al.*, 2005; MARAIS *et al.*, 2006).

A TBEP pode ocorrer durante a primo-infecção em decorrência de uma disseminação linfo-hematogênica do bacilo para diversos órgãos ou sistemas. Os sítios mais acometidos na TBEP são: pleural, meningoencefalite, ganglionar, osteoarticular, geniturinária e oftálmica (LEWINSOHN *et al.*, 2004; OHENE *et al.*, 2019). Em pessoas imunocomprometidas, como pacientes soropositivos para HIV, é descrita uma maior ocorrência de TBEP (ANTAS *et al.*, 2006; NAING *et al.*, 2013). Os pacientes com TB pleural podem apresentar tosse seca, dor torácica pleurítica (ventilatório-dependente) e dispneia dependendo do volume de líquido pleural (SHAW *et al.*, 2018). Na TB óssea e articular, as localizações mais acometidas são a coluna vertebral e as articulações do quadril e joelho. As manifestações incluem dor nas costas, sensibilidade na coluna, paraplegia, deformidades da coluna e, em casos mais graves, podem ocorrer *déficits* neurológicos irreversíveis (KHANNA; SABHARWAL, 2019; LEE, 2014). Na TB ganglionar, os linfonodos mais afetados são o cervical, mediastinais, auxiliar, inguinais epitroclear e mesentérico. Os principais sintomas incluem adenopatia, febre, emagrecimento e fistulização ganglionar (KETATA *et al.*, 2015). A TB no Sistema Nervoso Central (SNC) é a forma mais grave da TB, com alta morbimortalidade. Os achados clínicos incluem a meningite, tuberculoma, abscesso tuberculoso e infecções espinhais extradurais e intradurais (SCHALLER *et al.*, 2019).

A TB é frequentemente encontrada entre a população infectada pelo HIV. Os pacientes coinfectados por TB-HIV, apresentam pior prognóstico em comparação com pessoas não

infectadas pelo HIV, refletindo em falha do tratamento e maior morbidade e mortalidade (FEKADU *et al.*, 2020). A ocorrência de tuberculose nos pacientes HIV ocorre devido a reativação, reinfecção ou infecção primária. Os aspectos clínicos na TB-HIV dependem do grau de imunodepressão, são frequentemente acometidos os sítios extrapulmonares (KALAM AZAD; CHOWDHURY, 2022; QIAN *et al.*, 2018).

Em alguns pacientes TB-HIV após o início da terapia antirretroviral (TARV) ocorre uma ativação aberrante de respostas inflamatórias que levam a uma piora clínica ou radiológica paradoxal designada síndrome inflamatória de reconstituição imune (SIRI) (ABDOOL KARIM *et al.*, 2010). Na TB-SIRI, os indivíduos podem apresentar febre, linfoadenopatia, piora dos sintomas respiratórios, reações inflamatórias sistêmicas no(s) local(is) da infecção (AL TARIQ, 2018). Evolução clínica desfavorável e até mesmo fatal é observada em pacientes TB-SIRI que apresentam sintomas neurológicos durante a TARV. A SIRI apresenta-se em duas formas: SIRI paradoxal – ocorre geralmente após início da TARV em pacientes tratados previamente com terapia antituberculose, nesses pacientes os sintomas são recorrentes ou novos de TB; SIRI "desmascarada" – ocorre no início da TARV (até 3 meses) com manifestações clínicas e inflamação acentuadas em pacientes não tratados com terapia antituberculose ou sem diagnóstico prévio para TB no início da TARV (QUINN *et al.*, 2020).

1.5 RESPOSTA IMUNE

1.5.1 **Imunidade Inata**

Na infecção por MTB, os macrófagos alveolares (MA) residentes no pulmão compõem uma das primeiras barreiras de defesa após a inalação dos bacilos. Os MA são responsáveis por fagocitar o MTB, produzir citocinas e quimiocinas e recrutar outras células da corrente sanguínea, como macrófagos inflamatórios, neutrófilos e células dendríticas (COHEN *et al.*, 2018). O recrutamento dessas células para o sítio pulmonar, promove a formação de estruturas clássicas da TB, denominadas granulomas. O granuloma é um ambiente favorável ao crescimento e a persistência do bacilo, nesse local, o MTB fica em estado de latência e pode sobreviver durante décadas (RUSSELL *et al.*, 2009). No granuloma, também, é onde as células imunes se relacionam para promover e controlar a disseminação da infecção. Os granulomas iniciais consistem em agregados de fagócitos infectados. Quando maduro, observam-se bacilos, células gigantes, neutrófilos, células dendríticas e fibroblastos envoltos por células T e B (BOROS; REVANKAR, 2017). À medida que a infecção progride, potencialmente após algum grau

de imunossupressão, os granulomas começam a se decompor, levando à disseminação do bacilo e à reativação da TB (SILVA MIRANDA *et al.*, 2012). A reativação da TB causa manifestações clínicas e promove a transmissão da doença mediante da eliminação de MTB pelas vias aéreas (GUPTA *et al.*, 2012).

A imunidade inata desempenha um papel fundamental na TB. As células do sistema imune inato, como os neutrófilos, as células *natural killer* (NK) e os macrófagos inflamatórios são as primeiras células recrutadas para o sítio de multiplicação do bacilo (LIU; LIU; GE, 2017). No sítio de infecção, os neutrófilos secretam armadilhas extracelulares (NETs) que serão posteriormente fagocitados por macrófagos inflamatórios. Os macrófagos inflamatórios, por sua vez, tentam destruir o MTB, secretando óxido nítrico (NO) (HILDA *et al.*, 2020). As células dendríticas fagocitam MTB e migram para o linfonodo de drenagem, onde secretam antígenos, os quais serão apresentados pelas células dendríticas residentes, iniciando a ativação das células T *naive* (GUILLIAMS; LAMBRECHT; HAMMAD, 2013). As células NK produzem citocinas inflamatórias e mediam a citotoxicidade celular (KORBEL; SCHNEIDER; SCHAIBLE, 2008).

A fagocitose dos macrófagos alveolares contendo MTB, envolve diferentes receptores presentes nas células fagocíticas. Esses receptores são capazes de reconhecer opsoninas na superfície do MTB ou se ligam ao bacilo não opsonizado (HMAMA *et al.*, 2015). O MTB pode se ligar diretamente a receptores do complemento (CR), CR3, CR4 e o receptor de manose (RM) (TSOLAKI; VARGHESE; KISHORE, 2021). Esse último, considerado o melhor receptor, é uma glicoproteína transmembrana encontrada em macrófagos e células dendríticas que reconhece resíduos de manose terminal presentes nas micobactérias (TSOLAKI; VARGHESE; KISHORE, 2021). Quando existe o bloqueio dos RM e dos CR, os macrófagos podem internalizar o bacilo através do receptor do tipo scavenger (LIU *et al.*, 2019). Os receptores para a porção Fc de anticorpos (FcRs) parecem ter pouco desempenho na TB (TRAN; KIM; RELJIC, 2019).

1.5.2 **Imunidade Adaptativa**

A imunidade adaptativa contra TB é mediada principalmente por células T $CD4^+$ via apresentação dos antígenos em associação com moléculas do complexo principal de histocompatibilidade (MHC) classe II. As células T $CD8^+$, também participam da resposta imune, reconhecendo os antígenos ligados ao MHC classe I oriundos do citoplasma e podem ser estimuladas por vesículas contendo antígeno de MTB, oriundas do apoptose de macrófago infectados (DAY *et al.*, 2011; LIN; FLYNN, 2015).

1.5.2.1 Subtipos de células T

De acordo as citocinas secretadas e/ou a expressão de receptores de quimiocinas como CXCR3 e CCR6, os linfócitos podem ser classificados nos seguintes subgrupos: Th1 ($CXCR3^+CCR6^-$), Th17 ($CXCR3^-CCR6^+$), Th2 ($CXCR3^-CCR6^-$) e Th1Th17 ($CXCR3^+CCR6^+$) (MAZZONI *et al.*, 2019; SARAVIA; CHAPMAN; CHI, 2019). Os linfócitos Th1 são estimulados pelas citocinas IL-12 e IFN-γ e desempenham um papel importante na resposta protetora à TB, através da produção de TNF-α e IFN-γ (ROBERT; MIOSSEC; IMMUNOLOGY, 2021). O TNF-α desempenha um papel fundamental na contenção e proteção contra o bacilo no pulmão através da formação e manutenção do granuloma. O IFN-γ é uma citocina crucial no controle da infecção por tuberculose, é responsável por ativar as células inflamatórias e fagocíticas e inibir o crescimento bacteriano através da produção de espécies reativas de oxigênios e nitrogênios (KISUYA *et al.*, 2019). Diferentemente, os linfócitos T $CD4^+$ Th2 favorecem o estabelecimento da infecção mediado por citocinas anti-inflamatórias como IL-4, IL-5 e IL-10 (AMELIO *et al.*, 2017; HARRIS *et al.*, 2007). As células Th17 parecem contribuir para a proteção imunológica em pacientes com tuberculose, são subtipos de linfócitos de perfil pro-inflamatório, produtores de citocinas inflamatórias como IL-17A, IL-17F, IL-21 e IL-22. As células Th1Th17 (que coexpressam (CXCR3 e CCR6) foram relatadas como presentes no sangue periférico de indivíduos com ILTB. O fenótipo Th1Th17 também foi encontrado na maioria das células T $CD4^+$ específicos para MTB das vias aéreas de macacos infectados com TB (NIKITINA *et al.*, 2018; SHANMUGASUNDARAM *et al.*, 2020).

1.5.2.2 Maturação de células T

Os linfócitos T são classificados de acordo a sua maturação em: naive (T_N), memória central (T_{CM}), memória efetora (T_{EM}) e efetora (T_E) (RAPHAEL; JOERN; FORSTHUBER, 2020). Os linfócitos T naive reconhecem os antígenos do bacilo, ativam-se e assim promovem a ocorrência da expansão clonal e posterior migração dos linfócitos T efetores para o foco da infecção. As células T_{EM} residem em tecidos periféricos e produzem citocinas como IFN-γ ou IL-4 rapidamente após a ativação em resposta ao reencontro com o antígeno, como na reinfecção. As células T_{CM}, residem inicialmente nos tecidos linfoides, produzem IL-2, são células de vida longa com autorrenovação e capacidade proliferativa homeostática(ANDO *et al.*, 2020; BROERE; VAN EDEN, 2019). Na TB as células T_{CM} são encontradas mesmo após o controle e erradicação (WANG *et al.*, 2010)

1.5.2.3 Resposta imune celular na TB-SIRI

As células T $CD4^+$ IFN-γ^+ participam ativamente na patogênese da TB-SIRI. Um perfil predominantemente Th1 em $CD4^+$ IFN-γ^+ foi evidenciado no grupo com TB-SIRI em comparação com aqueles que não desenvolveram SIRI. Em um grupo de indivíduos coinfectados com TB-HIV e que desenvolveram SIRI precocemente, apresentaram menor frequência de $CD4^+$ T_E em comparação ao grupo que não desenvolveu SIRI. Na SIRI de início tardio foi observado uma menor frequência de células CD4 T_{EM} comparado ao grupo que não desenvolveu SIRI (GOOVAERTS, ODIN *et al.*, 2015; KUMAR, 2017).

Em indivíduos TB-SIRI também foi observado frequências mais altas de células T $CD8^+$, além disso, os níveis dessas células, foram correlacionadas com o resultado a baciloscopia antes do tratamento. Indivíduos com TB-SIRI exibiram frequências mais altas de T$CD8^+$ T_{EM} e frequência reduzidas de células T $CD8^+$ T_M do que não-SIRI (TIBÚRCIO *et al.*, 2022). Em outro estudo, os pacientes com TB-SIRI que desenvolveram precocemente SIRI apresentaram uma menor frequência de células $CD8^+$ T_{CM} em comparação com controles não SIRI. Diferentemente, os pacientes com TB-SIRI de início tardio demostraram frequências menores de $CD8^+$ T_{EM} e elevada frequência de células $CD8^+$ T_E em comparação com controles não-SIRI. Ambos os pacientes com TB-SIRI de início precoce e tardio apresentaram frequência mais baixas de subconjuntos $CD8^+$ T_N e T_{CM} em comparação com os pacientes TB-HIV que não desenvolveram SIRI (GOOVAERTS, ODIN *et al.*, 2015).

1.6 DIAGNÓSTICO DA TUBERCULOSE

Deve-se investigar TBP, em pessoas que apresentam tosse persistente por duas semanas ou mais; contactantes de pacientes com diagnóstico de TBA, soropositivos para o HIV; indivíduos com *diabetes mellitus;* pessoas privadas de liberdade, em situação de rua, vivendo em albergues ou instituições de longa permanência, indígenas; profissionais de saúde; pessoas expostas a sílica, dentre outras populações vulneráveis (ORGANIZAÇÃO MUNDIAL DA SAÚDE, 2021).

O diagnóstico de TBP decorre da identificação do MTB em amostra biológica, mediante realização de baciloscopia, cultura ou métodos moleculares (DELOGU; SALI; FADDA, 2013; FERRI *et al.*, 2014). As amostras utilizadas no diagnóstico geralmente são escarro, lavado broncoalveolar ou outras relacionadas com o trato respiratório. O escarro é a amostra mais utilizada devido a sua facilidade de obtenção. A obtenção dessa amostra é realizada pelo próprio

paciente em coletor estéril, após a higienização oral, antes do café da manhã, por indução de uma tosse profunda (diafragmática) (DATTA *et al.*, 2017; PENNA, 2011). O volume de escarro deve ser maior que 3mL, sendo o volume ideal de 5-10mL, isento de saliva (LEWINSOHN *et al.*, 2017). No entanto, alguns indivíduos com suspeita clínica/radiológica da TB pulmonar ou baciloscopia de escarro negativa apresentam dificuldade na expectoração espontânea, dessa forma, a amostra poderá ser obtida através técnica da indução com solução salina hipertônica, lavado broncoalveolar ou broncoscopia (BROWN *et al.*, 2007). A broncoscopia, apesar de ser mais invasiva dentre as técnicas rotineiras, é capaz também de detectar alterações das estruturas brônquicas, principalmente em casos de hemoptise (CONDE *et al.*, 2000; MCWILLIAMS *et al.*, 2002).

A baciloscopia ou a pesquisa de bacilos álcool-ácido resistentes (BAAR) é um exame rápido, de baixo custo, capaz de diagnosticar pacientes com TBA (LEWINSOHN *et al.*, 2017). O exame consiste na pesquisa direta de bacilos, microscopicamente identificados pelas suas características morfotintoriais após a coloração de Zeel-Nielsen. A sensibilidade da baciloscopia direta em escarro chega em média a 80% na presença de lesões cavitadas e extensas, porém em pacientes com lesão mínima a acurácia é reduzida, apresentando sensibilidade de 40-60% (CUDAHY; SHENOI, 2016). Além disso, a baciloscopia apresenta menor sensibilidade em pacientes coinfectados por HIV (20-60%) (MÉNDEZ-SAMPERIO, 2017). A microscopia de fluorescência pode incrementar a capacidade de detecção do bacilo em 10%, quando comparada à microscopia com luz convencional. Um aumento de 10-20% na sensibilidade da baciloscopia também pode ser obtido após centrifugação e/ou sedimentação do escarro (WORKINEH *et al.*, 2017).

O padrão ouro para o diagnóstico de TB consiste na detecção do MTB por métodos moleculares ou cultura. A cultura microbiológica consiste em semear a amostra respiratória em meios sólidos como o Löwenstein-Jensen ou Ogawa-Kudoh e avaliar em um período de duas até oito semanas, observando há presença ou não de crescimento micobacteriano (PENNA, 2011). Como alternativa para otimizar o elevado tempo de diagnóstico da cultura convencional, foi desenvolvido o sistema de diagnóstico de TB em meio líquido, o *Mycobacterium Growth Indicator Tube* (MGIT). No entanto, essa metodologia apresenta elevado custo para a sua execução, o que inviabiliza implementação em larga escala nos serviços de saúde. Após a confirmação do crescimento do bacilo, métodos bioquímicos e fenotípicos ou técnicas moleculares são necessários para distinguir as espécies do complexo *Mycobacterium tuberculosis* das não tuberculosas (PENNA, 2011). A partir da identificação do complexo *Mycobacterium tuberculosis* é necessário realizar o teste de sensibilidade a antimicrobianos

(DROBNIEWSKI; RÜSCH-GERDES; HOFFNER, 2007). Os métodos disponíveis para o teste de sensibilidade a antimicrobianos são os métodos das proporções, os quais utilizam meio sólido e tem seu resultado em até 42 dias de incubação. Os antimicrobianos rotineiramente testados são estreptomicina, isoniazida, rifampicina, etambutol e pirazinamida (MIOTTO *et al.*, 2018). Para os casos de tuberculose multidroga resistente (TB-MDR, multidrug-resistant) são testados fármacos de segunda linha (PENNA, 2011). Testes moleculares como o *line probe assay* ou o GeneXpert são capazes de detectar o MTB pela presença do ácido nucleico presente no material respiratório. O *line probe assay*, além de identificar o complexo *M. tuberculosis*, detecta a resistência à rifampicina e à isoniazida e, em teste separado, também pode avaliar resistência à fluoroquinolonas e a drogas injetáveis (MORGAN *et al.*, 2005). A sensibilidade e a especificidade desse teste foram de 96,7% e 98,8% na avaliação de resistência à rifampicina, respectivamente, enquanto, em relação a resistência à isoniazida, essas foram de 90,2% e 99,2%, respectivamente (NATHAVITHARANA *et al.*, 2017). Métodos de imunodiagnóstico para a TB existentes incluem o teste tuberculínico (teste de Mantoux ou PPD - *purified protein derivative*) ou ensaios de liberação de interferon-gama (IGRA - Interferon Gamma Release Assay). Esses testes são capazes de detectar a resposta do hospedeiro frente a infecção por MTB, porém apresentam limitações que dificultam a sua utilização no diagnóstico da doença ativa, por exemplo, ambos são incapazes de distinguir doença latente da TBA, além disso o PPD também pode ser positivo em pessoas não infectadas e vacinadas recentemente com o BCG (HAAS; BELKNAP, 2019)

Nos casos suspeitos de TBEP, o diagnóstico é frequentemente presuntivo, realizado a partir da coleta de amostra clínica dependendo do local suspeito da doença e requer procedimentos invasivos, pois o diagnóstico clínico não é suficiente, exigindo exames complementares a fim de investigar e elucidar a suspeita. Baciloscopia e a cultura do material do local suspeito da doença apesar de indicados, apresentam baixa sensibilidade por se tratar de uma forma paucibacilar (LEWINSOHN *et al.*, 2017). Outros exames complementares a partir de amostras clínicas podem auxiliar o diagnostico, como métodos moleculares, histopatológicos e exames de imagem (SILVA *et al.*, 2021). Na TB ganglionar, o diagnóstico é realizado através de aspirado por punção por agulha ou ressecção do linfonodo. Na TB pleural, o líquido pleural apresenta-se como um exsudato, com predomínio de linfócitos, mas com baixo rendimento para a pesquisa de BAAR (<5%) e cultura de micobactérias. Níveis elevados de adenosina desaminase no líquido pleural são considerados como fortemente sugestivos do diagnóstico de TB pleural (ARAKAKI-SANCHEZ; BRITO, 2011). A cultura de escarro induzido

pode ser positiva em até 50% dos casos, mesmo que a única alteração visível na radiografia de tórax seja o derrame pleural (CONDE *et al.*, 2000).

Metodologias baseados no sangue como transcriptoma, proteoma, metaboloma e ensaios celulares têm sido propostos como ferramentas promissoras para o diagnóstico de TB (DUTTA *et al.*, 2020; GARAY-BAQUERO *et al.*, 2020; PENG; CHEN; ZHANG, 2020; YONG *et al.*, 2019). No entanto, a abordagem de ensaios baseados em sangue através das "ômicas" apresenta limitações que dificultam a sua utilização principalmente em ambientes com recursos limitados, devido aos altos custo operacionais e infraestrutura.

Estudos focados em ensaios celulares demostraram que marcadores de ativação, diferenciação ou proliferação expressos em células T $CD4^+$ IFN-γ^+ e/ou TNF-α^+ podem contribuir para o diagnóstico, monitoramento e predição do tratamento da TB (VICKERS *et al.*, 2020). O HLA-DR, é um receptor expresso na superfície celular do antígeno do complexo de histocompatibilidade principal de classe II humano (MHC-II). O CD38 é uma glicoproteína transmembranar expressa em várias células apresenta propriedades ectoenzimáticas, catalisa a síntese e hidrólise de NAD ou ADP-ribose cíclica. O Ki-67 é uma proteína nuclear expressa em célula ciclada é utilizada como marcador de proliferação celular (HIZA *et al.*, 2022; ORCIANI *et al.*, 2008; TIPPALAGAMA, RASHMI *et al.*, 2021).

A expressão de CD27, IFN-γ, CD38, HLA-DR e Ki-67 em células T $CD4^+$ específicos para MTB foram capazes de distinguir TBA e ILTB antes e após o tratamento (ACHARYA *et al.*, 2020; ADÉKAMBI *et al.*, 2015). A expressão de CD38 e HLA-DR em T $CD4^+$ dos pacientes TBA reduziram rapidamente no primeiro mês de tratamento, enquanto a expressão de CD27 e Ki-67 reduziu mais lentamente e esse resultado está correlacionado com a carga bacteriana (PRIYANTO *et al.*, 2021). A expressão de CD27 em células T $CD4^+$ também foi capaz de distinguir vacinados com BCG dos pacientes com TBA e ILTB (ADEKAMBI *et al.*, 2012; PETRUCCIOLI *et al.*, 2015)

2 **JUSTIFICATIVA**

A tuberculose (TB) é uma das doenças infectocontagiosas que mais causa mortes no mundo, constituindo-se um problema de saúde pública. A fim de combater a infecção no organismo, a célula T $CD4^+$ tem um papel importante na proteção contra TB, pois controla a infecção primária e promove a formação de granuloma. No entanto, em indivíduos imunocompetentes, como crianças e coinfectados com HIV, essa resposta celular não é totalmente eficaz e pode ocorrer a ativação e disseminação do bacilo. Apesar do seu papel protetor, a célula T $CD4^+$ também está associada a patogênese da TB em indivíduos coinfectados com HIV que desenvolvem a síndrome inflamatória da reconstituição imune. Nesses pacientes, a restauração das células $TCD4^+$ após a terapia antiretroviral, promove uma resposta imune exagerada aos bacilos da TB ou seus antígenos residuais.

Dessa forma, são necessárias novas abordagens que caracterizem o papel desempenhado pelas células T $CD4^+$ e suas subpopulações frente as diferentes apresentações clínicas da TB. Essa caracterização, pode contribuir futuramente com o diagnóstico, monitoramento e predição do tratamento de pacientes com TB com ou sem HIV.

3 **PARTE I**

3.1 OBJETIVOS

3.1.1 **Objetivo geral**

Avaliar a capacidade dos marcadores de ativação celular em diferenciar TB pulmonar (TBP) e extrapulmonar (TBEP) e infecção latente (ILTB).

3.1.2 **Objetivos específicos**

- Comparar a expressão de CD38, HLA-DR e Ki-67 em células T $CD4^+$ IFN-γ^+ entre indivíduos com TB ativa e TBL;
- Verificar a acurácia de células T $CD4^+$ IFN-γ^+ expressando CD38, HLA-DR, Ki-67 em distinguir TBEP de ILTB e TBP;
- Avaliar a expressão de CD38, HLA-DR e Ki-67 em células T $CD4^+$ IFN-γ^+ entre indivíduos HIV com TBP e TBEP.

3.2 MANUSCRITO I

Differential expression of activation markers by *Mycobacterium tuberculosis*-specific $CD4^+$ T cell distinguishes extrapulmonary from pulmonary tuberculosis and latent infection

O trabalho avalia a capacidade dos marcadores de ativação celular em diferenciar tuberculose pulmonar e extrapulmonar e infecção latente.

Resumo

Os indivíduos com tuberculose pulmonar (TBP) ou extrapulmonar (TBEP) apresentaram frequências mais altas de células T $CD4^+$ que expressam CD38, HLA-DR ou Ki-67 em comparação com indivíduos que possuíam tuberculose latente (ILTB). As frequências de células HLA-DR^+ ou Ki-67^+ distinguiram com precisão TBP de TBEP. Os marcadores $CD38^+$, HLA-DR^+ ou Ki-67^+ foram capazes em distinguir indivíduos com infecção ativa de latente ou indivíduos com TBP de TBEP independentemente da infecção por HIV.

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Clinical Infectious Diseases

MAJOR ARTICLE

Differential Expression of Activation Markers by *Mycobacterium tuberculosis*-specific CD4$^+$ T Cell Distinguishes Extrapulmonary From Pulmonary Tuberculosis and Latent Infection

Paulo S. Silveira-Mattos,[1,2,3] Beatriz Barreto-Duarte,[1,2,4] Beatriz Vasconcelos,[1,2,5] Kiyoshi F. Fukutani,[1,2,3] Caian L. Vinhaes,[1,2,3] Deivide Oliveira-de-Souza,[1,2,3] Chris C. Ibegbu,[6,7] Marina C. Figueiredo,[8] Timothy R. Sterling,[8,a] Jyothi Rengarajan,[6,7,a] and Bruno B. Andrade[1,2,3,4,5,8,9,a]

[1]Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Bahia, Brazil; [2]Multinational Organization Network Sponsoring Translational and Epidemiological Research Initiative, Fundação José Silveira, Salvador, Bahia, Brazil; [3]Curso de Medicina, Faculdade de Tecnologia e Ciências, Salvador, Bahia, Brazil; [4]Universidade Salvador, Laureate Universities, Salvador, Bahia, Brazil; [5]Escola Bahiana de Medicina e Saúde Pública, Salvador, Bahia, Brazil; [6]Emory Vaccine Center, Emory University School of Medicine, Atlanta, Georgia, USA; [7]Division of Infectious Disease, Department of Medicine, Emory University School of Medicine, Atlanta, Georgia, USA; [8]Division of Infectious Diseases, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee, USA; and [9]Wellcome Centre for Infectious Diseases Research in Africa, Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa

Background. Diagnosis of active tuberculosis (ATB) currently relies on detection of *Mycobacterium tuberculosis* (Mtb). Identifying patients with extrapulmonary TB (EPTB) remains challenging because microbiological confirmation is often not possible. Highly accurate blood-based tests could improve diagnosis of both EPTB and pulmonary TB (PTB) and timely initiation of anti-TB therapy.

Methods. A case-control study was performed using discriminant analyses to validate an approach using Mtb-specific CD4$^+$T-cell activation markers in blood to discriminate PTB and EPTB from latent TB infection (LTBI) as well as EPTB from PTB in 270 Brazilian individuals. We further tested the effect of human immunodeficiency virus (HIV) coinfection on diagnostic performance. Frequencies of interferon-γ$^+$CD4$^+$T cells expressing CD38, HLADR, and/or Ki67 were assessed by flow cytometry.

Results. EPTB and PTB were associated with higher frequencies of CD4$^+$T cells expressing CD38, HLADR, or Ki67 compared with LTBI (all P values < .001). Moreover, frequencies of HLADR$^+$ (P = .03) or Ki67$^+$ (P < .001) cells accurately distinguished EPTB from PTB. HIV infection did not affect the capacity of these markers to distinguish ATB from LTBI or EPTB from PTB.

Conclusions. Cell activation markers in Mtb-specific CD4$^+$T cells distinguished ATB from LTBI and EPTB from PTB, regardless of HIV infection status. These parameters provide an attractive approach for developing blood-based diagnostic tests for both active and latent TB.

Keywords. tuberculosis; biomarker; extrapulmonary TB; T cells; immune activation.

Diagnosis of active tuberculosis (ATB) disease currently relies on microbiologic tests such as acid-fast smear and culture and on molecular polymerase chain reaction–based assays such as GeneXpert [1] that detect *Mycobacterium tuberculosis* (Mtb) in patients' sputum. However, the sensitivity of these tests can be low, particularly in extrapulmonary TB (EPTB), in which the bacillary burden is low [2]. Thus, better methods are needed to identify EPTB as well as to discriminate between EPTB, pulmonary TB (PTB), and asymptomatic latent TB infection (LTBI).

EPTB involves organs other than the lungs, such as the lymph nodes (LNs), the pleura, and meninges, and occurs with increased frequency in immunocompromised persons, including those living with human immunodeficiency virus (HIV) [3]. Diagnosis of EPTB is often more difficult than PTB because patients are more likely to have negative sputum-based tests. Indeed, radiographic-based diagnosis and empirical data on response to anti-TB therapy are commonly used to guide diagnosis of EPTB [4]. Thus, more sensitive and specific diagnostic assays for EPTB that are faster and less invasive would be a great advance for the field.

Current diagnostic tests for LTBI (eg, the tuberculin skin test and interferon gamma [(IFN)-γ] release assays) are unable to distinguish between LTBI and ATB and have decreased sensitivity in persons living with HIV. A diagnostic test that could accurately distinguish between latent and active TB, including among persons living with HIV, would substantially improve our ability to accurately diagnoses and treat these 2 disease states.

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[a]T. R. S., J. R., and B. B. A. equally contributed to this work.

Correspondence: B. B. Andrade, Laboratório de Inflamação e Biomarcadores, Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão, 121, Candeal, Salvador, Bahia 40296–710, Brazil (bruno.andrade@fiocruz.br).

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We have previously identified a blood-based assay in which Mtb-specific $CD4^+$T cells are examined for activation and proliferation markers for diagnosis of active TB [5]. In this assay, the frequency of Mtb-specific $CD4^+$ T cells expressing the immune activation markers CD38 and HLADR as well as the intracellular proliferation marker Ki67, can accurately identify ATB and successfully distinguish ATB from LTBI in persons from Georgia in the United States [5] and the Western Cape, South Africa [6]. Subsequently, it was demonstrated that these markers can also identify ATB in individuals living with HIV [6]. However, the performance of these diagnostic assays for identifying EPTB has not been previously explored. In the present study, we sought to extend our previously published findings on PTB to additional populations by evaluating cryopreserved peripheral blood mononuclear cell (PBMC) samples from Brazilian patients with EPTB, PTB, and LTBI. We report that $CD38^+$ IFN-γ^+, $HLADR^+$IFN-γ^+, and Ki-67^+IFN-γ^+ $CD4^+$ T cells successfully distinguished ATB from LTBI and EPTB from PTB, regardless of HIV status.

METHODS

Clinical Study Design

A case-control study was performed using cryopreserved PBMC samples and corresponding clinical and epidemiological data obtained from participants enrolled in a translational study performed at the Instituto Brasileiro para Investigação da Tuberculose (IBIT) and at the Hospital Especializado Octavio Mangabeira (HEOM), Salvador, Bahia, northeast Brazil, between December 2015 and January 2018. The parent study was focused on characterization of inflammatory markers in different clinical forms of TB and recruited 1792 individuals with presumptive TB at the referral primary care clinic at IBIT. These patients underwent clinical assessments and radiological (chest x-ray) examination. In addition, acid-fast bacilli (AFB) screening in sputum smears (by microscopy) and sputum cultures (Lowenstein–Jensen solid cultures) was performed in all patients. At this stage, 235 (13%) individuals were diagnosed with culture-confirmed PTB, and 215 (12%) had PTB excluded and were suspected to have EPTB. Further investigation to confirm EPTB was conducted at a TB referral site at HEOM by performing LN fine needle aspirates (with AFB screening and culture) for TB lymphadenitis and by pleural fluid drainage with lung biopsy for pleuropulmonary TB. Among the confirmed EPTB cases (n = 211, 7%), there were 102 with TB lymphadenitis, 105 with pleuropulmonary TB, 1 case of TB meningitis, 2 had spinal TB, and 1 had abdominal tuberculomas. All individuals were tested for HIV; those who tested positive had $CD4^+$ T-cell counts and HIV viral loads (RNA copies/μL) assessed. All patients who screened positive for HIV were diagnosed at the time of study enrollment and had not been treated with antiretroviral therapy previously. The parent study also included participants who were asymptomatic contacts of TB index cases. At the time of study enrollment, individuals not living with HIV who tested positive for QuantiFERON TB Gold-in-Tube (QFT) enzyme-linked immunosorbent assay (Qiagen) were considered to have LTBI, and individuals who were QFT-negative were considered uninfected healthy controls (HCs).

At the time of study enrollment and prior to initiation of anti-TB treatment, 10 mL of venous blood was collected in sodium heparin tubes for isolation of PBMCs from a subset of participants who consented to blood collection. Cells were cryopreserved in liquid nitrogen at the biorepository of the Laboratory of Inflammation and Biomarkers, Fundação Oswaldo Cruz, Salvador, Brazil. For the immunological assays performed in the present study, selected samples from individuals with confirmed PTB and EPTB were matched on age (±5 years) and sex, with subgroups of patients living with and without HIV as well as within HCs and those with LTBI. For this study, only patients with TB lymphadenitis without pulmonary involvement were included in the EPTB group. Samples used for flow cytometry studies and characteristics of the corresponding study participants are shown in Table 1. Sample sizes were determined based on calculations of study power of 80% (alpha error, 5%) to detect differences in median frequencies of T-cell subsets >2% between active and latent TB, based on a previous study from our group [5].

Flow Cytometry

Cryopreserved PBMCs were thawed and resuspended in 1640 Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum at 10^6 cells per well in 96-well plates and rested for 2 hours at 37°C in 5% CO_2. Cells were washed and resuspended in complete media with Brefeldin-A (Biolegend, San Diego, CA) and Monensin (Biolegend, San Diego, CA) to block cytokine secretion and stimulated with ESAT-6 and CFP-10 peptide pools (10 μg/mL) overnight at 37°C in 5% CO_2. Cells were then stained for cell surface markers with the following panel of antibodies: CD3 APC-CY7 (clone SK7), CD4 PerCp-Cy5.5 (clone L200), HLADR PE-Cy7 (clone L243), and CD38 PE (clone HB7), all from BD Biosciences. Cells were then fixed and permeabilized using the Foxp3 Fixation and Permeabilization Buffer (eBioscience). Intracellular staining was performed to detect IFN-γ Alexa Fluor 700 (clone B27) and Ki67 FITC (clone B56), all from BD Bioscience. Acquisition of stained cells was performed using a BD LSRFortessa cell analyzer (BD Bioscience, San Jose, CA) and analyzed using FlowJo software (BD Bioscience, San Jose, CA). Overall gating strategies together with representative plots are shown in Supplementary Figure 1.

Statistical Analyses

Median values with interquartile ranges were compared using the Mann-Whitney *U* test. Receiver operator characteristic (ROC) curve analysis was used to test the ability of frequencies

Table 1. Characteristics of the Study Participants

Characteristic	Healthy Controls	Latent Tuberculosis Infection	PTB/HIV−	PTB/HIV+	EPTB/HIV−	EPTB/HIV+	*P* Value
N	20	50	50	50	50	50	
Age, median (interquartile range), y	25 (20–32)	27 (20–33)	29 (19–34)	27 (19–31)	25 (21–29)	26 (20–32)	>.999
Male, no. (%)	10 (50)	25 (50)	25 (50)	25 (50)	25 (50)	25 (50)	>.999
Non-white race, no. (%)	17 (85)	45 (90)	45 (90)	48 (96)	43 (86)	49 (98)	.198
Illicit drug use, no. (%)	2 (10)	1 (2)	6 (12)	12 (14)	3 (6)	18 (36)	**<.001**
Smoking, no. (%)	1 (5)	3 (6)	5 (10)	10 (20)	3 (6)	7 (14)	.152
Alcohol abuse, no. (%)	5 (25)	10 (20)	12 (14)	22 (44)	21 (42)	18 (36)	.050
Prior tuberculosis, no. (%)	0 (0)	0 (0)	0 (0)	1 (2)	1 (2)	1 (2)	NA
Acid-fast bacilli smear grade, no. (%)							
0	20 (100)	50	0 (0)	0 (0)	0 (0)	0 (0)	<.001
1+/scanty	0 (0)	0 (0)	1 (2)	30 (60)	15 (30)	35 (70)	
2+	0 (0)	0 (0)	24 (48)	15 (30)	30 (60)	13 (26)	
≥3+	0 (0)	0 (0)	25 (50)	5 (10)	5 (10)	2 (4)	

The Kruskal-Wallis test was used to compare continuous variables between the groups and the distributions of age, while the Pearson χ^2 test was used to compare frequencies. All tuberculosis patients had a positive culture for *Mycobacterium tuberculosis*. *P* values in bold font are statistically significant. Acid-fast bacilli smear grade was compared between PTB and EPTB groups with or without HIV using the Pearson χ^2 test (the healthy control and latent tuberculosis infection groups were excluded from this analysis). Smear grade from sputum samples for PTB patients or lymph node aspirates for EPTB.

Abbreviations: EPTB, extrapulmonary tuberculosis; HIV, human immunodeficiency virus; NA, nonapplicable; PTB, pulmonary tuberculosis.

of $CD38^+$, $HLADR^+$, and $Ki67^+$ $CD4^+$ T cells to distinguish ATB from LTBI and PTB from EPTB in individuals not living with HIV. The overall accuracy of the biomarkers was examined by comparing the area under the curve with C-statistics. The Fisher exact test was used to compare frequencies of virologically suppressed individuals living with HIV between PTB and EPTB groups. Spearman correlation rank analysis was performed to test the correlations between $CD4^+$T-cell counts and frequency of IFN-γ^+ $CD4^+$ T cells expressing CD38, HLADR, or Ki67 in patients living with HIV. A *P* value of < .05 was considered statistically significant after adjustment for multiple comparisons using the Holm-Bonferroni method. The statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software Inc) and JMP 14.0 software.

Ethics Statement

This study was conducted according to the principles expressed in the Declaration of Helsinki and approved by the Maternidade Climério de Oliveira Ethics Committee, Federal University of Bahia. Written informed consent was obtained from all participants.

RESULTS

Characteristics of the Study Population

The study groups were similar with regard to age and sex and most of the clinical and epidemiological characteristics. The highest frequency of reported illicit drug use was observed in EPTB patients living with HIV (n = 18, 36%, *P* < .001; Table 1). Additional analyses revealed that patients with active TB (PTB or EPTB) who had HIV coinfection exhibited lower smear grade values more frequently compared with those not living with HIV (Table 1).

Higher Frequencies of Mtb-specific $CD4^+$ T Cells Expressing CD38, HLADR, or Ki67 in Brazilian Patients With ATB Compared With LTBI

Frequencies of IFN-γ producing Mtb-specific $CD4^+$ T cells expressing CD38, HLADR, and Ki67 were compared between ATB and LTBI patients. We found higher frequencies of ESAT-6/CFP-10–specific IFN-γ^+CD4 T-cells expressing the immune activation markers CD38 and HLADR as well as the intracellular proliferation marker Ki67 in ATB patients (Figure 1A). Next, we used cutoff values for these markers, which were established in a previous study from our group [5], and reexamined the discriminatory power in our study sample. Importantly, ROC curve analysis confirmed that each biomarker had the potential to identify ATB cases with high overall accuracy (Figure 1B). These results validated the use of the $CD4^+$ T-cell activation markers as potential diagnostic biomarkers for ATB in this study population.

Evaluating the Predictive Value of $CD38^+$IFN-γ^+, $HLADR^+$IFN-γ^+, and $Ki67^+$IFN-γ^+CD4^+ T Cells in Distinguishing EPTB from LTBI and PTB

Next, we investigated whether expression of CD38, HLADR, and Ki-67 on antigen-specific IFN-γ^+ CD4 T cells could identify patients with EPTB. We stimulated PBMCs from individuals not living with HIV with EPTB and compared their activation profiles to those with PTB and LTBI and with HCs. $CD4^+$ T cells from EPTB, PTB, and LTBI groups exhibited similar frequencies of IFN-γ–producing $CD4^+$ T cells in response to in vitro stimulation with ESAT-6/CFP-10 peptides (Figure 2A). However, when the activation and proliferation markers CD38, HLADR, and Ki67 were assessed within the gate of IFN-γ^+ $CD4^+$ T cells in the stimulated conditions, major differences were observed between the groups, with both disease groups displaying significantly higher frequencies of cells expressing these markers compared with the LTBI group

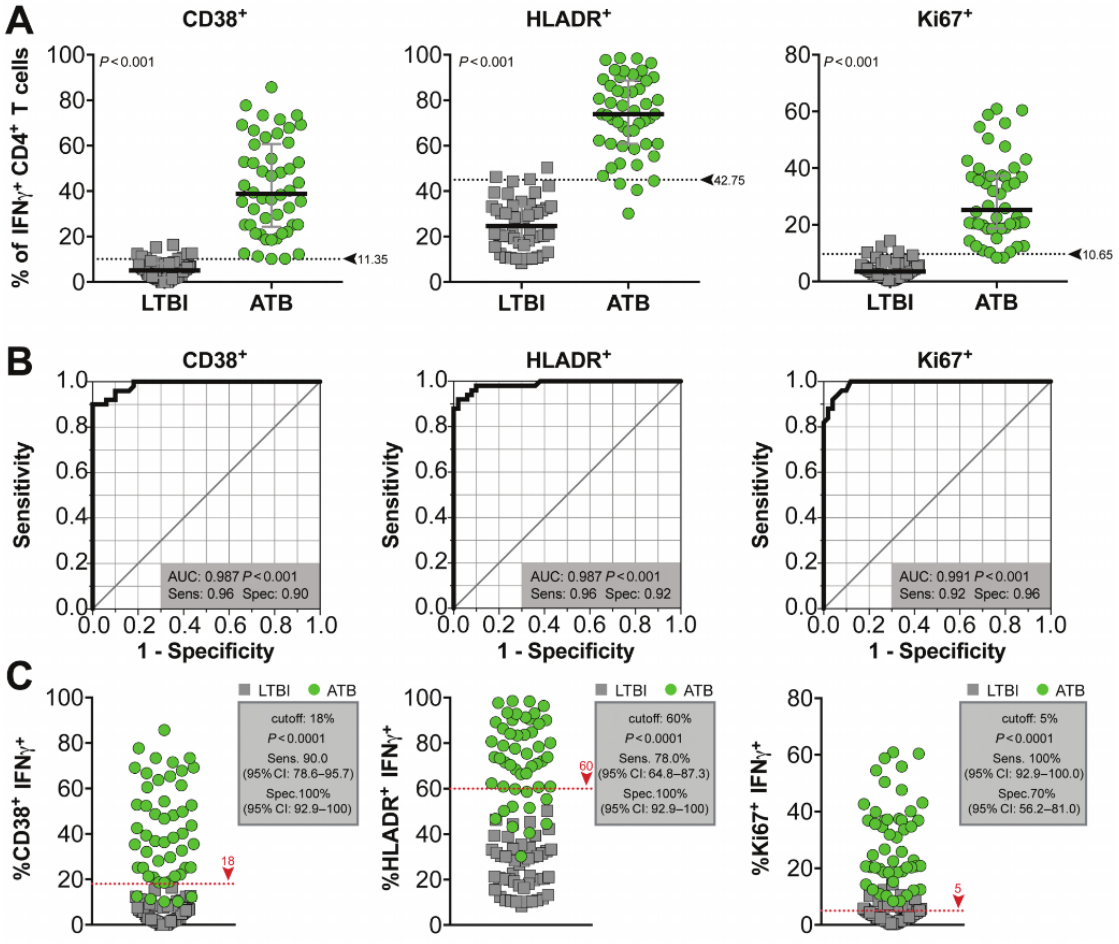

Figure 1. Expression of CD38, HLADR, and Ki67 on IFN-γ+CD4+ T cells distinguishes active from latent tuberculosis (TB). *A*, Frequencies of CD38+, HLADR+, and Ki67+cells within IFN-γ+ CD4+ T cells were examined in peripheral blood mononuclear cells stimulated with ESAT6-CFP10 peptide pools (10 μg/mL) from individuals with latent TB infection (LTBI; n = 50) or active TB (ATB; n = 100). Lines represent median values and interquartile ranges. Data were analyzed using the Mann-Whitney *U* test. *B*, Receiver operator characteristic (ROC) curve analyses of the frequency of CD38+, HLADR+, or Ki67+IFN-γ+ CD4+ T cells were used to test accuracy to distinguish ATB from LTBI. In (*A*), dashed lines represent the discrimination thresholds obtained in ROC curve analysis. *C*, Graphs in (*A*) were merged. The red, dashed lines represent the discrimination threshold for each marker and show cutoff values of 18%, 60%, and 5% for CD38+IFN-γ+, HLADR+IFN-γ+, and Ki67+IFN-γ+, respectively. Such thresholds were published previously [5]. Abbreviations: AUC, the area under the curve; CI, confidence interval; IFN-γ, interferon gamma; Sens, sensitivity; Spec, specificity.

(Figure 2A). Frequencies of CD4+ T cells expressing any of the 3 biomarkers tested were significantly higher in EPTB patients compared with those with LTBI (Figure 2A). Interestingly, the frequencies of cells expressing HLADR or Ki67, but not CD38, were higher in patients with EPTB compared with those with PTB (Figure 2A). ROC analysis confirmed that the frequency of cells expressing these markers was able to distinguish EPTB from PTB with high accuracy. The highest performance was achieved when the discriminant model was composed of data on simultaneous expression of the CD38, HLADR, and Ki67 (Figure 2B).

Frequencies of Activated Mtb-specific IFN-γ+CD4+ T Cells Expressing CD38, HLADR, and Ki67 in PTB and EPTB Are Comparable in Individuals Living With and Without HIV

Next, we sought to determine whether the ability of Mtb-specific T cells expressing CD38, HLADR, and Ki67 to identify PTB and EPTB is altered by HIV status. We stratified the PTB and EPTB groups according to HIV status and determined the frequencies of CD38+ IFN-γ+, HLADR+ IFN-γ+, and Ki67+IFN-γ+ CD4+ T cells in each group. As shown in Figure 3A, HIV status did not substantially alter the frequencies of cells expressing CD38, HLADR, or Ki67. Previous studies have suggested that progression of HIV disease gradually impairs the capacity to

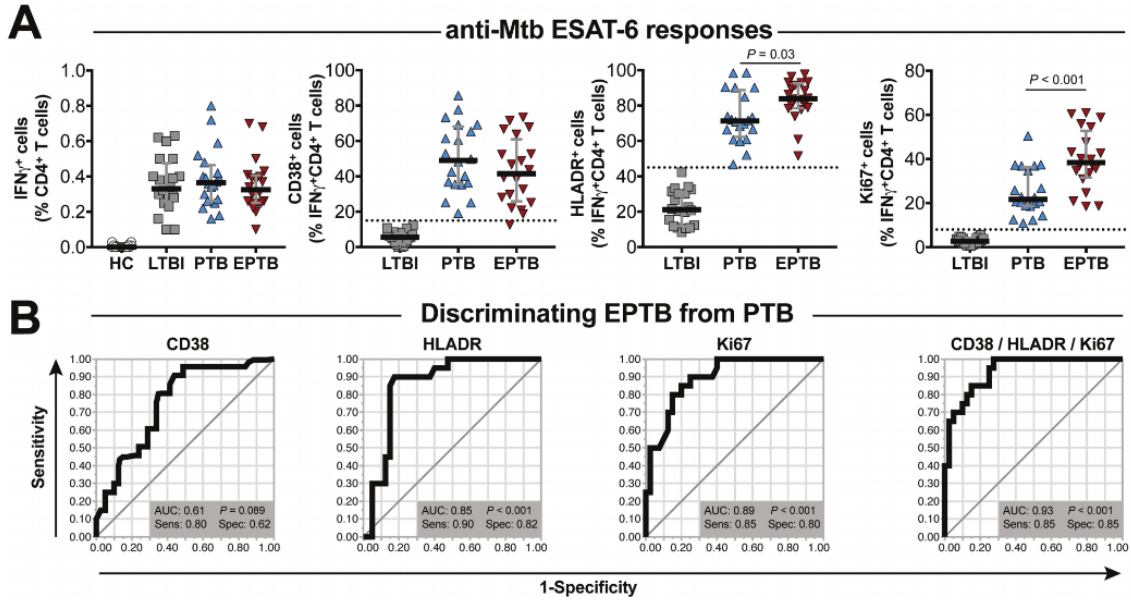

Figure 2. In individuals without human immunodeficiency virus (HIV), EPTB can be distinguished from PTB based on frequencies of IFN-γ$^+$CD4$^+$ T-cell lymphocytes expressing HLADR$^+$ and Ki67$^+$. *A*, Frequencies of total IFN-γ$^+$CD4$^+$ T cells as well as of CD38$^+$, HLADR$^+$, and Ki67$^+$ cells within IFN-γ$^+$ CD4$^+$ T-lymphocytes from peripheral blood mononuclear cells stimulated with ESAT6-CFP10 peptide pools (10 μg/mL) obtained from HIV-unexposed healthy controls (n = 20), LTBI (n = 50), EPTB (n = 50), or PTB patients (n = 50). Lines represent median values and interquartile ranges. Data from EPTB and PTB were compared using the Mann-Whitney *U* test. *B*, Receiver operator characteristic curve analyses of frequencies of CD38$^+$, HLADR$^+$, and Ki67$^+$ and when all parameters were considered simultaneously to distinguish EPTB and PTB patients. Abbreviations: AUC, area under the curve; EPTB, extrapulmonary tuberculosis; IFN-γ, interferon gamma; LTBI, latent tuberculosis infection; Mtb, *Mycobacterium tuberculosis*; PTB, pulmonary tuberculosis; Sens, sensitivity; Spec, specificity.

restrain Mtb growth, thus, favoring bacterial dissemination and extrapulmonary manifestations of TB [7, 8]. In our study, the frequency of virologically suppressed patients was just slightly lower in EPTB patients compared with those with PTB, without reaching statistical significance (Figure 3B). Nevertheless, median total CD4$^+$ T-cell counts were lower in EPTB vs PTB (P = .039; Figure 3C), although all patients living with HIV included in the study had total CD4$^+$ T-cell counts above 350 cells/μL and were thus not highly immunosuppressed. We further tested correlations between the total CD4$^+$ T-cell count values and the frequencies of Mtb-specific CD4$^+$ T cells expressing CD38, HLADR, or Ki67 in the subgroup of patients living with HIV. We found that the frequency of CD38$^+$ Mtb-specific CD4$^+$ T cells was positively correlated with total CD4$^+$ T-cell counts only in EPTB patients, with all other associations not reaching statistical significance (Figure 3D). Figure 3E shows the Spearman correlation plot of such significant association (r = .42, P = .039).

DISCUSSION

In the present study, we explored the quantification of Mtb-specific CD4$^+$ T cells expressing activation markers CD38 and HLADR and the intracellular proliferation marker Ki67 to diagnose ATB in a Brazilian population. Our primary results extend results from our previous studies in patients from Georgia in the United States and the Western Cape, South Africa [5], to Brazilian patients. We demonstrate that ATB patients from Brazil express higher frequencies of Mtb-specific CD4$^+$ T cells expressing CD38, HLADR, and Ki67 when compared with individuals with LTBI. To reliably validate results from our earlier studies in our Brazilian population, we used cutoff values previously established for each marker. Using these cutoffs, we observed that quantification of cells expressing these activation markers could reliably distinguish active from latent TB in this study population with high specificity and sensitivity, as reported previously [9]. Interestingly, similar to IFN-γ release assays, IFN-γ$^+$ CD4$^+$ T cells did not distinguish ATB from LTBI. Our findings represent an important step toward validating these 3 blood-based diagnostic biomarkers and demonstrate their high reliability when used in patient populations from different geographical locations.

Importantly, our studies also show that these biomarkers are useful for diagnosing EPTB. Extrapulmonary disease accounts for about 20%–50% of reported TB cases [10]. The most frequent locations are the pleura, LNs, bones and joints, the central nervous system, and gastrointestinal or genitourinary areas. Such anatomical sites represent a challenge for direct visualization of Mtb and therefore hinder accurate diagnosis.

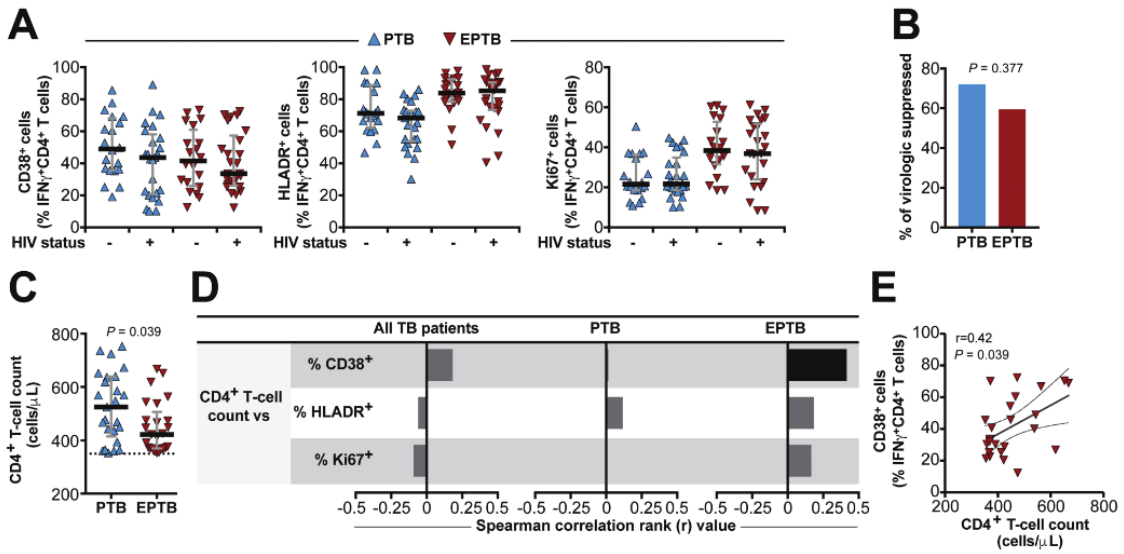

Figure 3. Frequencies of IFN-γ$^+$ CD4$^+$ T cells expressing CD38, HLADR, or Ki67 are not substantially altered in active TB patients living with HIV. *A*, Frequencies of IFN-γ$^+$ CD4$^+$ T cells expressing CD38, HLADR, or Ki67 from peripheral blood mononuclear cells stimulated with ESAT6-CFP10 peptide pools (10 μg/mL) obtained from with PTB and or EPTB stratified by HIV status (50 patients living with HIV and 50 patients not living with HIV for each disease group). Lines represent median values and interquartile ranges. Data were compared between patients living with HIV and patients not living with HIV using the Mann-Whitney *U* test. *B*, The proportion of patients living with HIV presenting with virological suppression (HIV RNA <80 RNA copies/μL) was compared between PTB and EPTB groups using the Fisher exact test. *C*, CD4$^+$T-cell counts were compared between PTB and EPTB patients living with HIV using the Mann-Whitney *U* test. *D*, Spearman correlations between CD4$^+$ T-cell counts and frequency of IFN-γ+ CD4+ T lymphocytes expressing CD38, HLADR, or Ki67 in patients living with HIV. Bars represent the strength of correlation (r values). Black bars indicate a statistically significant correlation, while gray bars were nonsignificant. *E*, The representative plot of correlation between CD4$^+$T-cell counts and frequency of IFN-γ$^+$ CD4$^+$ T cells expressing CD38 in EPTB patients living with HIV lines represent linear regression with a 95% confidence interval. Abbreviations: EPTB, extrapulmonary TB; HIV, human immunodeficiency virus; IFN-γ, interferon gamma; PTB, pulmonary TB; TB, tuberculosis.

According to the World Health Organization, diagnosis of EPTB is currently based on a positive culture from sputum or extrapulmonary sites, positive histology, or strong clinical evidence consistent with active EPTB. Notably, 2 of these 3 criteria require invasive techniques that can delay the diagnosis of TB [11, 12]. Thus, blood-based markers are attractive for the diagnosis of EPTB and represent a desirable alternative tool for diagnosing PTB and EPTB. Several recent studies have explored nonsputum-based approaches for diagnosing ATB, including transcriptome [13], metabolome [14], proteome [15], and cellular assays [16]. We recently found that a combination of 3 plasma markers can distinguish EPTB from PTB and also from HCs in children [17]. In adults, plasma markers have also been shown to differ between PTB and EPTB [9]. However, these studies were exploratory and lacked specificity for Mtb infection. The present study significantly advances the field because it not only validates previously reported cellular biomarkers for identifying ATB in a Brazilian population [5] but also expands its potential use by accurately identifying EPTB cases. Thus, our results demonstrate that ATB patients can be identified by higher frequencies of CD38$^+$ IFN-γ$^+$, HLADR$^+$ IFN-γ$^+$, and Ki67$^+$ IFN-γ$^+$ CD4$^+$ and that persons with EPTB are distinguished by even higher values of such markers compared with those with either LTBI or PTB disease alone.

Diagnosis of TB in individuals living with HIV remains challenging. HIV-induced immunosuppression leads to reduced frequency of cavitation, further dampening the sensitivity of sputum-based [18] or radiographic-based assessments. Although clinical/empiric diagnoses are often used, these approaches can also be problematic since clinical manifestations of TB (pulmonary and extrapulmonary) are usually atypical. Moreover, the delayed time to diagnosis in individuals living with HIV via microbiological-based techniques directly affects clinical prognosis, with increased odds of death and treatment failure [19, 20]. Our finding that the higher frequencies of Mtb-specific CD4$^+$ T cells expressing CD38, HLADR, and Ki67 present in ATB patients with PTB and EPTB disease compared with those with LTBI were not significantly influenced by HIV infection status is therefore of great interest. Our studies in HIV-coinfected patients corroborate previously reported observations on PTB from South Africa [21, 22] and extend the utility of these biomarkers to diagnosis of EPTB in populations living with and without HIV in Brazil. Thus, the Mtb-specific T-cell–based assay described here can be used as a blood-based diagnostic tool for both PTB and EPTB for all individuals with presumptive TB, independent of their HIV coinfection status. Although flow cytometry–based TB diagnostic tests may not be feasible in some programmatic settings, efforts are underway to

develop reasonably priced commercial assays that could be used in clinical reference laboratories.

Our study has some limitations. We performed a cross-sectional investigation and examined samples obtained from a single time point, which precluded us from making conclusions about the utility of using these biomarkers for assessing response to treatment. However, we have previously reported on this in other populations [5]. The number of individuals investigated in this study was relatively small but was determined by the study power calculations, and the groups were carefully matched to reduce the influence of potential confounding factors. In addition, because we did not have drug-susceptibility test results, we were unable to investigate whether drug-susceptible vs drug-resistant Mtb strains affected immune responses; this is an important avenue for future investigation. Among the persons living with HIV who were investigated here, $CD4^+$ T-cell counts were ≥350 cells/μL, so no highly immunosuppressed patients were examined. Future studies are necessary to test whether the performance of the biomarkers evaluated here are similar at lower $CD4^+$ T-cell counts. All patients with PTB in our study exhibited positive AFB in sputum smears. A potential area of interest for future studies is testing the performance of these biomarkers in persons with lower mycobacterial loads (eg, AFB-negative). Despite these limitations, our study presents robust data that demonstrate that biomarkers on $CD4^+$ T cells can distinguish EPTB from LTBI as well as from PTB cases.

In summary, we have validated a reliable, fast, noninvasive blood-based approach that accurately identified patients with active TB compared to LTBI and also distinguished PTB from EPTB—and both independent of HIV status. These observations are relevant to guide further development of point-of-care diagnosis for both active and latent TB.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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Potential conflicts of interest. The authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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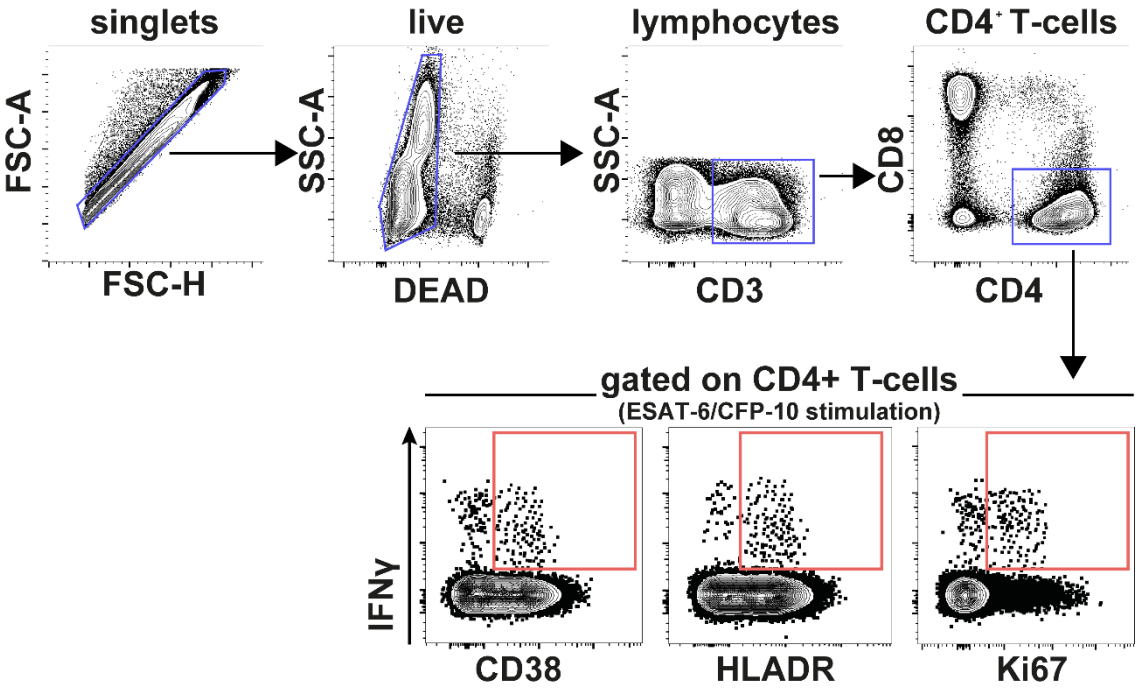
singlets
live
lymphocytes
CD4+ T-cells
FSC-A
FSC-H
SSC-A
DEAD
SSC-A
CD3
CD8
CD4
gated on CD4+ T-cells
(ESAT-6/CFP-10 stimulation)
IFNγ
CD38
HLADR
Ki67

4 **PARTE II**

4.1 OBJETIVOS

4.1.1 **Objetivo geral**

Analisar as alterações fenotípicas nas células T $CD4^+$ em indivíduos coinfectados por HIV-TB antes e depois do início da TARV.

4.1.2 **Objetivos Específicos**

- Observar a expressão dos marcadores de memória CD27 e CD45RO em linfócitos T $CD4^+$;
- Investigar a expressão dos receptores de quimiocinas CXCR3 e CCR6 em linfócitos T $CD4^+$;
- Avaliar simultaneamente os marcadores de memória e receptores de quimiocinas;
- Verificar a associação entre os subconjuntos de células T $CD4^+$ com níveis circulantes de várias citocinas pró-inflamatórias e quimiocinas, bem como a frequência de subtipos de monócitos;
- Associar as mudanças na inflamação sistêmica e as frequências dos subtipos de células T $CD4^{+.}$

4.2 MANUSCRITO II

Differential expression of CXCR3 and CCR6 on $CD4^+$ T-lymphocytes with distinct memory phenotypes characterizes tuberculosis-associated immune reconstitution inflammatory syndrome.

Este trabalho identifica alterações fenotípicas nas células T $CD4^+$ durante a coinfecção por MTB-HIV com SIRI e sua relação com a inflamação sistêmica.

Resumo:

Os indivíduos coinfectados por tuberculose-HIV com SIRI nas 2–6 semanas após o início da terapia antiretroviral apresentaram maior frequência de células T $CD4^+$ *naive* ($CD27^+CD45RO^-$), bem como menor frequência de células T $CD4^+$ de memória efetora ($CD27^-$ $CD45RO^+$) do que os indivíduos não SIRI. Nos pacientes SIRI-TB a TARV promoveu uma expansão das células T $CD4^+$ expressando $CXCR3^+$ e $CCR6^-$. Além disso, houve uma expansão

e restauração funcional dos linfócitos T $CD4^+$ de memória central ($CD27^+$ $CD45RO^+$) $CXCR3^+$ $CCR6^-$ e citocinas correspondentes, com redução nas células $CXCR3^-CCR6^+$ após iniciação com a TARV apenas naqueles que desenvolveram SIRI-TB.

Este trabalho foi publicado no periódico internacional *Scientific Reports* (Fator de Impacto JCR 2020 = 4.3).

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Differential expression of CXCR3 and CCR6 on CD4$^+$ T-lymphocytes with distinct memory phenotypes characterizes tuberculosis-associated immune reconstitution inflammatory syndrome

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Paulo S. Silveira-Mattos[1,2], Gopalan Narendran[3], Kevan Akrami[1,4], Kiyoshi F. Fukutani[1,2], Selvaraj Anbalagan[3], Kaustuv Nayak[3], Sudha Subramanyam[3], Rajasekaran Subramani[3], Caian L. Vinhaes[1,2], Deivide Oliveira-de Souza[1,2], Lis R. Antonelli[5], Kumar Satagopan[6], Brian O. Porter[7], Alan Sher[8], Soumya Swaminathan[3], Irini Sereti[7] & Bruno B. Andrade[1,2,9,10,11,12]

Immune reconstitution inflammatory syndrome (IRIS) occurs in up to 40% of individuals co-infected with pulmonary tuberculosis (PTB) and HIV, primarily upon antiretroviral therapy (ART) initiation. Phenotypic changes in T-cells during TB-IRIS and their relationship with systemic inflammation are not fully understood. In this prospective cohort study, we followed 48 HIV-positive patients with PTB from South India before and after ART initiation, examining T-lymphocyte subsets and inflammatory biomarkers in peripheral blood. Quantification of naïve (CD27$^+$CD45RO$^-$) as well as effector memory CD4$^+$ T cells (CD27$^-$CD45RO$^+$) at weeks 2–6 after ART initiation could distinguish TB-IRIS from non-IRIS individuals. Additional analyses revealed that ART reconstituted different quantities of CD4$^+$ T lymphocyte subsets with preferential expansion of CXCR3$^+$ CCR6$^-$ cells in TB-IRIS patients. Moreover, there was an expansion and functional restoration of central memory (CD27$^+$CD45RO$^+$) CXCR3$^+$CCR6$^-$ CD4$^+$ lymphocytes and corresponding cytokines, with reduction in CXCR3$^-$CCR6$^+$ cells after ART initiation only in those who developed TB-IRIS. Together, these observations trace a detailed picture of CD4$^+$ T cell subsets tightly associated with IRIS, which may serve as targets for prophylactic and/or therapeutic interventions in the future.

[1]Instituto Gonçalo Moniz, Salvador, Bahia, Brazil. [2]Multinational Organization Network Sponsoring Translational and Epidemiological Research (MONSTER) Initiative, Fundação José Silveira, Salvador, Bahia, Brazil. [3]National Institute for Research in Tuberculosis, Chennai, India. [4]Division of Infectious Diseases, Department of Medicine, University of California, San Diego, United States of America. [5]Laboratório de Biologia e Imunologia de Doenças Infecciosas e Parasitárias, Instituto René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, Minas Gerais, Brazil. [6]Government Hospital of Thoracic Medicine, Tambaram, Chennai, India. [7]Clinical HIV Pathogenesis Section, Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, United States of America. [8]Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, United States of America. [9]Wellcome Trust Centre for Infectious Disease Research in Africa, Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, Republic of South Africa. [10]Escola Bahiana de Medicina e Saúde Pública (EBMSP), Salvador, Bahia, Brazil. [11]Universidade Salvador (UNIFACS), Laureate Universities, Salvador, Bahia, Brazil. [12]Division of Infectious Diseases, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee, United States of America. Paulo S. Silveira-Mattos and Gopalan Narendran contributed equally. Correspondence and requests for materials should be addressed to B.B.A. (email: bruno.andrade@fiocruz.br)

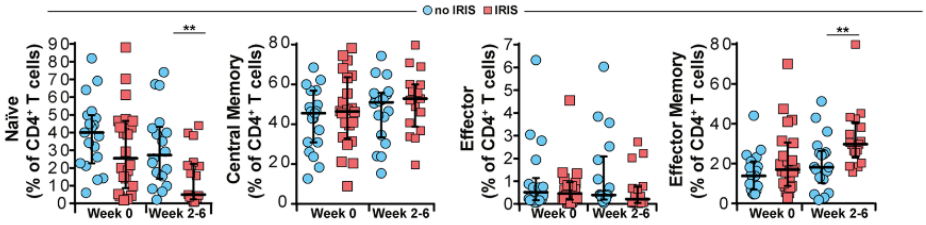

Figure 1. Surface expression of memory markers CD27 and CD45RO on $CD4^+$ T-lymphocytes from TB-HIV co-infected patients prior to and following ART initiation. Frequencies of $CD27^+CD45RO^-$ (naïve), $CD27^-CD45RO^+$ (central memory), $CD27^-$ $CD45RO^-$ (effector) and $CD27^-CD45RO^+$ (effector memory) $CD4^+$ T cells from whole blood obtained at week 0 (pre-ART) and at week 6 after ART initiation (in non-IRIS patients, n = 22) or at the time of TB-IRIS event (n = 26). Lines represent median values and interquartile ranges. Data were analyzed using the Mann-Whitney test or Wilcoxon matched-pairs test for paired analyses within each study group. $^{**}P < 0.01$ after adjustment for multiple measurements.

Immune reconstitution inflammatory syndrome (IRIS) is the paradoxical clinical or radiological worsening of a disease or condition that occurs after the initiation of antiretroviral therapy (ART) in HIV infected individuals despite effective virological suppression[1]. IRIS has a propensity to occur when HIV patients are concomitantly infected with other opportunistic pathogens such as *Mycobacterium tuberculosis* (Mtb)[1]. In this setting, previous studies have shown that immune reconstitution triggers aberrant activation of inflammatory responses leading to IRIS[2]. The reported incidence of tuberculosis (TB)-associated IRIS (TB-IRIS) ranges from 2%[3] to 54%[4], depending on factors such as the TB endemicity in the region, the degree of immunodeficiency and the mycobacterial antigen load prior to ART initiation[5].

The pathogenesis of IRIS remains unclear but appears to require two elements: (i) failure of the immune system to eliminate the pathogen (s), leading to persistent and high burden of infection concurrent with (ii) and abrupt immune recovery in response to ART[6]. IRIS is characterized by a heightened and dysregulated activation of pathogen-specific T-lymphocytes. Recent studies, including ours, have shown that frequency of Mtb-specific circulating $CD4^+$ T cells against Mtb is intimately associated with onset and occurrence of IRIS[7] when compared to individuals who do not develop such outcome[8–12].

Several risk factors have been associated with the development of IRIS such as increased levels of pro-inflammatory cytokines in peripheral blood, as well as degree of lymphopenia prior to ART, the latter being poorly understood[1,13,14]. It is known that lymphocyte depletion alone in the context of HIV and TB occurs due to a direct negative impact on bone marrow as well as apoptosis and lysis of cytotoxic T cells mediated by antibodies[15].

The detailed participation of T cells in TB-IRIS is not completely described. Since IRIS can sometimes occur prior to quantitative CD4 recovery, functional restoration, rather than a mere increase in T cell number, may play a role in its pathogenesis[8,9,15,16]. Here, we describe TB-IRIS in a TB and HIV treatment naïve population focusing on the relative frequency of various memory and T-helper subsets of $CD4^+$ lymphocytes as defined by chemokine receptor expression.

Results

TB-IRIS is associated with altered frequencies of naïve and effector memory $CD4^+$ T cells.

Surface expression of CD27 and CD45RO was used to define naïve, memory and effector phenotypes in $CD4^+$ T cells[7] in our study population prior to ART initiation and then at 2–6 weeks following treatment. At enrollment pre-ART, the frequency of naïve $CD4^+$ T cells ($CD27^+CD45RO^-$) was similar between TB-HIV co-infected patients who developed IRIS and those who did not (Fig. 1). Interestingly, the frequency of these cells was substantially lower in TB-IRIS patients at the time of the IRIS event, compared to non-IRIS patients at equivalent timepoints (Fig. 1). In addition, percentages of both central memory ($CD27^-CD45RO^+$) and effector ($CD27^-CD45RO^-$) cells were not different between TB-IRIS and non-IRIS patients at pre-ART as well as at week 2–6 post-ART initiation (Fig. 1). Of note, the frequency of effector memory CD4 cells ($CD27^-CD45RO^+$) was similar between the study groups at pre-ART but substantially increased during the IRIS events compared to that in non-IRIS patients points after ART initiation (Fig. 1). Our findings indicate that use of CD27 and CD45RO markers on $CD4^-$ T cells at pre-ART does not accurately predict and differentiate patients who will develop TB-IRIS from those who will not before ART commencement. However, quantification of naïve and effector memory $CD4^+$ T cells after ART initiation could potentially identify TB-IRIS from non-IRIS individuals.

Antiretroviral therapy initiation leads to substantial alterations of CXCR3 and CCR6 expressing $CD4^+$ T cell subsets in TB-HIV co-infected patients. We next evaluated the differential expression of CXCR3 and CCR6 on T cells. We found that there were major changes in the frequencies of $CD4^-$ T cell subsets differentially expressing CXCR3 and CCR6 following ART initiation in this study population (Fig. 2A). In patients who did not develop IRIS, we observed a substantial increase in frequency of $CXCR3^+CCR6^+$ and a significant decrease in the percentage of $CXCR3^-CCR6^-$ the lymphocytes from pre-ART to week 2–6 post-ART initiation (Fig. 2A). In contrast, ART did not impact the frequencies of $CXCR3^+CCR6^-$ and $CXCR3^-CCR6^+$ cells in non-IRIS patients (Fig. 2A). In the group of patients who experienced IRIS during follow-up, we noticed a dramatic increase in the frequencies of both $CXCR3^+CCR6^-$ cells and $CXCR3^+CCR6^+$ lymphocytes (Fig. 2A),

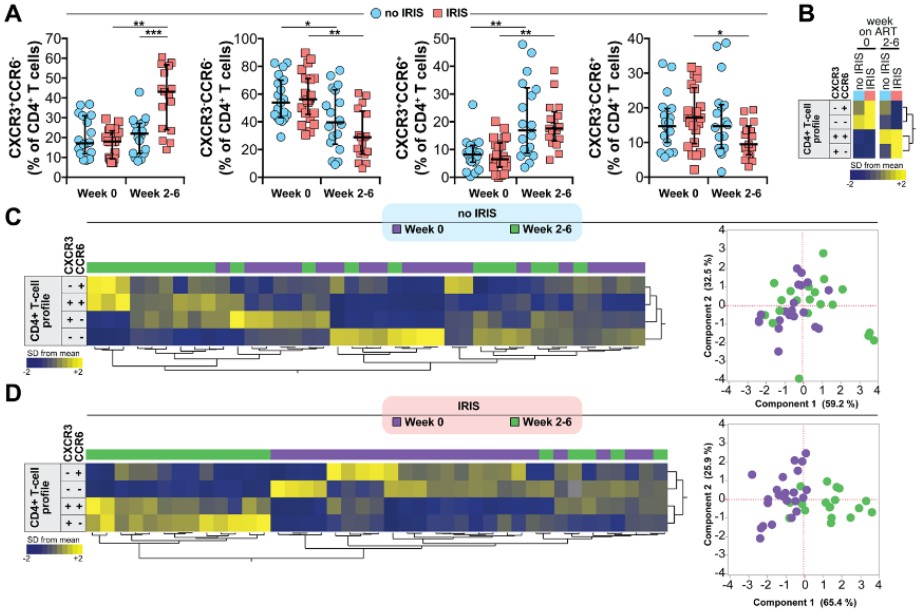

Figure 2. Expression of chemokine receptors CXCR3 and CCR6 in $CD4^+$ T-lymphocytes from TB-HIV co-infected patients prior to and following ART initiation. (**A**) Frequency of $CXCR3^-CCR6^-$, $CXCR3^-CCR6^-$, $CXCR3^+CCR6^+$ and $CXCR3^-CCR6^+$ $CD4^+$ T cells were evaluated in whole blood obtained at week 0 (pre-ART) and at week 6 after ART initiation (in non-IRIS patients, n = 22) or at the time of TB-IRIS event (n = 26). Lines represent median values and interquartile ranges. Data were analyzed using the Mann-Whitney test or Wilcoxon matched-pairs test for paired analyses within each study group. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, after adjustment for multiple measurements. (**B**) Hierarchical cluster analysis of the z-score normalized average frequency values of the $CD4^+$ T cell phenotypes is shown to summarize the overall trends of data variation between the study groups and timepoints. Yellow color represents the highest values whereas blue color indicates the lowest values observed for each cell type. Additional hierarchical cluster analyses of the z-score normalized frequency values of the $CD4^+$ T cell phenotypes per study participant at pre-ART (**C**) or at week 6 post-ART initiation/time of IRIS event (**D**) were performed to evaluate whether the overall profile of T cell frequencies could differentiate IRIS from non-IRIS patients. These results were confirmed by principal component analysis (PCA) (C and D, left panels).

while the percentages of $CXCR3^-CCR6^-$ and $CXCR3^-CCR6^+$ cells were significantly diminished (Fig. 2A) upon ART initiation.

We next performed a hierarchical cluster analysis of the median frequencies of CD4 T cells expressing CXCR3 and CCR6 in each study group and time point. This approach revealed that ART initiation was associated with a distinct expression profile independent of the patient groups (Fig. 2B). Further analyses using hierarchical clustering of individual values, as well as a principal component analysis (PCA), indicated that in non-IRIS patients, the differential expression of CXCR3 and CCR6 could not distinguish the study timepoints (pre-ART and week 2–6 post-ART initiation) (Fig. 2B). In the group of TB-IRIS patients, the frequencies of the different $CD4^+$ T cell subsets examined were distinct between the study time points (Fig. 2C). These findings argue that while the frequencies of $CD4^+$ T cell subsets differentially expressing CXCR3 and CCR6 are affected by ART, there is a unique expansion of $CXCR3^+CCR6^-$ and $CXCR3^+CCR6^+$ cells in TB-IRIS patients after ART initiation.

Assessment of CXCR3, CCR6 expression and memory cell markers identifies IRIS events after ART initiation. We tested the combination of memory/naïve and chemokine receptor markers that could better characterize TB-IRIS. A hierarchical cluster analysis of the median frequencies of cells expressing CD27, CD45RO, CXCR3 and CCR6 indicated that ART initiation selectively led to changes in the expression profiles in both groups of IRIS and non-IRIS patients (Fig. 3A). Furthermore, a PCA model indicated that the overall expression profile of these cell surface markers was similar between IRIS and non-IRIS groups at pre-ART, whereas important differences were evident at week 2–6 post ART between the IRIS and non-IRIS groups (Fig. 3B). The most significant variables driving these differences were the frequencies of effector and effector memory $CXCR3^+CCR6^+$cells as well as central memory $CXCR3^+CCR6^-$ lymphocytes (Fig. 3B). This finding suggests that these cells were highly associated with the occurrence of TB-IRIS in TB-HIV co-infected patients initiating ART.

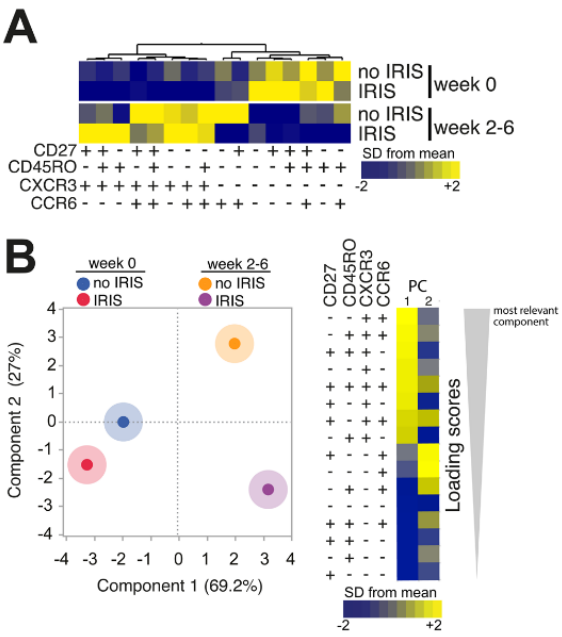

Figure 3. TB-IRIS patients can be distinguished from non-IRIS based on simultaneous assessment of memory markers and chemokine receptors. (**A**) Hierarchical cluster analysis of the z-score normalized average frequency values of the indicated $CD4^+$ T cell phenotypes is shown to summarize the overall trends of data variation between the study groups and timepoints. Yellow color represents the highest values whereas blue color indicates the lowest values observed for each cell type. (**B**) A PCA model was employed to test whether a combination of the memory cell markers and chemokine receptors could cluster patients with IRIS vs. non-IRIS at week 0 (pre-ART) and at week 6 post-ART initiation (right panel). Loading Scores reflect the strength that the combination of markers represents in each principal component shown (left panel).

Expansion of $CXCR3^+CCR6^-$ and of central memory $CXCR3^-CCR6^+$ lymphocytes is strongly associated with systemic inflammation typical of IRIS in TB-HIV co-infected patients receiving ART. We next ascertained whether subsets of $CD4^+$ T cells described above are correlated with plasma levels of a large panel of cytokines, chemokines and growth factors, frequencies of monocyte subsets, in the entire study population (n = 48) before and after ART initiation. Prior to ART initiation, there were no significant correlations between the frequency of $CD4^+$ T cell subsets and monocyte frequencies or with plasma biomarker concentrations (Fig. 4A,B), except for interleukin (IL)-7, which was negatively correlated with frequency of $CXCR3^+CCR6^-$ cells. In contrast, frequency of $CXCR3^+CCR6^-$ cells at week 2–6 of ART exhibited positive correlations with frequency of pro-inflammatory monocytes ($CD14^{++}CD16^-$) as well as levels of several key mediators of inflammation, including C-reactive protein (CRP), IL-6, interferon (IFN)-γ, IL-1β, IL-17, IL-18, soluble tissue factor (sTF) and tumor necrosis factor (TNF)-α (Fig. 4A). In addition, the percentage of central memory $CXCR3^-CCR6^-$ cells was also directly associated with systemic concentrations of pro-inflammatory mediators, including type I and II IFNs, IL-1β, IL-1Ra, IL-6, IL-15, IL-18 and TNF-α (Fig. 4B).

More detailed analyses were performed to better understand the relationships between changes in concentrations of inflammatory markers and frequencies of monocyte subsets from pre-ART to time of TB-IRIS event or equivalent timepoint and frequencies of the distinct $CD4^+$ T cell subsets depicted above. Using Spearman correlation matrices, we found that increases in levels of innate and adaptive immune activation markers were associated with frequencies of $CXCR3^-CCR6^-$ cells, but not of the other subsets (Fig. 5). Interestingly, changes in monocytes subsets, previously described as predictors of TB-IRIS[17], were associated with increased frequencies of $CXCR3^+CCR6^-$ cells in peripheral blood after ART initiation (Fig. 5). Indeed, increased frequency of $CD14^{++}CD16^-$ pro-inflammatory monocytes from baseline to week 2–6 of ART positively correlated with the proportion of $CXCR3^+CCR6^-$ cells in blood during the latter time point. On the converse, increasing frequency of the patrolling monocytes ($CD14^{dim}CD16^+$) during the first weeks after ART commencement was negatively associated with proportion of $CXCR3^-CCR6^-$ cells at the onset of IRIS event or equivalent time point (Fig. 5). These findings strongly suggest that $CXCR3^+CCR6^-$ $CD4^-$ T cells participate in the intricate pro-inflammatory changes, which occur upon immune reconstitution and favor occurrence of TB-IRIS.

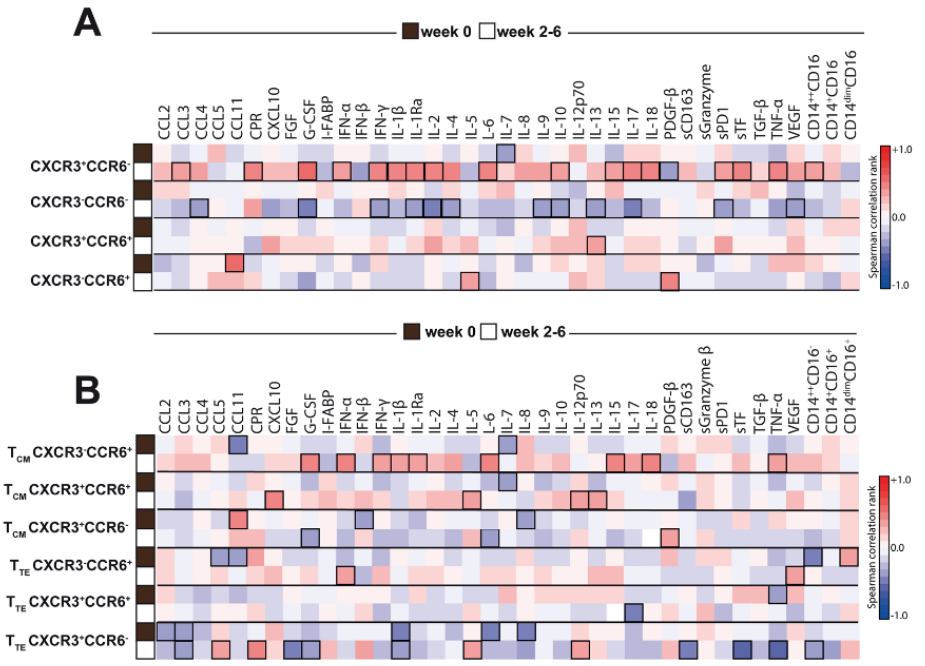

Figure 4. Dynamics of associations between CD4$^+$ T cell subtypes with circulating levels of several pro-inflammatory cytokines and chemokines, as well as frequency of monocyte subtypes. (**A**) Frequencies of CXCR3$^+$CCR6$^-$, CXCR3$^-$CCR6$^-$, CXCR3$^-$ CCR6$^+$ and CXCR3$^-$CCR6$^+$ lymphocytes were tested for correlations with several pro-inflammatory cytokines and chemokines at week 0 (pre-ART) and at week 6 post-ART initiation or at the time of IRIS event in the entire study population (n = 48). (**B**) Frequencies of CXCR3$^+$CCR6$^-$, CXCR3$^+$ CCR6$^+$ and CXCR3$^-$CCR6$^+$ CD4$^-$ T-lymphocytes expressing markers of central memory (CM) or T effector (TE) cells were tested for correlations with the same parameters shown in (**A**). A heat map was used to represent the strength of the associations (Spearman rank value). Statistically significant correlations ($P < 0.05$) after adjustment for multiple measurements are highlighted with bold squares.

Discussion

Our study highlighted the concept that differential expression of CXCR3 and CCR6 on effector and memory CD4$^+$ T cells was associated with development of TB-IRIS in HIV patients following ART initiation and can be linked with the inflammatory milieu, both soluble mediators and inflammatory monocytes, that characterizes this syndrome.

Phenotypic analysis of circulating CD4$^+$ T cells showed a higher frequency of effector memory (CD27$^-$ CD45RO$^+$) T cells and a decreased frequency of naïve (CD27$^-$ CD45RO$^-$) T cells in IRIS compared to non-IRIS patients at equivalent time points after ART initiation. While this difference could reflect advanced HIV infection, which is an important risk factor for IRIS subsequently developing in the presence of antigenemia as in TB[18], differences among CD4$^+$ T cell types were negligible at pre-ART in both groups. Alterations in the frequency of circulating memory T cells have been reported in TB-IRIS[19–22]. Haridas *et al.* demonstrated that the post-ART/TB-IRIS shift of the CD4$^+$ T cell memory compartment to an effector memory-dominated phenotype could help in controlling acute TB infection during the early stages of ART-mediated immune restoration, thereby conferring long-term enhanced protection against Mtb reinfection/reactivation/relapse[20].

Our findings complement those of Antonelli *et al.* studying a US patient cohort who described a higher proportion of effector cells at the time of an IRIS event and at 6 months post-ART and a higher percentage of naïve cells in the non-IRIS group. This reiterates the fact that non-IRIS patients reconstitute the naïve cell compartment faster while IRIS patients expanded initially predominantly CD4$^-$ effector T cells[7]. This is not unexpected considering that persistent antigenemia in TB could facilitate the expansion and survival of effector cells which are antigen-specific. Consistent with our observations, chronic and indolent infection (such as TB) with persistent antigenemia can provide the ideal environment and stimulus for the persistence of effector T cells.

It is well established that the differential expression profile of CXCR3 and CCR6 can define distinct T helper (Th) phenotypes[23,24]. Following this concept, Th1 cells are usually defined as population with CXCR3$^+$CCR6$^-$, whereas CXCR3$^-$CCR6$^-$ are hallmarks of Th2 cells. Recently, a new, CXCR3$^+$CCR6$^-$ CD4$^+$ subset referred to as Th1* has been described that appears to play a critical role in mycobacterial infections in humans[25]. Lastly, Th17 lymphocytes, defined as CXCR3$^-$CCR6$^+$, have also been implicated in TB immune responses and pathogenesis[26]. Although we have not directly tested cytokine production, the chemokine expression analyses reinforce the idea that a predominantly Th1 type of response and associated cytokine outburst underlies the clinical presentation of

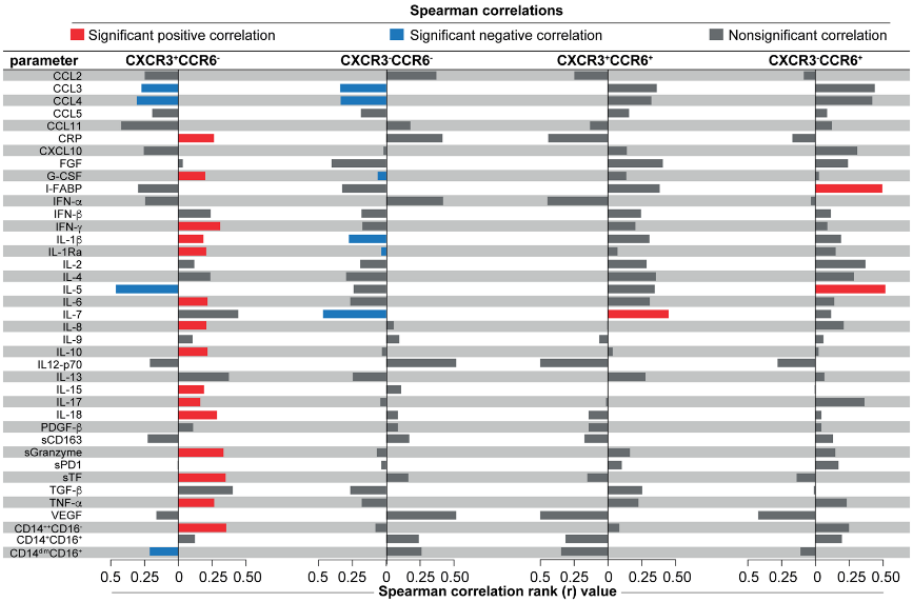

Figure 5. Associations between changes in systemic inflammation between before and after ART commencement and frequencies of $CD4^+$ T cell subtypes. For the entire study population, change in inflammation over time was estimated by calculating fold-differences of concentrations of each indicated plasma markers as well as for frequencies of the monocyte subsets, from pre-ART to week 2–6 after ART initiation (values at week 2–6 divided by values from pre-ART). Spearman analysis was used to test correlations between changes values of the inflammatory markers and frequencies of the indicated CD4+ T cell subsets in peripheral blood at the time of TB-IRIS event or equivalent timepoint. Colored bars indicate statistically significant correlations ($p < 0.05$) after adjustment for multiple measurements.

IRIS with a corresponding decline in Th2 response, more pronounced among IRIS patients compared to non-IRIS patients after ART initiation. Furthermore, we noticed a dramatic increase in the frequencies of Th1 and Th1* lymphocytes, while the percentages of Th2 and Th17 cells were significantly diminished upon ART initiation[27]. Th phenotypic characterization was equivalent in both the IRIS and non-IRIS groups at baseline prior to ART initiation, thereby aiding in the diagnosis of IRIS but not in its prediction.

Our study reaffirms that the tilt in balance from a Th2 to Th1 immune phenotype with ART administration may occur in IRIS. We previously demonstrated that after ART initiation, patients who experienced IRIS exhibit hyperactivation of T cells specific to antigens from opportunistic pathogens, leading to elevated levels of many pro- and anti-inflammatory cytokines and chemokines, resulting in a phenomenon known as cytokine storm or hypercytokinemia[28]. Consistent with our study, Meintjes *et al.* showed a higher frequency of IFN-γ secreting Th1 cells in patients with IRIS compared to non-IRIS patients, which induces the cytokine storm[29].

Prior studies have also demonstrated that in IRIS, there is an increase in Th1 cytokines such as IL-2, IL-12, IFN-γ and TNF-α[8,30]. Acute exacerbation of Mtb-specific Th1 responses were independent of $CD4^-$ T cell count, viral load and time of ART initiation[8]. This supports the idea that, rather than raising T cell numbers, ART contributes to a functional restoration of these cells. Quantification of $CD4^+$ T cells may not reflect the true cell count, as there may be transient sequestration of inflammatory cells at the tissue level that was not detected or a delayed increase in the frequency of these cells[15].

The role of innate immunity in the pathogenesis of TB-IRIS has been studied by several groups worldwide. There is strong indication that innate immune activation prior to ART commencement, with elevated levels of IL-6[4] and IL-18[31] followed by inflammasome activation[32–34] as well as expansion of inflammatory monocyte subsets[17], hallmarks patients at higher risk of IRIS. Whether pre-ART dysregulation of innate immune activation contributes to abnormal T-cell activation during IRIS is unknown. In the present study, we found that between the timepoints examined (baseline and week 2–6 after ART commencement), increases in plasma levels of inflammatory markers such as CRP, G-CSF, IL-1β, IL-1Ra, IL-6, IL-8, IL-18, TNF-α and sTF were directly associated with frequencies of $CXCR3^+CCR6^-$ $CD4^+$ T cells in the study population. This scenario was also associated with increases in proportion of $CD14^{++}CD16^-$ monocytes in peripheral blood. These observations make possible to hypothesize that expansion of $CXCR3^+CCR6^-$ $CD4^+$ T cells, rather than representing the main immunological basis of TB-IRIS, may be driven by an underlying augmentation of pro-inflammatory innate mediators prior to ART in TB-HIV patients with high microbial burden and who are at a very high risk of developing this syndrome.

The strengths of the current study were the selection of a uniform group of patients who had culture-positive, drug sensitive pulmonary TB, were naïve at baseline for anti-tuberculous treatment and ART, had an increase in their $CD4^-$ T cell population with immune recovery, and demonstrated a decline in HIV viral concentration of at least 0.5 log after the initiation of ART. With complete mycobacterial culture and drug sensitivity results, we excluded the possibility of TB treatment failure at the time of IRIS diagnosis by demonstrating negative cultures at IRIS diagnosis, thereby strengthening the validity of our findings. Since all patients were admitted during ART initiation, there was active surveillance for IRIS with clinical samples collected immediately after the onset of IRIS prior to the institution of anti-inflammatory agents, thereby avoiding distortion of lab parameters or T cell subsets. The main limitation of our study was the relatively small number of patients analyzed as the cohort was nested within a randomized controlled trial with stringent inclusion and exclusion criteria that precluded the recruitment and analysis of a larger sample.

Finally, in the current study we evaluated the expression of chemokine receptors and memory markers in $CD4^+$ T cells, along with their association with plasma biomarkers and monocyte subtypes that were shown to accurately help to diagnose IRIS. These findings confirm a prominent role of ART and Th1 effector cells in pathogenesis of IRIS.

Methods

Description of the patients. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all study participants. This study was approved by the Scientific Advisory Committee and Institutional Ethics Committee of the National Institute for Research in Tuberculosis (Chennai, India) and the main randomized clinical trial that provided patient information and samples for the present study was registered on Clinicaltrials.gov (NCT00933790).

Description of the patients. The Indian TB-IRIS cohort study was a retrospective observational analysis of cryopreserved samples from an investigation nested within a randomized controlled trial (NCT00933790) at the National Institute for Research in Tuberculosis (NIRT), Chennai, India, enrolling HIV-infected patients with newly diagnosed sputum culture-confirmed pulmonary TB, as previously reported. The parent randomized controlled clinical trial compared daily vs. intermittent anti-TB therapeutic regimens in HIV infected patients with pulmonary TB and has been already published[35]. Eligible participants in the TB-IRIS observational study were above 18 years of age, with newly diagnosed culture-positive rifampicin-sensitive TB, and who were ART-naïve[4]. They were initiated on ART within the intensive phase of anti-tuberculous therapy, as per prevailing national guidelines (National AIDS Control Organization, NACO). Clinical evaluations and blood collections were performed at baseline (pre-ART), at the time of the IRIS event (between weeks 2–6 post-ART initiation) or after 6 weeks of ART in the non-IRIS group, and after 6 months of ART in both groups. Mycobacterial loads in sputum cultures were assessed as described elsewhere[4]. IRIS was diagnosed by a panel of 3 doctors after ruling out drug resistance, as well as failure and occurrence of other opportunistic infections or common endemic infections. Modified INSHI criteria that included a 0.5 log decline in HIV viral load from baseline at the time of IRIS and a negative mycobacterial culture or decline in grade of TB infection from baseline were added for a definitive diagnosis of IRIS[18]. All patients were hospitalized for ART initiation and were discharged within two weeks. In this cohort, 48 individuals were enrolled and 26 (54%) developed IRIS during the study. The detailed clinical, laboratory, and microbiologic description of the study participants has been previously reported by our group[4].

Plasma biomarker measurements. Concentrations of IL-1β, IL-1Ra, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, IL-15, IL-17, IL-18, CCL2, CCL3, CCL4, CCL5, CCL11, CXCL10, IFN-γ, TNF-α, TGF-β, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) (Bio-Plex, Bio-Rad, Hercules, CA), C-reactive protein (CRP) (eBioscience, San Diego, CA), sCD163, soluble tissue factor (sTF) (R&D Systems, Minneapolis, MN) and intestinal fatty acid binding protein (I-FABP) (Hycult Biotech, The Netherlands) were assessed in cryopreserved plasma samples maintained at −80 °C.

Flow cytometry. The immunophenotyping of lymphocytes and monocytes was performed in whole blood collected in heparinized vacuum tubes. For "*ex vivo*" phenotyping, aliquots of 250 µL blood were stained with five panels of antibodies prepared in PBS 1% BSA for one hour at room temperature (RT) to characterize the lymphocyte populations. The panels with antibody clones and fluorochromes, as well as the gating strategies, are listed in Fig. S1. Antibodies were from eBioscience (San Diego, CA), Biolegend (San Diego, CA), BD Biosciences (San Jose, CA) and Life Technologies (Carlsbad, CA). Data were acquired on a BD FACS Canto II flow cytometer (BD Biosciences). The panel of T cells was defined based on surface expression of CD3 and CD4, different memory subpopulations to discriminate naïve, central and effector memory T cells using CD27 and CD45RO. The chemokine receptor expression was characterized used CXCR3 and CCR6. The immunophenotyping of subsets of monocytes was described previously[17]. All compensation and gating analyses were performed using FlowJo 9.5.3 (TreeStar, Ashland, OR).

Data analysis. Median values with IQR or frequencies of variables were compared using the Mann-Whitney test (when two groups were compared) or the Kruskal-Wallis test with Dunn's multiple comparisons ad hoc analysis (when three groups were compared). Fisher's exact test or Chi-square tests were used to compare two or three groups, respectively, for proportions. Paired changes from before ART initiation to week 6 or the time of IRIS development were compared using the Wilcoxon matched-paired T test. Using JMP 10.0 software, geometric mean values (log10) for each marker measured at week 0 and week 6 were calculated for the entire study population. To assess the overall pattern of expression of these markers in each clinical group and time point, heat maps were built using variation from the geometric mean value calculated for each candidate biomarker. A hierarchical

cluster analysis using the Ward's method was employed to reveal patterns of expression in plasma. Throughout the text, a p value of <0.05 was taken as statistically significant after adjustments for multiple measurements (Holm-Bonferroni's correction method). The statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., USA), STATA 9.0 (StataCorp, TX, USA), and JMP 10.0 software.

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Author Contributions

B.B.A., I.S., S. Swaminathan, B.O.P., A.S. conceptualized the study. B.B.A., I.S. supervised the immunological study. G.N., S. Swaminathan supervised the clinical study. I.S., S. Swaminathan, A.S. acquired funding for research. G.N., R.S., K.S., B.O.P., I.S., B.B.A. performed the clinical assessments. S.A., K.N., S. Subramanian, B.B.A., L.R.A. performed the experiments. P.S.S.M., B.B.A., K.F.F., C.L.V., D.O.S., K.A. analyzed the data. P.S.S.M., B.B.A. drafted the first version of the manuscript. All the authors revised the manuscript.

Additional Information

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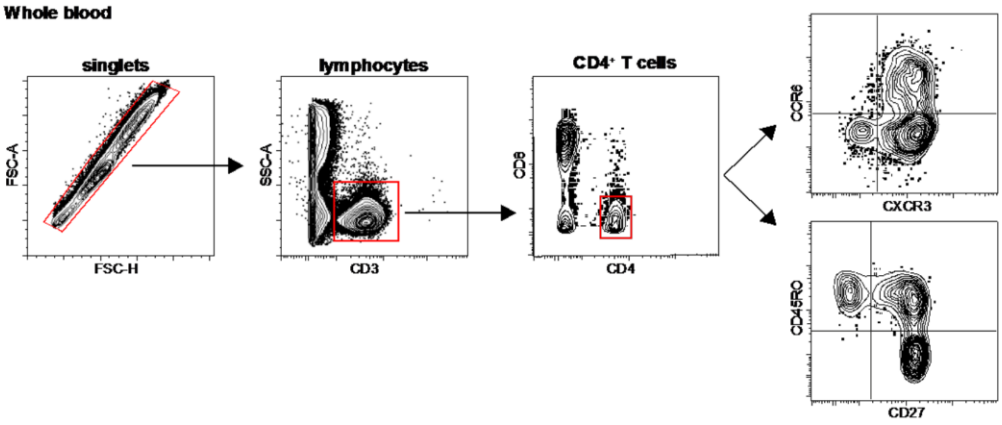

Figure S1. Overall gating strategy.

5 **DISCUSSÃO**

As limitações encontradas nos métodos microbiológicos tradicionais para o diagnóstico de TB levaram a busca de novas metodologias. Abordagens mais recentes exploraram metodologias não baseadas em escarro para diagnosticar TBA, incluindo transcriptoma, metaboloma, proteoma e ensaios celulares (HAAS *et al.*, 2016). Considerando a necessidade de novos testes para o diagnóstico de TB, este trabalho buscou inicialmente, avaliar a capacidade dos marcadores de ativação celular na diferenciação de diferentes formas clínicas: TBP, TBEP e ILTB.

Neste estudo, observamos que os pacientes com TBA possuíam maiores frequências de $CD4^+$ $IFN\gamma^+$ expressando marcadores de ativação: CD38, HLA-DR, e Ki-67 com alta especificidade e sensibilidade em comparação com os pacientes com ILTB. Nossos resultados foram semelhantes aos estudos anteriormente descritos em populações de pacientes dos EUA e África do Sul, demostrando assim um potencial emprego desses marcadores no diagnóstico da TB (ADEKAMBI *et al.*, 2015). Estudo recente também demostrou aumento na frequência de $HLA\text{-}DR^+$ em células T $CD4^+$ em pacientes com TBA (TIPPALAGAMA, R. *et al.*, 2021). Esses resultados reforçam o potencial emprego de marcadores de ativação em células T $CD4^+$ no diagnóstico da TB.

Segundo a OMS, o diagnóstico de TBEP é atualmente baseado em técnicas invasivas como cultura dos sítios extrapulmonares e histologia ou forte evidência clínica consistente com TBEP (GEADAS *et al.*, 2017; HAMILTON *et al.*, 2015);. Resultados desse estudo demostraram alterações nas frequências de $CD38^+$, $HLA\text{-}DR^+$, e $Ki\text{-}67^+$ em células T $CD4^+$ em pacientes com TBEP, TBA e ILTB. Portanto, foi possível distinguir pacientes com TBEP por valores mais elevados desses marcadores em comparação àqueles com ILTB ou TBP. Os marcadores $CD4^+$ $IFN\gamma^+$ expressando CD38, HLA-DR, e Ki-67 em conjunto ou não foram úteis para diagnosticar TBEP de TBP com alta precisão.

Assim como nosso estudo, Hiza *et al.* (2021) encontraram alta acurácia na distinção de diferentes formas da TB utilizando a frequência de células T $CD4^+$ $IFN\gamma^+$ expressando CD38 . A extensa ativação celular encontrada em pacientes com TBEP pode ser decorrente do cenário inflamatório, conforme evidenciado em estudos anteriores, onde, adultos e crianças com TBEP exibiram um perfil inflamatório distinto dos pacientes com TBP (ALBUQUERQUE *et al.*, 2019; VINHAES *et al.*, 2019).

O diagnóstico de TB em indivíduos coinfectados por HIV permanece desafiador. A imunossupressão induzida pelo HIV leva a uma menor frequência de lesões pulmonares, prejudicando ainda mais a sensibilidade de avaliações baseadas em escarro ou radiografias (GARDINER; KARP, 2015). Além disso, a infecção pelo HIV pode reduzir o desempenho dos ensaios imunológicos baseados no sangue devido a redução das células T $CD4^+$. Embora diagnósticos clínicos/empíricos sejam frequentemente utilizados, essas abordagens podem ser problemáticas, uma vez que as manifestações clínicas de TB (pulmonar e extrapulmonar) na coinfecção por HIV são geralmente atípicas. O atraso no diagnóstico de TB em indivíduos que vivem com o HIV afeta diretamente o prognóstico clínico, com aumento das chances de falha no tratamento e de óbito (DAS; DWIBEDI, 2016; OSEI; AKWEONGO; BINKA, 2015). Dessa forma, avaliamos se o HIV era capaz de influenciar na frequência das células T $CD4^+$ específicas de MTB expressando CD38, HLA-DR e Ki-67 em pacientes com TBP, TBEP e ILTB. Assim, os dados deste trabalho demonstraram que o *status* da infecção pelo HIV não influencia na ativação das células T $CD4^+$. Resultados similares foram obtidos em pacientes com TBP da África do Sul (DU BRUYN *et al.*, 2018; LESOSKY *et al.*, 2019).

Em conjunto, nossos dados sugerem que a ativação de células T $CD4^+$ antígeno-específicas seja capaz de detectar formas extrapulmonares ou paucibacilares de TB, como pacientes com HIV. Recentemente, foi demostrado que a ativação de T $CD4^+$ também pode identificar pacientes com maior risco de resultados de tratamentos ruins e avaliar a extensão da gravidade da doença (RIOU *et al.*, 2020). Futuramente, é provável que esses biomarcadores possam ser utilizados para inferir a carga microbiana e, potencialmente, a exposição ao antígeno *in vivo* durante diferentes estágios de infecção e da doença.

Os indivíduos com TB infectados pelo HIV após o início da terapia antirretroviral (TARV), tem propensão a ocorrer piora clínica ou radiológica paradoxal, denominada síndrome de reconstituição imunológica (SIRI) (BARBER *et al.*, 2012). A SIRI é caracterizada por uma ativação aumentada e desregulada de linfócitos T patógeno-específicos. Estudos recentes, mostraram que a frequência de células T $CD4^+$ circulantes contra MTB está intimamente associada ao aparecimento e ocorrência de SIRI quando comparado a indivíduos que não desenvolvem tal desfecho (ANTONELLI *et al.*, 2010; BOURGARIT *et al.*, 2006; BOURGARIT *et al.*, 2009; VIGNESH *et al.*, 2013; VIGNESH *et al.*, 2017). No entanto, a participação detalhada das células T no TB-SIRI não está completamente elucidada. Assim sendo, descrevemos nesse trabalho a participação de subtipos de linfócitos T $CD4^+$ em uma população com TB-SIRI.

A análise fenotípica de células T $CD4^+$ circulantes mostrou uma maior frequência de células T de memória efetora ($CD27^-$ $CD45RO^+$) e uma frequência diminuída de células T *naive* ($CD27^+$ $CD45RO^-$) em SIRI em comparação com pacientes não SIRI em pontos de tempo equivalentes após o início da TARV. Embora essa diferença possa refletir a infecção avançada por HIV, que é um importante fator de risco para o desenvolvimento subsequente de SIRI na presença de antigenemia como na TB (MEINTJES; LAWN*; et al.*, 2008), as diferenças entre os tipos de células T $CD4^+$ eram insignificantes no pré-TARV em ambos os grupos. Alterações na frequência de células T de memória circulação tem sido relatados em TB-SIRI (ESPINOSA *et al.*, 2013; GOOVAERTS, O. *et al.*, 2015; HARIDAS *et al.*, 2015; WILKINSON *et al.*, 2012). Haridas *et al.* (2015) demonstraram que a mudança pós-TARV/TB-SIRI do compartimento de memória das células T $CD4^+$ para um fenótipo de memória efetora dominante poderia ajudar no controle da infecção aguda por TB durante os estágios iniciais da restauração imunológica mediada por TARV, conferindo, assim, proteção aprimorada de longo prazo contra reinfecção/reativação/recidiva de MTB.

Nossos achados complementam o estudo de Antonelli *et al.* (2010) que relataram uma proporção maior de células efetoras no momento do evento da SIRI e 6 meses após a TARV e uma porcentagem maior de células *naive* no grupo não SIRI. Isso reitera o fato de que os pacientes não SIRI reconstituem o compartimento de células *naive* mais rápido, enquanto os pacientes SIRI expandiram inicialmente predominantemente células T efetoras $CD4^+$ (ANTONELLI *et al.*, 2010). Isso não é inesperado, considerando que a antigenemia persistente na TB pode facilitar a expansão e a sobrevivência das células efetoras que são específicas do antígeno. Consistente com nossas observações, a infecção crônica e indolente (como a TB) com antigenemia persistente pode fornecer o ambiente ideal e o estímulo para a persistência de células T efetoras.

Está bem estabelecido que o perfil de expressão diferencial de CXCR3 e CCR6 pode definir fenótipos T auxiliares (Th) distintos (FIGUEIREDO *et al.*, 2017; GOSSELIN *et al.*, 2010). Seguindo esse conceito, as células Th1 são geralmente definidas como população com $CXCR3^+$ $CCR6^-$, enquanto $CXCR3^-$ $CCR6^-$ são definidas como células Th2. As células $CXCR3^+$ $CCR6^+$ $CD4^+$ parecem desempenhar um papel crítico em infecções por micobactérias em humanos (BECATTINI *et al.*, 2015). Por último, linfócitos Th17, definidos como $CXCR3^-CCR6^+$, também têm sido implicados nas respostas imunes e na patogênese da TB (KORN *et al.*, 2009). Embora não tenhamos testado diretamente a produção de citocinas, as análises de expressão de quimiocinas reforçam a ideia de que um tipo de resposta predominantemente Th1 e a

tempestade de citocina (*Cytokine Storm*), estão associadas à apresentação clínica de SIRI com um declínio correspondente na resposta de Th2, mais pronunciado entre pacientes SIRI em comparação com não pacientes SIRI após o início do TARV (IMAMI *et al.*, 1999). Além disso, notamos um aumento dramático nas frequências de linfócitos Th1 e Th1*, enquanto as porcentagens de células Th2 e Th17 diminuíram significativamente após o início da TARV. Em macacos rhesus infectados com MTB e posteriormente desafiados com o vírus da imunodeficiência símia (SIV) tratados com TARV foi identificado um aumento significativo na frequência de células Th1* (GANATRA *et al.*, 2020). Em ambos os grupos (SIRI e não SIRI), no início da TARV, não encontramos diferença entre os subtipos de LT CD4, demostrando assim, que esses, não são capazes de prever a ocorrência da SIRI. Em tempo, o aumento da frequência da célula Th* independe do status da SIRI e esse achado provavelmente ocorre devido a utilização do ARV, que por sua vez, permite o restabelecimento das células TCD4 e a redução da carga viral.

Nosso estudo reafirma que o balanço de um fenótipo imune Th2 a Th1 com a administração de TARV pode ocorrer em SIRI. Demonstramos anteriormente que após o início da TARV, os pacientes que experimentaram SIRI exibiram hiperativação de células T específicas para antígenos de patógenos oportunistas, levando a níveis elevados de muitas citocinas e quimiocinas pró e anti-inflamatórias, resultando em um fenômeno conhecido como tempestade de citocinas ou hipercitocinemia (TADOKERA *et al.*, 2011). Consistente com nosso estudo, Meintjes; Wilkinson*; et al.* (2008) mostraram maior frequência de células Th1 secretoras de IFN-γ em pacientes com SIRI em comparação com pacientes não SIRI, induzindo uma tempestade de citocinas (MEINTJES; WILKINSON*; et al.*, 2008).

Estudos anteriores também demonstraram que na SIRI há aumento de citocinas Th1 como IL-2, IL-12, IFN-γ e TNF-α (BOURGARIT *et al.*, 2006; TAN *et al.*, 2008). A exacerbação aguda das respostas Th1 específicas de MTB foram independentes da contagem de células T $CD4^+$, carga viral e tempo de início da TARV. Isso apoia a ideia de que, em vez de aumentar o número de células T, a TARV contribui para a restauração funcional dessas células.

O papel da imunidade inata na patogênese da TB-SIRI tem sido estudado por vários grupos em todo o mundo. Há forte indicação de que a ativação imune inata ocorra antes do início da TARV, com níveis elevados de IL-6 e IL-18 seguido de ativação do inflamassoma, bem como expansão de subtipos de monócitos inflamatórios. (ANDRADE *et al.*, 2014; NAKANISHI, 2018; NARENDRAN *et al.*, 2013; OLIVER *et al.*, 2010; TAN *et al.*, 2015; TAN *et al.*, 2016)Estes fatores podem determinar pacientes com maior risco de SIRI. No entanto, até o

momento não foi investigado se a desregulação pré-TARV da ativação do sistema imune inato contribui para a ativação anormal das células T durante a SIRI. No presente estudo, descobrimos que entre os intervalos de tempo examinados no estudo (*baseline* e semana 2-6 após o início da TARV), houve um aumento nos níveis plasmáticos de marcadores inflamatórios como PCR, G-CSF, IL-1β, IL-1Ra, IL-6, IL-8, IL-18, TNF-α e sTF. Estes achados foram diretamente associados às frequências de células T $CD4^{+}CXCR3^{+}CCR6^{-}$ e de monócitos $CD14^{++}$ $CD16^{-}$ no sangue periférico da população estudada. Essas observações possibilitam a hipótese de que a expansão de $CXCR3^{+}$ $CCR6^{-}$ $CD4^{+}$, em vez de representar a principal base imunológica da TB-SIRI, podem ser impulsionadas por um aumento subjacente de mediadores inatos pró-inflamatórios antes da TARV em pacientes TB-HIV com alta carga microbiana e que estão em um nível muito alto risco de desenvolver esta síndrome.

Os pontos fortes do estudo atual foram a seleção de um grupo uniforme de pacientes que apresentavam TB pulmonar, com cultura positiva, sensível a medicamentos, sem tratamento antituberculose e TARV (no início do estudo), aumento de células T $CD4^{+}$ com recuperação imune e um declínio na concentração viral de pelo menos 0,5 log após o início da TARV. Excluímos a possibilidade de falha no tratamento da TB no momento do diagnóstico de SIRI, através da identificação de culturas negativas, fortalecendo assim a validade de nossos achados. Como todos os pacientes foram admitidos durante o início da TARV, houve vigilância ativa para SIRI com amostras clínicas coletadas imediatamente após o início da SIRI, antes da administração de medicamentos anti-inflamatórios, evitando assim distorção dos parâmetros laboratoriais ou subtipos de células T $CD4^{+}$.

Finalmente, no presente estudo identificamos que a expressão de receptores de quimiocinas e marcadores de memória em células T $CD4^{+}$, juntamente com sua associação com biomarcadores plasmáticos e subtipos de monócitos auxilia com precisão no diagnóstico de SIRI. Esses achados confirmam um papel proeminente da TARV e das células efetoras Th1 na patogênese da SIRI.

6 **CONCLUSÕES**

Os dados dos estudos que fazem parte dessa tese demostram as seguintes conclusões:

- As células T $CD4^+$ específicas de MTB expressando CD38, HLA-DR e Ki-67 distinguiram TB ativa de ILTB e TBEP da TBP, independentemente do status de infecção pelo HIV.
- A expressão diferencial de CXCR3 e CCR6 em células T $CD4^+$ efetoras e de memória foi associada ao desenvolvimento de TB-SIRI em pacientes com HIV após o início da TARV sendo fortemente associada à inflamação sistêmica.

As conclusões relatadas nessa tese contribuem para a compreensão de mecanismos imunológicos da TB que poderão ser aplicados no desenvolvimento de testes de diagnóstico sanguíneo ou servir como alvos para profilaxia e/ou intervenções terapêuticas no futuro.

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ANEXOS

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Plasma levels of C-reactive protein, matrix metalloproteinase-7 and lipopolysaccharide-binding protein distinguish active pulmonary or extrapulmonary tuberculosis from uninfected controls in children

Victor V.S. Albuquerque[a,b,c,1], Nathella Pavan Kumar[d,1], Kiyoshi F. Fukutani[a,b,1], Beatriz Vasconcelos[a,b,c], Maria B. Arriaga[a,b], Paulo S. Silveira-Mattos[a,b], Subash Babu[d,e,1], Bruno B. Andrade[a,b,c,e,f,g,1,*]

[a] Instituto Gonçalo Moniz, Salvador, Bahia, Brazil
[b] Multinational Organization Network Sponsoring Translational and Epidemiological Research (MONSTER) Initiative, Fundação José Silveira, Salvador, Bahia, Brazil
[c] Escola Bahiana de Medicina e Saúde Pública (EBMSP), Salvador, Bahia, Brazil
[d] National Institutes of Health, NIRT, International Center for Excellence in Research, Chennai, India
[e] Wellcome Trust Centre for Infectious Disease Research in Africa, Institute of Infectious Disease and Molecular Medicine, University of Cape Town, South Africa
[f] Universidade Salvador (UNIFACS), Laureate Universities, Salvador, Bahia, Brazil
[g] Division of Infectious Diseases, Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN, United States

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ABSTRACT

The immune profile associated with distinct clinical forms of tuberculosis (TB) has been extensively described for adult populations. Nevertheless, studies describing immune determinants of pulmonary or extrapulmonary TB (PTB or EPTB, respectively) in children are scarce. Here, we retrospectively assessed plasma levels of several mediators of inflammation in age and sex-matched children from South India presenting with PTB (n = 14) or EPTB (n = 22) as well as uninfected healthy controls (n = 19) to identify biomarkers that could accurately distinguish different TB clinical forms. Furthermore, we performed exploratory analyses testing the influence of sex on the systemic inflammatory profile. The analyses identified a biosignature of 10 biomarkers capable of distinguishing the three clinical groups simultaneously. Machine-learning decision trees indicated that C-reactive protein (CRP), matrix metalloproteinase (MMP)-7 and lipopolysaccharide-binding protein (LBP) were the markers that, when combined, displayed the highest accuracy in identifying the clinical groups. Additional exploratory analyses suggested that the disease signatures were highly influenced by sex. Therefore, sex differentially impacted status of systemic inflammation, immune activation and tissue remodeling in children with distinct clinical forms of TB. Regardless of such nuances related to biological sex, MMP-7, CRP and LBP were strong discriminators of active TB and thus could be considered as biomarkers useful in discrimination different TB clinical forms. These observations have implications on our understanding of the immunopathology of both clinical forms of TB in pediatric patients. If validated by other studies in the future, the combination of identified biomarkers may help development of point-of-care diagnostic or prognostic tools.

1. Introduction

Tuberculosis (TB) is a major cause of death worldwide [1,2]. Most studies on TB diagnosis are performed primarily in adult populations and then applied to children. Since both groups are described to present distinct immune and inflammatory profiles [3], the established tests are not as accurate for diagnosis of childhood TB [3]. Children, especially those below 5 years old, and adolescents are thought to present relatively weaker immune responses against infection compared to adults [4]. These immune differences are likely involved in increased susceptibility to *Mycobacterium tuberculosis* (*M.tb*) infection [5]. Once infected with *M.tb*, pediatric populations who develop active TB frequently present with extrapulmonary forms of the disease [6]. Recent studies estimated that TB is one of the main causes of childhood

* Corresponding author at: Laboratório de Inflamação e Biomarcadores, Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão, no. 121, Candeal, Salvador, Bahia 40269-710, Brazil.
E-mail address: bruno.andrade@bahia.fiocruz.br (B.B. Andrade).
[1] Equal contributions.

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Determinants of losses in the latent tuberculosis cascade of care in Brazil: A retrospective cohort study

Nélia C.N. Araújo[a,b,c,d,1], Constança M.S. Cruz[a,e,1], María B. Arriaga[c,f,1], Juan M. Cubillos-Angulo[c,f,1], Michael S. Rocha[b,c], Paulo S. Silveira-Mattos[c,f,g], Gisela M. Matos[a], Izabella M.B. Marques[a], Isa Carolina P. Espirito Santo[a], Luiza L. Almeida[a], Caroline M. Andrade[a], Leonardo A. Souza[a], Eduardo M. Netto[b,d], Bruno B. Andrade[a,c,f,g,h,*]

[a] Escola Bahiana de Medicina e Saúde Pública, Salvador, Bahia, Brazil
[b] Instituto Brasileiro para Investigação da Tuberculose (IBIT), Fundação José Silveira, Salvador, Bahia, Brazil
[c] Multinational Organization Network Sponsoring Translational and Epidemiological Research (MONSTER), Salvador, Bahia, Brazil
[d] Complexo Hospitalar Universitário Professor Edgard Santos, Universidade Federal da Bahia, Salvador, Bahia, Brazil
[e] Obras Sociais Irmã Dulce, Salvador, Bahia, Brazil
[f] Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Bahia, Brazil
[g] Curso de Medicina, Faculdade de Tecnologia e Ciências, Salvador, Bahia, Brazil
[h] Universidade Salvador (UNIFACS), Laureate Universities, Salvador, Bahia, Brazil

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ABSTRACT

Background: The present study evaluated factors associated with losses in the latent tuberculosis infection (LTBI) cascade of care in contacts of tuberculosis (TB) patients, in a referral center from a highly endemic region in Brazil.
Methods: Contacts of 1672 TB patients were retrospectively studied between 2009 and 2014. Data on TB screening by clinical investigation, radiographic examination and tuberculin skin test (TST) were extracted from medical records. Losses in the cascade of care and TB incidence within 2-year follow-up were calculated.
Results: From a total of 1180 TB contacts initially identified, only 495 were examined (58% loss), and 20 were diagnosed with active TB at this stage. Furthermore, 435 persons returned for TST result interpretation and 351 (~81%) were TST positive. Among those with positive TST, 249 (73%) were treated with isoniazid for 6 months whereas 51 abandoned therapy early. Three individuals who did not receive LTBI treatment, one with incomplete treatment and another who completed treatment developed active TB. A logistic regression analysis revealed that increases in age were associated with losses in the LTBI cascade independent of other clinical and epidemiological characteristics.
Conclusions: Major losses occur at initial stages and older patients are at higher risk of not completing the LTBI cascade of care.

Introduction

The majority of new cases of Tuberculosis (TB) occur in 30 countries with high disease burden such as Brazil, India, China, and South Africa (WHO, 2019). Furthermore, approximately 1.5 million deaths attributable to TB globally were reported in 2018 (WHO, 2019). Factors that may underlie the slow improvement of TB control include inaccurate diagnosis and loss to follow up of patients or household contacts undergoing anti-TB treatment (Zelner et al., 2018). In Brazil, despite significant investment from the government, the reported reduction in TB incidence (−1.34% per year) is considered insufficient to meet targets established by the World Health Organization (WHO) to reduce the incidence of TB by 90% by 2035 and eliminate TB (less than 1 incident case per 1,000,000 per year) by 2050 (Houben and Dodd, 2016). To achieve

* Corresponding author at: Laboratório de Inflamação e Biomarcadores, Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão, No. 121, Candeal, Salvador, Bahia 40269-710, Brazil.
E-mail address: bruno.andrade@fiocruz.br (B.B. Andrade).
[1] NCNA, CMSC, MBA and JMCA equally contributed to the work.

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MAJOR ARTICLE

Host Inflammatory Biomarkers of Disease Severity in Pediatric Community-Acquired Pneumonia: A Systematic Review and Meta-analysis

Catarina D. Fernandes,[1,2,3,*] María B. Arriaga,[1,2,5,*] Maria Carolina M. Costa,[1,2,4] Maria Clara M. Costa,[1,2,4] Maria Heloina M. Costa,[5] Caian L. Vinhaes,[1,2,4] Paulo S. Silveira-Mattos,[1,2,4,5] Kiyoshi F. Fukutani,[1,2,4,*] and Bruno B. Andrade[1,2,3,4,6,7,8,*]

[1]Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Bahia, Brazil, [2]Multinational Organization Network Sponsoring Translational and Epidemiological Research (MONSTER) Initiative, Fundação José Silveira, Salvador, Bahia, Brazil, [3]Universidade Salvador (UNIFACS), Laureate Universities, Salvador, Bahia, Brazil, [4]Faculdade de Tecnologia e Ciências (FTC), Salvador, Bahia, Brazil, [5]Universidade Federal da Bahia (UFBA), Salvador, Bahia, Brazil, [6]Division of Infectious Diseases, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee, USA, [7]Wellcome Centre for Infectious Disease Research in Africa, Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa, and [8]Escola Bahiana de Medicina e Saúde Pública, Salvador, Bahia, Brazil

Background. Community-acquired pneumonia (CAP) is the leading cause of death in children. Identification of reliable biomarkers offers the potential to develop a severity quantitative score to assist in clinical decision-making and improve outcomes.

Methods. A systematic review and meta-analysis was performed in PubMed and EMBASE on November 13, 2018, to examine the association between host inflammatory biomarkers and CAP severity in children. The inclusion criteria were case–control, cross-sectional, and cohort studies that examined candidate serum biomarkers. We extracted outcomes of interest, means, and standardized mean differences (SMDs) of plasma and serum levels of biomarkers together with information on disease severity. Meta-analysis was performed. This review was registered in the PROSPERO international registry (CRD42019123351).

Results. Two hundred seventy-two abstracts were identified, and 17 studies were included. Among the biomarkers evaluated, levels of C-reactive protein (CRP; SMD, 0.63; 95% confidence interval [CI], 0.35 to 0.91), interleukin (IL)-6 (SMD, 0.46; 95% CI, 0.25 to 0.66), IL-8 (SMD, 0.72; 95% CI, 0.15 to 1.29), neutrophil count (SMD, 0.27; 95% CI, 0.07 to 0.47), and procalcitonin (SMD, 0.68; 95% CI, 0.20 to 1.15) were substantially increased in severe CAP. In contrast, IL-2 concentrations (SMD, −0.24; 95% CI, −0.45 to −0.03) were higher in nonsevere CAP. Study heterogeneity was reported to be high ($I^2 > 75\%$), except for IL-2, IL-5, IL-6, and IL-12p70, which were classified as moderate ($I^2 = 50\%–74\%$). Only neutrophil and white blood cell counts were described by studies exhibiting a low level of heterogeneity.

Conclusions. Our results suggest that host biomarkers, and especially CRP, IL-6, IL-8, and procalcitonin levels, have the potential to predict severe CAP in pediatric populations.

Keywords. biomarkers; children; inflammation; pneumonia; severity.

Community-acquired pneumonia (CAP) is the leading cause of death in children [1]. In pediatric populations, especially in children under 5 years old, CAP-associated mortality is substantially higher than HIV/AIDS, malaria, and measles combined [1]. Severe CAP and lethal CAP are more frequent in low-resource settings, resulting in significant disease burden [2]. Nevertheless, such conditions are also very common in well-developed countries in Europe and North America [3]. These observations demonstrate that the morbimortality linked to CAP has a devastating impact on the public health system globally, irrespective of the economic status of the affected populations.

Assessment of CAP severity is critical for improving site-of-care decisions and implementation of adequate clinical management in a timely fashion [3–5]. In adults, several prognostic scores are available for patients with pneumonia, and studies of these models have indicated that its use may reduce broad-spectrum antibiotic use and decrease hospitalization among low-risk individuals [3–5]. Unfortunately, absence of a standardized and validated pediatric CAP severity quantitative score remains one of the biggest challenges in the field [3–5].

The most used guidelines for the management of CAP worldwide are from the World Health Organization (WHO) [6], the British Thoracic Society [7], and the Pediatric Infectious Diseases Society/Infectious Diseases Society of America [8]. Importantly, such guidelines do not consider markers in the blood to assess severity, but only clinical parameters. The parameters found in

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*Equal contribution

Correspondence: Bruno B. Andrade, MD, PhD, Laboratório de Inflamação e Biomarcadores, Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão, No. 121, Candeal, Salvador, Bahia 40269-710, Brazil (bruno.andrade@fiocruz.br).

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Original Investigation | Pediatrics

Clinical and Biochemical Features of Hypopituitarism Among Brazilian Children With Zika Virus–Induced Microcephaly

Leda L. Ferreira, MD, PhD; Juan P. Aguilar Ticona, MD; Paulo S. Silveira-Mattos, PhD; María B. Arriaga, MSci; Thaisa B. Moscato, BBiomedSc; Gildásio C. Conceição, BBiomedSc; Antonio Carlos dos Santos, MD, PhD; Federico Costa, PhD; Crésio A.D. Alves, MD, PhD; Sonir R. Antonini, MD, PhD

Abstract

IMPORTANCE The Zika virus infects progenitor neuron cells, disrupts cerebral development, and, in mice, drives hypothalamic defects. Patients with microcephaly caused by congenital Zika infection present with midline cerebral defects, which may result in hypopituitarism.

OBJECTIVE To analyze postnatal growth and the presence of clinical and biochemical features associated with hypopituitarism in children with congenital Zika infections.

DESIGN, SETTING, AND PARTICIPANTS In this prospective cohort study at 2 public referral hospitals in Bahia, Brazil, specializing in the treatment of congenital Zika infection, clinical data and growth parameters of 65 patients with the infection were evaluated. Data were analyzed from April 2017 through July 2018.

EXPOSURE Congenital Zika infection.

MAIN OUTCOMES AND MEASURES Length, weight, and head circumference were measured at birth and during follow up (ie, at 27 months of life) for each patient. Basal levels of free thyroxine, thyrotropin, cortisol, corticotropin, prolactin, insulin-like growth factor 1, insulin-like growth factor binding protein 3, urine and plasma osmolality, electrolytes, glucose, and insulin were evaluated at the age of 26 months to 28 months. All patients underwent central nervous system computed tomography scans and ophthalmic and otoacoustic evaluations at the time of this investigation or had done so previously.

RESULTS Among 65 patients (38 [58.4%] male; median [interquartile range] age at enrollment, 27 [26-28] months), 61 patients presented with severe brain defects (93.8%), including corpus callosum agenesis or hypoplasia (ie, midline brain defects; 25 patients [38.5%]) and optic nerve atrophy (38 patients [58.5%]). Most patients presented with severe neurodevelopmental delay (62 of 64 patients [96.9%]). Past or present clinical signs of hypopituitarism were rare, occurring in 3 patients (4.6%). Severe microcephaly, compared with mild or moderate microcephaly, was associated with a shorter length by median (interquartile range) *z* score at birth (−1.9 [−2.5 to −1.0] vs −0.3 [−1.0 to 0]; $P < .001$), but this difference did not persist at 27 months (−1.6 [−2.3 to −0.3] vs −2.9 [−4.0 to −1.2]; $P = .06$). Growth hormone deficiency or hypothyroidism were not observed in any patients, and glucose and insulin levels were within reference ranges for all patients. Low cortisol levels (ie, below 3.9 µg/dL) were observed in 4 patients (6.2%). These 4 patients presented with low (ie, below 7.2 pg/mL) or inappropriately low (ie, below 30 pg/mL) corticotropin levels. Low corticotropin levels (ie, below 7.2 pg/mL) were observed in 6 patients (9.2%). Diabetes insipidus was evaluated in 21 patients; it was confirmed in 1 patient (4.8%) and suggested in 3 patients (14.3%).

(continued)

Key Points

Question Do patients with microcephaly and central nervous system malformations caused by congenital Zika virus infection present with hormone deficiencies?

Findings In this cohort study of 65 patients with congenital Zika virus infection, most patients presented with severe brain defects. Severe developmental delay and prenatal growth impairment with no postnatal catch-up growth occurred frequently, but they were not associated with growth hormone deficiency or hypothyroidism, and few of these patients presented with central adrenal insufficiency or diabetes insipidus in the third year of life.

Meaning These findings suggest that hypopituitarism is infrequent within the first years of life in children with congenital Zika infections.

+ Supplemental content

Author affiliations and article information are listed at the end of this article.

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Tuberculosis-associated anemia is linked to a distinct inflammatory profile that persists after initiation of antitubercular therapy

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Leonardo Gil-Santana[1,2,3], Luís A. B. Cruz[1,2,3], María B. Arriaga[1,2], Pryscila F. C. Miranda[4], Kiyoshi F. Fukutani[1,2,5], Paulo S. Silveira-Mattos[1,2], Elisangela C. Silva[4,6], Marina G. Oliveira[4], Eliene D. D. Mesquita[7], Anneloek Rauwerdink[8], Frank Cobelens[8], Martha M. Oliveira[9], Afranio Kritski[4] & Bruno B. Andrade[1,2,3,5,10]

Pulmonary tuberculosis (PTB) is associated with chronic inflammation and anemia. How anemia impacts systemic inflammation in PTB patients undergoing antitubercular therapy (ATT) is not fully understood. In the present study, data on several blood biochemical parameters were retrospectively analyzed from 118 PTB patients during the first 60 days of ATT. Multidimensional statistical analyses were employed to perform detailed inflammatory profiling of patients stratified by anemia status prior to treatment. Anemia was defined as hemoglobin levels <12.5 g/dL for female and <13.5 g/dL for male individuals. The findings revealed that most of anemia cases were likely caused by chronic inflammation. A distinct biosignature related to anemia was detected, defined by increased values of uric acid, C-reactive protein, and erythrocyte sedimentation rate. Importantly, anemic patients sustained increased levels of several biochemical markers at day 60 of therapy. Preliminary analysis failed to demonstrate association between persistent inflammation during ATT with frequency of positive sputum cultures at day 60. Thus, TB patients with anemia exhibit a distinct inflammatory profile, which is only partially reverted at day 60 of ATT.

Tuberculosis (TB) remains the major cause of death from infection by a single pathogen[1]. *Mycobacterium tuberculosis* infection drives a chronic pulmonary disease characterized by persistent granulomatous inflammation with substantial lung tissue damage[2]. The chronic inflammation observed in pulmonary TB patients is reflected by increased circulating levels of acute phase proteins, such as C-reactive protein (CRP) as well as of inflammatory cytokines[3,4]. In fact, patients with more severe clinical forms of TB disease have been shown to exhibit a distinct inflammatory profile associated with balance between different cytokines and lipid mediators[5]. Understanding the mechanisms driving increased susceptibility to persistent pathological inflammation in TB may help development of new treatment strategies to optimize patient care.

Many patients with active pulmonary TB exhibit decreased levels of hemoglobin, which can directly impact TB-associated morbidity. Anemia can be defined as hemoglobin (Hb) levels below 12.5 g/dL for women and 13.5 g/dL for men[6]. Anemia can have many causes, including iron deficiency and chronic inflammation. These two distinct mechanisms of anemia present different laboratorial definitions. Anemia caused by iron deficiency

[1]Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Brazil. [2]Multinational Organization Network Sponsoring Translational and Epidemiological Research (MONSTER) Initiative, Fundação José Silveira, Salvador, Brazil. [3]Curso de Medicina, Faculdade de Tecnologia e Ciências, Salvador, Brazil. [4]Tuberculosis Academic Program, Medical School, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. [5]Universidade Salvador (UNIFACS), Laureate Universities, Salvador, Brazil. [6]Recognize the Biology Laboratory, Center of Bioscience and Biotechnology, State University of North Fluminense Darcy Ribeiro, Rio de Janeiro, Brazil. [7]Ary Parreira Institute, State Secretary of Health of Rio de Janeiro, Rio de Janeiro, Brazil. [8]Amsterdam Institute for Global Health and Development, Amsterdam University Academic Medical Centre, Amsterdam, The Netherlands. [9]Centro de Desenvolvimento Tecnológico em Saúde (CDTS), Fundação Oswaldo Cruz, Rio de Janeiro, Brazil. [10]Escola Bahiana de Medicina e Saúde Pública (EBMSP), Salvador, Brazil. Leonardo Gil-Santana and Luís A. B. Cruz contributed equally. Correspondence and requests for materials should be addressed to B.B.A. (email: Bruno.andrade@bahia.fiocruz.br)

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Hydroxyurea alters circulating monocyte subsets and dampens its inflammatory potential in sickle cell anemia patients

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Caroline C. Guarda[1,2], Paulo S. M. Silveira-Mattos[2,3,4,5], Sètondji C. M. A. Yahouédéhou[1,2], Rayra P. Santiago[1,2], Milena M. Aleluia[1], Camylla V. B. Figueiredo[1,2], Luciana M. Fiuza[1,2], Suellen P. Carvalho[1,2], Rodrigo M. Oliveira[1,2], Valma M. L. Nascimento[6], Nívea F. Luz[3], Valéria M. Borges[2,3], Bruno B. Andrade[2,3,4,5,7,8] & Marilda S. Gonçalves[1,2]

Sickle cell anemia (SCA) is a hemolytic disease in which vaso-occlusion is an important pathophysiological mechanism. The treatment is based on hydroxyurea (HU), which decreases leukocyte counts and increases fetal hemoglobin synthesis. Different cell types are thought to contribute to vaso-occlusion. Nevertheless, the role of monocytes subsets remains unclear. We investigated frequencies of monocytes subsets in blood and their response to HU therapy, testing their ability to express pro-inflammatory molecules and tissue factor (TF). We identified major changes in monocyte subsets, with classical monocytes ($CD14^{++}CD16^{-}$) appearing highly frequent in who were not taking HU, whereas those with patrolling phenotype ($CD14^{dim}CD16^{+}$) were enriched in individuals undergoing therapy. Additionally, HU decreased the production of TNF-α, IL1-β, IL-6, IL-8 as well as TF by the LPS-activated monocytes. Likewise, frequency of TF-expressing monocytes is increased in patients with previous vaso-occlusion. Moreover, activated monocytes expressing TF produced several pro-inflammatory cytokines simultaneously. Such polyfunctional capacity was dramatically dampened by HU therapy. The frequency of classical monocytes subset was positively correlated with percentage cytokine producing cells upon LPS stimulation. These findings suggest that classical monocytes are the subset responsible for multiple pro-inflammatory cytokine production and possibly drive inflammation and vaso-occlusion in SCA which is damped by HU.

Sickle cell anemia (SCA) is a genetic disease associated with important alterations of morphology and function of red blood cells (RBC) which cause a wide range of clinical manifestations linked to vascular injury and coagulation abnormalities[1]. The SCA is characterized by homozygosity of the hemoglobin S (HbS), and patients with this disease exhibit the most severe clinical forms[1]. Of note, polymerization of HbS triggers biochemical and morphological changes in sickle erythrocytes, which interact with other erythrocytes, as well as with reticulocytes, leukocytes, platelets and endothelial cells leading to vaso-occlusive events (VOE)[1,2], which is the main pathophysiological mechanism underlying SCA. VOE is thought to be caused at least by three components: (i) activation of endothelial cells and leukocytes due to adherence of sickle erythrocytes; (ii) nitric oxide (NO) consumption by arginase and free hemoglobin as result of intravascular hemolysis; (iii) activation of coagulation cascades due to activation of endothelium and leukocytes, which drive blood flow obstruction and eventually

[1]Laboratório de Investigação em Genética e Hematologia Translacional, Instituto Gonçalo Moniz, FIOCRUZ-BA, Salvador, Bahia, Brazil. [2]Faculdade de Medicina da Bahia, Universidade Federal da Bahia, Salvador, Brazil. [3]Laboratório de Inflamação e Biomarcadores, Instituto Gonçalo Moniz, FIOCRUZ-BA, Salvador, Bahia, Brazil. [4]Multinational Organization Network Sponsoring Translational and Epidemiological Research (MONSTER) Initiative, Salvador, Bahia, Brazil. [5]Curso de Medicina, Faculdade de Tecnologia e Ciências, Salvador, Bahia, Brazil. [6]Fundação de Hematologia e Hemoterapia do Estado da Bahia (HEMOBA) Salvador, Bahia, Brazil. [7]Universidade Salvador (UNIFACS), Laureate Universities, Salvador, Bahia, Brazil. [8]Escola Bahiana de Medicina e Saúde Pública, Salvador, Bahia, Brazil. Caroline C. Guarda, Paulo S. M. Silveira-Mattos, Bruno B. Andrade and Marilda S. Gonçalves contributed equally. Correspondence and requests for materials should be addressed to B.B.A. (email: bruno.andrade@fiocruz.br) or M.S.G. (email: mari@bahia.fiocruz.br)

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RESEARCH ARTICLE

Persistent inflammation during anti-tuberculosis treatment with diabetes comorbidity

Nathella Pavan Kumar[1†], Kiyoshi F Fukutani[2,3,4†], Basavaradhya S Shruthi[5], Thabata Alves[2,6], Paulo S Silveira-Mattos[2,4], Michael S Rocha[2], Kim West[7], Mohan Natarajan[8], Vijay Viswanathan[5], Subash Babu[1], Bruno B Andrade[2,3,4,6‡], Hardy Kornfeld[7‡*]

[1]National Institutes of Health, National Institute for Research in Tuberculosis, International Center for Excellence in Research, Chennai, India; [2]Multinational Organization Network Sponsoring Translational and Epidemiological Research (MONSTER), Fundação José Silveira, Salvador, Brazil; [3]Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Brazil; [4]Faculdade de Tecnologia e Ciências, Salvador, Brazil; [5]Prof. M. Viswanathan Diabetes Research Center, Chennai, India; [6]Universidade Salvador, Laureate Universities, Salvador, Brazil; [7]University of Massachusetts Medical School, Worcester, United States; [8]National Institute for Research in Tuberculosis, Chennai, India

Abstract Diabetes mellitus (DM) increases risk for pulmonary tuberculosis (TB) and adverse treatment outcomes. Systemic hyper-inflammation is characteristic in people with TB and concurrent DM (TBDM) at baseline, but the impact of TB treatment on this pattern has not been determined. We measured 17 plasma cytokines and growth factors in longitudinal cohorts of Indian and Brazilian pulmonary TB patients with or without DM. Principal component analysis revealed virtually complete separation of TBDM from TB individuals in both cohorts at baseline, with hyper-inflammation in TBDM that continued through treatment completion at six months. By one year after treatment completion, there was substantial convergence of mediator levels between groups within the India cohort. Non-resolving systemic inflammation in TBDM comorbidity could reflect delayed lesion sterilization or non-resolving sterile inflammation. Either mechanism portends unfavorable long-term outcomes including risk for recurrent TB and for damaging immune pathology.

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***For correspondence:** Hardy.Kornfeld@umassmed.edu

†These authors contributed equally to this work
‡These authors also contributed equally to this work

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Introduction

It is now well established that diabetes mellitus (DM) is associated with increased the risk to become infected with *Mycobacterium tuberculosis* (*Mtb*), to progress from latent infection to active pulmonary tuberculosis (TB) disease, and to suffer adverse TB outcomes including delayed sputum conversion on treatment, treatment failure, death, and recurrent TB (***Critchley et al., 2017***). The biochemical and cellular mechanisms of increased TB susceptibility in DM are incompletely understood. Emerging evidence ties the complication of diabetic immunopathy to the well-studied diabetic complications of microvascular, macrovascular and renal disease driven by pathways primary driven by chronic hyperglycemia and related oxidative stress (***Giacco and Brownlee, 2010***; ***Martinez and Kornfeld, 2014***).

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ORIGINAL RESEARCH
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Lutzomyia longipalpis Saliva Induces Heme Oxygenase-1 Expression at Bite Sites

Nivea F. Luz[1†], Thiago DeSouza-Vieira[2†], Waldione De Castro[2], Aislan Carvalho Vivarini[3], Lais Pereira[2], Riam Rocha França[1,4], Paulo S. Silveira-Mattos[1,4], Diego L. Costa[5], Clarissa Teixeira[6], Claudio Meneses[2], Viviane S. Boaventura[1,4], Camila I. de Oliveira[1,4], Ulisses Gazos Lopes[3], Naomi Aronson[7], Bruno B. Andrade[1,4,8], Claudia I. Brodskyn[1,4], Jesus G. Valenzuela[2], Shaden Kamhawi[2‡] and Valeria M. Borges[1,4*‡]*

[1] Gonçalo Moniz Institute, Oswaldo Cruz Foundation, Salvador, Brazil, [2] Vector Molecular Biology Section, Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, United States, [3] Laboratory of Molecular Parasitology, Center of Health Science, Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, [4] Faculdade de Medicina da Bahia, Universidade Federal da Bahia, Salvador, Brazil, [5] Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, United States, [6] Oswaldo Cruz Foundation (Fiocruz-PI), Teresina, Brazil, [7] Infectious Diseases Division, Uniformed Services University of the Health Sciences, Bethesda, MD, United States, [8] Multinational Organization Network Sponsoring Translational and Epidemiological Research Initiative, Fundação José Silveira, Salvador, Brazil

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David M. Mosser, University of Maryland, College Park, United States

***Correspondence:**
Shaden Kamhawi
skamhawi@niaid.nih.gov
Valeria M. Borges
vborges@bahia.fiocruz.br

†These authors have contributed equally to this work

‡These senior authors have contributed equally to this work

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Sand flies bite mammalian hosts to obtain a blood meal, driving changes in the host inflammatory response that support the establishment of *Leishmania* infection. This effect is partially attributed to components of sand fly saliva, which are able to recruit and activate leukocytes. Our group has shown that heme oxygenase-1 (HO-1) favors *Leishmania* survival in infected cells by reducing inflammatory responses. Here, we show that exposure to sand fly bites is associated with induction of HO-1 *in vivo*. Histopathological analyses of skin specimens from human volunteers experimentally exposed to sand fly bites revealed that HO-1 and Nrf2 are produced at bite sites in the skin. These results were recapitulated in mice ears injected with a salivary gland sonicate (SGS) or exposed to sand fly bites, indicating that vector saliva may be a key factor in triggering HO-1 expression. Resident skin macrophages were the main source HO-1 at 24–48 h after bites. Additionally, assays *in vivo* after bites and *in vitro* after stimulation with saliva both demonstrated that HO-1 production by macrophages was Nrf2-dependent. Collectively, our data demonstrates that vector saliva induces early HO-1 production at the bite sites, representing a major event associated with establishment of naturally-transmitted *Leishmania* infections.

Keywords: ***Lutzomyia longipalpis*, sand fly bite, saliva, skin, macrophages, heme oxygenase-1, Nrf2**

INTRODUCTION

The leishmaniases are a group of diseases caused by protozoan parasites from more than 20 *Leishmania* species (1). There are three main forms of the disease: visceral leishmaniasis (VL, also known as kala azar), cutaneous leishmaniasis (CL), and mucosal leishmaniasis (MCL). While CL is the most common form of the disease, VL is the most serious and can be fatal if untreated. Most

REVISTA DE Biología Tropical

Biometría testicular y características seminales en felinos neotropicales (Carnivora: Felidae) sometidos a cateterismo uretral

Mónica Madrigal-Valverde[1,2]*, Rodrigo F. Bittencourt[1], Antonio de Lisboa Ribeiro Filho[1], Maicon P. Lents[1], Milena C. de Azevedo[1], Renata O. Barreto[1], Nayara A. Teixeira[1], Paulo M. S. Mattos[4,5], Vitor P. Curvelo[6], Marta C. Gomes[6], Vínicius Dantas [6], Gediendson Ribeiro de Araujo[7] & Anthony Valverde-Abarca[2]

1. Escola de Medicina Veterinária e Zootecnia, Universidade Federal da Bahia, CEP 40170-110 Salvador, Bahía, Brasil; madrigalvalverdem@gmail.com, rfb@ufba.br, alisboa@ufba.br, maicon_pl@hotmail.com, renata.obrt@hotmail.com, naay_aquino@hotmail.com
2. Instituto Tecnológico de Costa Rica. Escuela de Agronomía. Campus Tecnológico Local San Carlos, Apto. Postal 223-21001 Alajuela, Costa Rica; mmadrigal@itcr.ac.cr, anvalverde@itcr.ac.cr
3. Hospital Veterinario Renato R. de Madeiros Neto, CEP 40170-110, Salvador, Bahía, Brasil; milenacastro92@hotmail.com
4. Instituto Gonçalo Moniz, Fundação Osvaldo Cruz, CEP 40296-710, Salvador, Bahía, Brasil; os.mattos@outlook.com
5. Facultade de Medicina da Bahia, Universidade Federal da Bahia, CEP 40110905 Salvador, Bahía, Brasil; os.mattos@outlook.com
6. Parque Zoobotânico Getúlio Vargas, CEP 40170-110, Salvador, Bahía, Brasil; vitor.curvelo@inema.ba.gov.br, marta.gomes@inema.ba.gov.br, vinicius.dantas@inema.ba.gov.br
7. Biosciences Institute, Federal Univesity of Mato Grosso do Sul, 79074460 Campo Grande, MS, Brazil, gediendson@gmail.com

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Abstract: Testicular biometry and seminal traits in neotropical cats (Carnivora: Felidae) by uretral catheterization. Introduction: The urethral catheterization associated with the application of alfa- 2 adrenergic agonist drug is a recent development and application technique for the collection of feline semen, with advantages over electro-ejaculation harvesting and epididymal harvesting. **Objetives:** Seminal collection in neotropical felines using the urethral catheterization technique. Determine the testicular and seminal parameters of samples collected by the urethral catateriza tion technique. Methods: The technique has been tested on *Panthera onca* and others small cats. In this study, individuals of *Panthera onca*, *Puma yagouaroundi* and *Leopardus wiedii* were used in captivity at Getulio Vargas Zoobotanical Park, Salvador, Bahia, Brazil. Medetomidine hydrochloride (0.1 mg/kg) and ketamine (5.0 mg/kg) were used as sedatives, the semen was collected by urethral catheterization. In the process were checked the testicular biometry was performed with percutaner and ultrasonography technique. The fresh samples were evaluated for conventional microscopy in the parameters of volume, sperm vigor, total motility, progressive motility, structural and functional integrity of the plasmatic membrane and sperm morphology. The samples were subjected to a freeze - thaw process using three different extenders. The thawed samples were tested using conventional microscopy, automated system analysis and flow cytometry. Results: In this study, the urethral harvest technique was successfully used in the three species. In the literature used don't have report about seminal parameters in *P. yagouaroundi* and urethral seminal collection in *L. wiedii*. The data presented describe the spermatic morphocinetics of the included species in a pioneer study. There were no differences ($P > 0.05$) in the seminal quality evaluating the extender, nor differences in the integrity of pre

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Can amides be alternative cryoprotectors for the preservation of feline semen?

Mónica Madrigal-Valverde [a,b], Rodrigo F. Bittencourt [c,*], Antônio de Lisboa Ribeiro Filho [c], Gediendson R. Araujo [d], Maicon P. Lents [c], Elisiane S. Santos [c], Adrielle S. Lima [c], Paulo Mattos [e,f]

[a] School of Agronomy, Costa Rica Institute of Technology, CTLSC, 223-21001, San Carlos, Costa Rica
[b] Animal Science Department, University of Costa Rica, Campus Rodrigo Facio, 1501-2060, San José, Costa Rica
[c] School of Veterinary and Animal Science Medicine, Federal University of Bahia, Av. Adhemar de Barros, 500, Salvador, Bahia, 40170-110, Brazil
[d] Federal University of Mato Grosso do Sul, Avenida Senador Filinto Muller - de 932/933 ao fim, Vila Ipiranga, 79074460, Campo Grande, MS, Brazil
[e] Instituto Gonçalo Moniz, Fundação Osvaldo Cruz, CEP 40296-710, Salvador, Bahía, Brazil
[f] Faculdade de Medicina da Bahia, Universidade Federal da Bahia, CEP 40110905, Salvador, Bahía, Brazil

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ABSTRACT

Sperm cryopreservation is a tool for the conservation of the genetic material of animals of genetic importance or for species preservation. In the case of domestic cats, this can be used to generate information about seminal harvest, evaluation and preservation, which is especially important due to its applicability to wild felids. This study evaluated seminal samples harvested by urethral catheterisation from 13 adult domestic cats. Samples were cryopreserved with experimental groups of extenders were defined by the penetrating cryoprotectant: 6% glycerol (GLY6%), 3% dimethylacetamide (DMA3%) and 3% dimethylformamide (DMF3%). The samples were thawed and evaluated by conventional microscopy and by computer-assisted sperm analysis (CASA). The structural and functional membrane integrity was assessed by supravital tests (EOS), hypoosmotic swelling tests (HOST) and flow cytometry (FC). There was a correlation ($P < 0.05$) between total motility and EOS ($r = 0.54$), HOST and FC ($r = -0.62$) and total motility and flow cytometry ($r = 0.63$), indicating that these are complementary parameters that increase the accuracy of the feline sperm quality evaluation post-thaw. The results regarding the structural and functional integrity of the sperm plasma membrane did not differ ($P > 0.05$) among groups. However, the DMA3% group had a lower ($P < 0.05$) percentage of morphological changes in the sperm tail compared to samples cryopreserved with GLY6% and DMF3%. Additionally, DMA3% provided lower values of immobile sperm post-thaw when compared to DMF3%. DMA is an interesting alternative to GLY and superior to DMF for the cryopreservation of feline semen at the studied concentrations.

1. Introduction

The pharmacological induction of ejaculation is a methodology used to harvest semen in domestic animals, primarily using alpha-2 adrenergic agonist drugs [30]. The harvest technique reported via urethral catheterisation consists of the introduction of a thin probe via the penile urethra after animal sedation [36] associated with an alpha-2 adrenergic agonist drug that acts as both an analgesic and a sedative, allowing adrenoreceptor stimulation in different ducts and the release of semen into the penile urethra, where it is harvested with the probe [35,50].

This recently developed technique provides an interesting option for seminal harvest in wild cats [1,26,29]. Since seminal harvest is fundamental for assisted reproduction programs in cats and sperm cryopreservation enables the artificial insemination of females [15,44] this represents an important component of the genome resource bank. Thus, the technique has huge potential for the genetic management of endangered felids [10]. However, the technique requires the development of cryopreservation protocols that are suitable for field use, with applicability in different species and acceptable sperm mobility and viability post-thawing [43]. The use of frozen-thawed semen for artificial insemination (AI) in cats has allowed for viable pregnancy rates, depending on the AI method used. In transcervical AI, a pregnancy rate of 41.7% has been recorded, while with vaginal AI there is a 0% chance of pregnancy [12]. With intrauterine AI, the observed pregnancy rate is

* Corresponding author. Av. Euclydes da Cunha, 403, apt 1103, Graça, Salvador, Bahia, 40.150-120, Brazil.
E-mail address: rfb@ufba.br (R.F. Bittencourt).

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ORIGINAL RESEARCH

Evaluation of in vitro and in vivo Efficacy of a Novel Amphotericin B-Loaded Nanostructured Lipid Carrier in the Treatment of *Leishmania braziliensis* Infection

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Jéssica Rebouças-Silva [1,2,*]
Maraine Catarina Tadini [3,4,*]
Danielle Devequi-Nunes [1,5]
Ana Luíza Mansur [3]
Paulo S Silveira-Mattos [1,2]
Camila I de Oliveira [2,6]
Fábio R Formiga [7,8]
Andresa A Berretta [9]
Franciane Marquele-Oliveira [3]
Valéria M Borges [1,2]

[1]Laboratory of Inflammation and Biomarkers, Gonçalo Moniz Institute, Oswaldo Cruz Foundation, Salvador, Bahia, Brazil; [2]Postgraduate Program in Pathology, Faculty of Medicine of Bahia, Federal University of Bahia (UFBA), Salvador, Bahia, Brazil; [3]Eleve Science Research and Development, Ribeirão Preto, São Paulo, Brazil; [4]Department of Chemistry, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil; [5]Laboratory of Pharmaceutical Formulations, SENAI Institute of Innovation in Advanced Health Systems, Salvador, Bahia, Brazil; [6]Laboratory of Vector-Borne Infectious Diseases, Gonçalo Moniz Institute, Oswaldo Cruz Foundation, Salvador, Bahia, Brazil; [7]Aggeu Magalhães Institute, Oswaldo Cruz Foundation (FIOCRUZ), Recife, Pernambuco, Brazil; [8]Postgraduate Program in Applied Cellular and Molecular Biology, University of Pernambuco (UPE), Recife, Pernambuco, Brazil; [9]Laboratory of Research, Development and Innovation, Apis Flora Industrial e Comercial Ltda, Ribeirão Preto, São Paulo, Brazil

*These authors contributed equally to this work

Correspondence: Valéria M Borges; Franciane Marquele-Oliveira
Email vborges@bahia.fiocruz.br; franciane.oliveira@elevescience.com.br

Background: Leishmaniasis is a neglected disease, and the current therapeutic arsenal for its treatment is seriously limited by high cost and toxicity. Nanostructured lipid carriers (NLCs) represent a promising approach due to high drug loading capacity, controlled drug release profiles and superior stability. Here, we explore the efficacy of a unique pH-sensitive amphotericin B-loaded NLC (AmB-NLC) in *Leishmania braziliensis* infection in vitro and in vivo.

Methods and Results: AmB-NLC was assessed by dynamic light scattering and atomic force microscopy assays. The carrier showed a spherical shape with a nanometric size of 242.0 ± 18.3 nm. Zeta potential was suggestive of high carrier stability (−42.5 ± 1.5 mV), and the NLC showed ~99% drug encapsulation efficiency (EE%). In biological assays, AmB-NLC presented a similar IC_{50} as free AmB and conventional AmB deoxycholate (AmB-D) (11.7 ± 1.73; 5.3 ± 0.55 and 13 ± 0.57 ng/mL, respectively), while also presenting higher selectivity index and lower toxicity to host cells, with no observed production of nitric oxide or TNF-α by in vitro assay. Confocal microscopy revealed the rapid uptake of AmB-NLC by infected macrophages after 1h, which, in association with more rapid disruption of AmB-NLC at acidic pH levels, may directly affect intracellular parasites. Leishmanicidal effects were evaluated in vivo in BALB/c mice infected in the ear dermis with *L. braziliensis* and treated with a pentavalent antimonial (Sb^{5+}), liposomal AmB (AmB-L) or AmB-NLC. After 6 weeks of infection, AmB-NLC treatment resulted in smaller ear lesion size in all treated mice, indicating the efficacy of the novel formulation.

Conclusion: Here, we preliminarily demonstrate the effectiveness of an innovative and cost-effective AmB-NLC formulation in promoting the killing of intracellular *L. braziliensis*. This novel carrier system could be a promising alternative for the future treatment of cutaneous leishmaniasis.

Keywords: leishmaniasis, neglected disease, nanoparticles, drug delivery

Introduction

Leishmaniasis is a widespread group of neglected vector-borne tropical diseases caused by *Leishmania* spp. (protozoa: Trypanosomatidae). The disease mainly manifests in two forms: visceral leishmaniasis (VL), which is potentially lethal, and the more common form, cutaneous leishmaniasis (CL). It is estimated that between 700,000 and 1,000,000 new cases are reported annually worldwide; about

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ORIGINAL RESEARCH
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Generation and Characterization of a Dual-Reporter Transgenic *Leishmania braziliensis* Line Expressing eGFP and Luciferase

Rohit Sharma[1], *Paulo S. Silveira-Mattos*[1], *Vinicius C. Ferreira*[1], *Francys A. Rangel*[1], *Laise B. Oliveira*[1], *Fabiana S. Celes*[1], *Sayonara M. Viana*[1], *Mary E. Wilson*[2] *and Camila I. de Oliveira*[1,3]*

[1] Instituto Gonçalo Moniz, FIOCRUZ, Salvador, Brazil, [2] Departments of Microbiology and Immunology and Internal Medicine, University of Iowa, and the Veterans' Affairs Medical Center, Iowa City, IA, United States, [3] INCT—Instituto de Investigação em Imunologia, São Paulo, Brazil

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Rodolfo Cordeiro Giunchetti, Federal University of Minas Gerais, Brazil

***Correspondence:**
Camila I. de Oliveira
camila@bahia.fiocruz.br

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In this study, we generated a transgenic strain of *Leishmania braziliensis,* an etiological agent associated with a diversity of clinical manifestations of leishmaniasis ranging from localized cutaneous to mucocutaneous to disseminated disease. Transgenic parasites expressing reporter proteins are valuable tools for studies of parasite biology, host-pathogen interactions, and anti-parasitic drug development. To this end, we constructed an *L. braziliensis* line stably expressing the reporters eGFP and luciferase (eGFP-LUC *L. braziliensis)*. The integration cassette co-expressing the two reporters was targeted to the ribosomal locus (*SSU*) of the parasite genome. Transgenic parasites were characterized for their infectivity and stability both *in vitro* and *in vivo*. Parasite maintenance in axenic long-term culture in the absence of selective drugs did not alter expression of the two reporters or infection of BALB/c mice, indicating stability of the integrated cassette. Infectivity of eGFP-LUC, *L. braziliensis,* both *in vivo* and *in vitro* was similar to that obtained with the parental wild type strain. The possibility of *L. braziliensis* tracking and quantification using fluorescence and luminescence broadens the scope of research involving this neglected species, despite its importance in terms of public health concerning the leishmaniasis burden.

Keywords: *L. braziliensis*, transgenic, dual reporters, luciferase, eGFP

INTRODUCTION

Leishmaniasis is a neglected tropical disease caused by the protozoan pathogen *Leishmania spp.* that is transmitted by sand flies. Nearly 1.3 million new cases occur each year (Burza et al., 2018) and 350 million people face risk of infection. The two most common forms of leishmaniasis are visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL). In Brazil, *Leishmania braziliensis* is the leading cause of CL, which mostly manifests as localized lesions on the skin, but may also metastasize to mucosal sites (Bittencourt and Netto, 1995). Another common manifestation is disseminated leishmaniasis (Carvalho et al., 1994), which is characterized by the presence of large numbers of papular and acneiform ulcers affecting different parts of the body. In contrast to American Cutaneous Leishmaniasis, disseminated leishmaniasis is associated with impaired

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Higher values of triglycerides:HDL-cholesterol ratio hallmark disease severity in children and adolescents with sickle cell anemia

R.S. Teixeira[1,2*], M.B. Arriaga[3,4*], R. Terse-Ramos[2], T.A. Ferreira[2], V.R. Machado[1], M.R. Rissatto-Lago[5], P.S. Silveira-Mattos[3,4], N. Boa-Sorte[1,5], A.M.T. Ladeia[1+], and B.B. Andrade[1,3,4,6*+]

[1]Escola Bahiana de Medicina e Saúde Pública, Salvador, BA, Brasil
[2]Departamento de Pediatria, Faculdade de Medicina, Universidade Federal da Bahia, Salvador, BA, Brasil
[3]Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, BA, Brasil
[4]Multinational Organization Network Sponsoring Translational and Epidemiological Research (MONSTER) Initiative, Fundação José Silveira, Salvador, BA, Brasil
[5]Departamento de Ciências da Vida, Universidade Estadual da Bahia, Salvador, BA, Brasil
[6]Universidade Salvador, Laureate International Universities, Salvador, BA, Brasil

Abstract

Dyslipidemia has been described in sickle cell anemia (SCA) but its association with increased disease severity is unknown. Here, we examined 55 children and adolescents with SCA as well as 41 healthy controls to test the association between the lipid profiles in peripheral blood and markers of hemolysis, inflammation, endothelial function, and SCA-related clinical outcomes. SCA patients exhibited lower levels of total cholesterol ($P<0.001$), low-density lipoprotein cholesterol (LDL-c) ($P<0.001$), and high-density lipoprotein cholesterol (HDL-c) ($P<0.001$), while displaying higher triglyceride (TG) levels and TG/HDL-c ratio values ($P<0.001$). TG/HDL-c values were positively correlated with lactate dehydrogenase (P=0.047), leukocyte count (P=0.006), and blood flow velocity in the right (P=0.02) and left (P=0.05) cerebral artery, while being negatively correlated with hemoglobin levels ($P<0.04$). Acute chest syndrome (ACS) and vaso-occlusive events (VOE) were more frequent in SCA patients exhibiting higher TG/HDL-c values (odds ratio: 3.77, P=0.027). Multivariate logistic regression analysis confirmed independent associations between elevated TG/HDL-c values and SCA. Thus, children and adolescents with SCA exhibited a lipid profile associated with hemolysis and inflammatory parameters, with increased risk of ACS and VOE. TG/HDL-c is a potential biomarker of severity of disease.

Key words: Sickle cell disease; Lipoproteins; Cholesterol; Triglycerides; Hydroxyurea; Endothelial function

Introduction

Sickle cell anemia (SCA) is a hemoglobinopathy of autosomal recessive inheritance that manifests with hemolysis, vaso-occlusive crises, progressive organic damage, and early death (1). SCA pathophysiology is complex and involves activation of leukocytes and intricate interactions between abnormal erythrocytes and the vascular endothelium (2,3). In SCA, the dyslipidemia profile characterized by lower levels of total cholesterol, low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), and high levels of triglycerides (TG) has been described, but the impact of these changes in the clinical picture needs to be better understood (4). In some clinical settings, serum cholesterol is an important parameter for assessment of disease severity and/or progression, with lower values usually being indicative of increased risk of death (5). Previous studies in adult patients with SCA have described a positive correlation between levels of TG and circulating concentrations of markers of hemolysis and inflammation, whereas TG/HDL-c ratio values are associated with endothelial dysfunction (6). Of note, hypertriglyceridemia in this patient population is considered a risk factor for pulmonary hypertension (6), a leading cause of

Correspondence: B.B. Andrade: <bruno.andrade@bahia.fiocruz.br>

*These authors contributed equally to the work.
+These senior authors contributed equally as supervisors of the work.

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Changes in inflammatory protein and lipid mediator profiles persist after antitubercular treatment of pulmonary and extrapulmonary tuberculosis: A prospective cohort study

Caian L. Vinhaes[a,b,c,1], Deivide Oliveira-de-Souza[a,b,c,1], Paulo S. Silveira-Mattos[a,b,c], Betania Nogueira[b], Ruiru Shi[d], Wang Wei[d], Xing Yuan[d], Guolong Zhang[e], Ying Cai[f], Clifton E. Barry III[f], Laura E. Via[f], Kiyoshi F. Fukutani[a,b,c], Bruno B. Andrade[a,b,c,g,h,i,j,*,2], Katrin D. Mayer-Barber[k,*,2]

[a] Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador 40296-710, Brazil
[b] Multinational Organization Network Sponsoring Translational and Epidemiological Research (MONSTER) Initiative, Fundação José Silveira, Salvador 40210-320, Brazil
[c] Curso de Medicina, Faculdade de Tecnologia e Ciências (FTC), Salvador 40290-150, Brazil
[d] Henan Chest Hospital, Zhengzhou 450000, China
[e] Henan Anti-TB Institute, Sino-US Joint Research Laboratory for Tuberculosis of Henan CDC, Zhengzhou 450000, China
[f] Tuberculosis Research Section, Laboratory of Clinical Immunology and Microbiology, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda 20892, MD, USA
[g] Escola Bahiana de Medicina e Saúde Pública (EBMSP), Salvador 40290-000, Brazil
[h] Universidade Salvador (UNIFACS), Laureate Universities, Salvador 41720-200, Brazil
[i] Wellcome Centre for Infectious Disease Research in Africa, Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town 7925, South Africa
[j] Division of Infectious Diseases, Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232, USA
[k] Inflammation and Innate Immunity Unit, Laboratory of Clinical Immunology and Microbiology, NIAID, NIH, Bethesda 20892, MD, USA

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ABSTRACT

Background: The identification of meaningful biomarkers of tuberculosis (TB) has potential to improve diagnosis, disease staging and prediction of treatment outcomes. It has been shown that active pulmonary TB (PTB) is associated with qualitative and quantitative changes in systemic immune profile, suggesting a chronic inflammatory imbalance. Here we characterized the profile of PTB and extrapulmonary TB (EPTB) in a prospective cohort study.

Methods: We measured a panel of 27 inflammatory cytokines, soluble receptors, and lipid mediators in peripheral blood from patients with PTB or EPTB from a prospective clinical study in China. Multidimensional analyses were performed to describe associations between plasma levels of biomarkers and different TB disease presentation profiles.

Results: *Mycobacterium tuberculosis* infection induced changes in both the expression and correlation profiles of plasma mediators of inflammation in patients with PTB compared to those with EPTB. Increases in mycobacterial loads in sputum smears were associated with rises in concentrations of several molecules involved in TB pathogenesis, such as IL-1β, IFN-α, IL-10 and PGF2α. Moreover, PTB patients presenting with severe disease exhibited a distinct inflammatory profile hallmarked by heightened levels of TNF-α, IL-1β, IL17, IL-18 and IL-27. Interestingly, while antitubercular treatment (ATT) resulted in early changes of plasma concentrations of markers in PTB, changes were delayed in EPTB patients. Exploratory analyses of the molecular degree of perturbation (MDP) of the inflammatory mediators before and during ATT suggested the occurrence of infection

Abbreviations: AFB, acid fast bacilli; ATT, antitubercular treatment; BCG, bacillus Calmette-guérin; CCA, canonical correlation analysis; LXA, lipoxin; EPTB, extrapulmonary tuberculosis; HC, healthy control; IFN, interferon; IL, interleukin; IQR, interquartile range; LXA, lipoxin; MDP, molecular degree of perturbation; MPO, myeloperoxidase; PG, prostaglandin; PTB, pulmonary tuberculosis; TB, tuberculosis; TNF, tumor necrosis factor

* Corresponding authors at: Laboratório de Inflamação e Biomarcadores, Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão, no. 121, Candeal, Salvador, Bahia 40269-710, Brazil (B.B. Andrade). National Institute of Allergy and Infectious Diseases/National Institutes of Health, Building 33, Room 2W10A, 33 North Drive, Bethesda, MD 20892, USA (K.D. Mayer-Barber).
E-mail addresses: bruno.andrade@fiocruz.br (B.B. Andrade), mayerk@niaid.nih.gov (K.D. Mayer-Barber).
[1] C.L.V. and D.O.S. equally contributed to the work.
[2] B.B.A. and K.D.M.B. equally contributed to the work.

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