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

TESE DE DOUTORADO

RESPOSTA INDUZIDA PELO *MYCOBACTERIUM TUBERCULOSIS* COM INTERRUPÇÃO NO *OPERON MCE1* E OS LIPÍDIOS DA PAREDE CELULAR: UMA ANÁLISE PARA IDENTIFICAÇÃO DE BIOMARCADORES

JÉSSICA DIAS PETRILLI

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JÉSSICA DIAS PETRILLI

Orientador: Prof. Dr.Sérgio Arruda

Co-orientador: Dr. Adriano Queiroz

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

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JÉSSICA DIAS PETRILLI

FOLHA DE APROVAÇÃO

Salvador, 22 de outubro de 2019.

COMISSÃO EXAMINADORA

Dr. Eduardo Martins Netto
Pesquisador
UFBA

Dr. Antonio Ricardo Khouri Cunha
Pesquisador
IGM/FIOCRUZ

Dra. Theolis Costa Barbosa Bessa
Pesquisadora
IGM/FIOCRUZ

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“A persistência é o menor caminho do êxito. ”

Charles Chaplin.

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RESUMO

INTRODUÇÃO. *Mycobacterium tuberculosis (M. tuberculosis)* apresenta características peculiares quando comparado a outros patógenos. A interrupção no *operon mce1 (Δmce1)* do *M. tuberculosis*, um putativo transportador de lipídios, causa reorganização lipídica da sua parede celular e reduz assim a capacidade do bacilo de induzir uma resposta inflamatória adequada para controlar a infecção. O grande desafio da TB consiste em interromper o ciclo de transmissão do bacilo e, portanto, uma melhor compreensão dos mecanismos de patogênese do *M. tuberculosis* pode ser a chave para a interrupção desse ciclo. **OBJETIVO.** Avaliar a resposta induzida pelo *M. tuberculosis Δmce1* e selvagem (WT), e pelo extrato apolar de lipídios da parede celular em modelo *in vitro*. **MATERIAL E MÉTODOS.** Foram utilizadas para avaliação da resposta induzida pelo bacilo na formação do granuloma e os extratos lipídicos da parede celular das duas cepas células do sistema imunológico. Em busca de biomarcadores associados com a TB, uma análise da expressão gênica de células de sangue total de pacientes com TB ativa, TB latente, outras doenças pulmonares e indivíduos saudáveis foi realizada. **RESULTADOS.** Os dados demonstram que o *M. tuberculosis* Δmce1 é capaz de induzir a formação do granuloma, no entanto apresenta capacidade reduzida de controlar a infecção *in vitro* por um período prolongado. Os lipídios do *M. tuberculosis* Δmce1 foram associados com a capacidade reduzida da cepa em montar uma resposta inflamatória, uma vez que o extrato apolar de lipídios da cepa induziu uma expressão reduzida de genes associados com a resposta inflamatória. Após análise de sangue total, genes associados a inflamação apresentaram-se como importantes alvos para avaliação de biomarcadores de diagnóstico da TB e progressão da doença. **CONCLUSÃO.** *M. tuberculosis* Δmce1 demonstrou ter uma capacidade imunogênica reduzida em comparação com a cepa selvagem e o rearranjo lipídico da sua parede celular está envolvido na resposta orquestrada pelo sistema imunológico do hospedeiro, revelando que os lipídios têm um importante papel no curso da doença.

Palavras-chave: Tuberculose pulmonar, Lipídios, Resposta Imune, Expressão Gênica.

PETRILLI, Jéssica Dias. Mycobacterium tuberculosis-induced response with interruption in operon mce1 and cell wall lipids: an analysis to identify biomarkers. 127 f. il. Tese (Doutorado em Biotecnologia em Saúde e Medicina Investigativa) – Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, 2019.

ABSTRACT

INTRODUCTION. Mycobacterium tuberculosis (*M. tuberculosis*) has peculiar characteristics when compared to other pathogens. The disruption in the operon mce1 (*Δmce1*) of M. *tuberculosis*, a putative lipid transporter, causes lipid reorganization of its cell wall and thus reduces the bacillus's ability to induce an appropriate inflammatory response to control the infection. The great challenge of TB is to interrupt the bacillus transmission cycle. Therefore, a better understanding of the pathogenesis mechanisms of *M. tuberculosis* may be the key to interrupting this cycle. **OBJECTIVE.** Evaluate the response induced by *M. tuberculosis* Δmce1 and wild (WT), and apolar cell wall lipid extract in an in vitro model. **MATERIAL AND METHODS.** *M. tuberculosis* strains, Δmce1 and WT, were used to evaluate the bacillus-induced response in granuloma formation and lipid extracts from the cell wall of the two strains were used to evaluate the induced response in immune cells. In search of biomarkers associated with TB, an analysis of whole blood cell gene expression of patients with active TB, latent TB, other lung diseases, and healthy individuals was performed. **RESULTS.** The data demonstrate that *M. tuberculosis* Δmce1 is able to inducing granuloma formation, however it has reduced ability to control infection for a prolonged period. *M. tuberculosis* Δmce1 lipids were associated with the reduced ability of the strain to mount an inflammatory response because its apolar lipid extract induced a decreased expression of genes associated with the inflammatory response. After analysis of whole blood, genes associated with inflammation were presented as important targets for evaluation of diagnostic biomarkers of TB and disease progression. **CONCLUSION.** *M. tuberculosis* Δmce1 has been shown to have a reduced immunogenic capacity compared to the wild type strain and its cell wall lipid rearrangement is involved in the response orchestrated by the host immune system, revealing that lipids play an important role in the course of the disease.

Palavras-chave: Pulmonary tuberculosis, Lipids, Immune Response, Gene Expression.

LISTA DE ABREVIATURAS

AM	Ácidos micólicos
AML	Ácidos micólicos livres
CoA	Coenzima A
CD	*cluster* de diferenciação
CXCL	Ligante de quimiocina
DAT	Diaciltrealose
DCs	Células dendríticas
FIOCRUZ	Fundação Oswaldo Cruz
HIV	Vírus da imunodeficiência humana
H37Rv	Cepa virulenta do *Mycobacterium tuberculosis*
IBIT	Instituto Brasileiro de Investigação da Tuberculose
IFN-γ	Interferon-gama
IGM	Instituto Gonçalo Moniz
IGRA	Ensaio de liberação de interferon gama
IL	Interleucina
LAM	Lipoarabinomanana
LASP	Laboratório Avançado de Saúde Pública
LM	Glicolipídios lipomanana
LXR	*Hepatocyte nuclear factor*
ManLAM	Liboarabinomanana manosilado
Mce1	*Mammalian cell entry 1*
Mce1A	*Mammalian cell entry protein 1A*
MHC	Complexo principal de histocompatibilidade
Mincle	Lectina tipo C
MMPs	Metaloproteases
M. tuberculosis	*Mycobacyerium tuberculosis*
MyD88	Fator de diferenciação mielóide 88
OMS	Organização Mundial de Saúde

PAMPs	Padrões moleculares associados a patógenos
PAT	Poliaciltrealose
PBMC	Células mononucleares do sangue periférico
PDIM	Dimicocerato de fitilcerol
PGL	Glicolipídio fenólico
PIMs	Fosfatidilinositol manosídeo
PPAR-γ	*Peroxisome proliferator-activated receptor gamma*
TR4	*Testicular receptor 4*
PRRs	Receptores de reconhecimento padrão
RAR	R*etinoic acid receptor*
RN	Receptores nucleares
RNAL	Receptores nucleares ativados por lipídios
SL	Sulfolipídio
TAT	Triacilglicerol
TB	Tuberculose
TBL	Tuberculose latente
TCR	Receptores de células T
TDM	Dimicolato de trealose
Th1	Linfócitos T auxiliaries do tipo 1
TLR	Receptores semelhantes ao *toll*
TNF	Fator de necrose tumoral
TST	*Tuberculin skin test*
WT	*Wild type*

SUMÁRIO

1 INTRODUÇÃO

Apesar de dispor de esquemas eficazes de tratamento contra o *Mycobacterium tuberculosis* (*M. tuberculosis*), o agente etiológico da tuberculose (TB), a TB ainda é considerada um grave problema de saúde pública. O grande desafio para o controle da TB consiste principalmente na dificuldade em predizer quais daqueles indivíduos que possuem tuberculose latente (TBL) irão progredir para a forma ativa da doença. Embora algumas condições do hospedeiro, como a imunossupressão, por exemplo, possam levar o indivíduo com TBL a desenvolver a forma ativa da doença, alguns indivíduos progridem da forma latente para a forma ativa sem que tenham apresentado quaisquer fatores associados ao hospedeiro que justifique este desfecho. Fatores relacionados ao patógeno, como sua composição lipídica, tem mostrado potencial papel imunogênico no curso da infecção sendo assim alvo de estudos sobre a imunopatogênese da TB.

Os lipídios se tornaram importantes alvos para imunorregulação da patogênese da TB devido a grande parte da capacidade codificante do genoma do *M. tuberculosis* ser dedicado a biossíntese e degradação de lipídios (COLE et al., 1998). Existem cerca de mais 5.000 espécies lipídicas expressos na parede do *M. tuberculosis* com distintas funções associada a resposta imune ou a patogênese da TB. Estudos demonstraram que o *operon mce1* está associado com a expressão de diferentes conjuntos lipídicos na parede celular do bacilo ao longo da infecção e essa remodelação da parede celular possivelmente está associada a diferentes desfechos da infecção (CANTRELL et al., 2013; QUEIROZ et al., 2015).

O *operon mce1* foi descrito pela primeira vez em 1993 (ARRUDA et al., 1993) e até então diversos estudos vem sendo desenvolvidos para compreender o papel deste operon na patogênese da TB. Estudos demonstraram que o *M. tuberculosis* com interrupção no *operon mce1 (Δmce1)* é incapaz de estimular a formação de um granuloma organizado em pulmões de camundongos, com diminuição da produção de citocinas inflamatórias e interleucina-10 (IL-10), o que leva os camundongos a uma morte mais rápida (LIMA et al., 2007; SHIMONO et al., 2003a). *Operon mce1* é reprimido nas primeiras quatro semanas de infecção pela cepa WT do *M. tuberculosis*, o que a torna funcionalmente equivalente ao *M. tuberculosis Δmce1* (UCHIDA et al., 2007).

Portanto, especula-se que os lipídios expressos na parede celular do *M. tuberculosis* WT são possivelmente os lipídios expressos na fase inicial da doença, enquanto que os lipídios expressos pelo *M. tuberculosis Δmce1* seriam expressos na fase tardia.

Assim, a parede celular do *M. tuberculosis* é uma estrutura dinâmica que constantemente se remodela durante o curso da infecção e as mudanças no conteúdo lipídico da parede celular do bacilo podem induzir diferentes repostas no hospedeiro. Deste modo, este trabalho buscou estudar as respostas induzidas pela cepa com interrupção no *operon mce1* e o papel dos lipídios expressos na parede celular, proporcionando conhecimento para estudos na identificação de biomarcadores que possam ser utilizados no diagnóstico da TB ativa e latente ou como possíveis alvos terapêuticos.

2 REVISÃO DA LITERATURA

2.1 ASPECTOS GERAIS DA TUBERCULOSE PULMONAR

Tuberculose (TB) é uma das doenças infectocontagiosas crônicas mais antigas que acomete o ser humano. Apesar de dispor de esquemas eficazes de tratamento contra o *M. tuberculosis*, agente etiológico da TB. A TB ainda é considerada um grave problema de saúde pública. De acordo com a Organização Mundial de Saúde (OMS), estima-se que 1.7 bilhões de pessoas estejam infectados pelo bacilo da TB. No último boletim epidemiológico divulgado, a OMS listou 30 países responsáveis pelas maiores taxas de TB no mundo, em que o Brasil ocupa o 19º lugar com 91 mil novos casos em 2017 (OMS, 2018).

O Brasil tem o maior número de casos registrados de TB na América do Sul, em 2017 foram registrados 228.493 casos de TB nas américas, sendo 86.858 (32%) casos notificados no Brasil (OMS, 2018). A TB é uma das doenças infecciosas que mais acomete a população brasileira, constituindo uma das principais causas de morbimortalidade no país. De acordo com o último boletim epidemiológico do ministério da saúde, a Bahia apresentou em 2018, 27,5 de novos casos por 100.000 habitantes, colocando o Estado no sétimo lugar do Nordeste e o 19º lugar do país em número de casos novos de TB (BRASIL, 2019). Esses dados epidemiológicos refletem do grande desafio do controle da TB, que consiste principalmente na dificuldade em predizer quais daqueles indivíduos que possuem TBL irão progredir para a forma ativa da doença.

A infecção por *M. tuberculosis* acontece através das vias áreas, a partir da inalação de partículas viáveis contendo o bacilo que ficam suspensas no ar por períodos prolongados, provenientes da fala, do espirro e principalmente da tosse de pacientes bacilíferos. Uma vez inalado, a depender da capacidade imune e características do próprio hospedeiro, a resposta imune do indivíduo pode controlar a infecção pelo bacilo e estabelecer um estado de latência (TBL) ou pode eliminar completamente o bacilo. No entanto, uma vez que a resposta imune não consiga eliminar ou controlar a infecção pelo bacilo, o hospedeiro pode desenvolver a forma ativa da TB (COOPER, 2009; VOLPE et al., 2006). Cerca de 5 a 10% dos indivíduos infectados pelo bacilo

irão desenvolver a TB ativa e as chances de desenvolver são maiores nos dois primeiros anos pós infecção (PAI et al., 2016; VYNNYCKY; FINE, 1997).

Uma vez que o indivíduo consegue controlar a infecção pelo bacilo e estabelece a TBL, o indivíduo encontra-se infectado pelo bacilo, porém não apresenta os sinais e sintomas característicos da TB e não são capazes de transmitir a doença. Estes indivíduos apresentam resultados negativo para os testes diagnósticos da TB ativa, porém apresentam reação cutânea (≥ 5 mm) positiva para o teste tuberculínico (TST) e níveis de Interferon-γ (INF-γ) maiores que 0,35 UI/mL pelo ensaio de liberação de INF-y (IGRA). O indivíduo infectado pode permanecer na forma latente da TB durante toda a sua vida, no entanto em alguns casos o bacilo pode sofrer uma reativação ou o indivíduo pode sofrer uma nova infecção, podendo desenvolver a forma ativa da doença (ANDREWS et al., 2012; BARRY et al., 2009; BRASIL, 2018)

A TB ativa pode ocorrer logo após o contágio com a fonte infectante (TB primária) ou após alguns anos com a transição do estado de latência para a forma ativa (TB secundária). As chances de um indivíduo desenvolver a forma ativa da doença são maiores nos dois primeiros anos pós-infecção. Durante a forma ativa da TB, os indivíduos apresentam sintomas como febre, sudorese noturna, perda de peso e principalmente tosse prolongada por mais de duas semanas (PAI et al., 2016). Os fatores que levam ao desenvolvimento da TB ativa ou TBL ainda são desconhecidos, entretanto a depender da eficiência da resposta imune do hospedeiro contra o bacilo, o sistema imune consegue controlar a infecção e estabelecer a TBL.

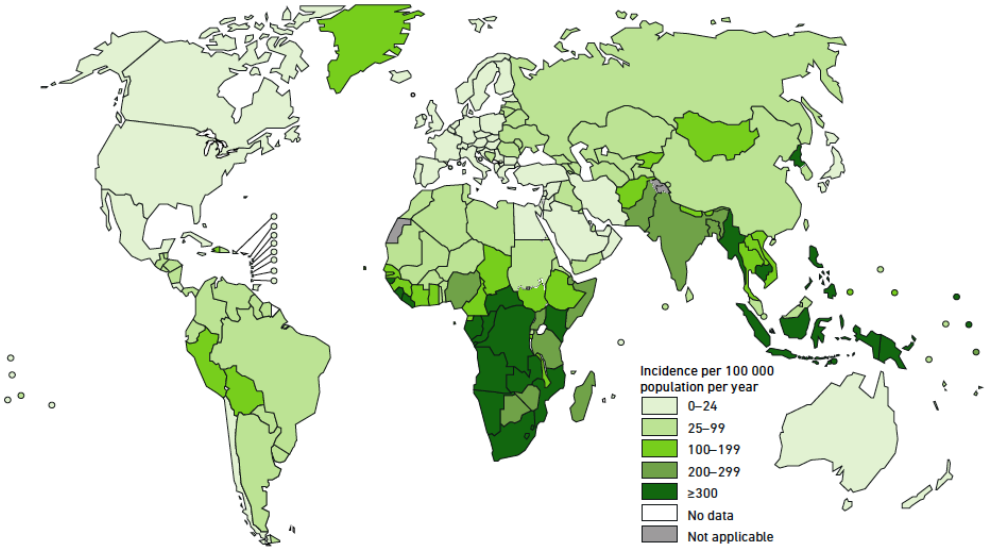

Figura 1. Número estimados de novos casos de TB no mundo durante o ano de 2017. WHO, 2018.

2.2 IMUNOPATOGÊNESE DA TUBERCULOSE PULMONAR

Após ser inalado, o bacilo atinge os alvéolos pulmonares e infectam as células residentes, principalmente os macrófagos alveolares. Através da sinalização celular, as células de defesa como monócitos e neutrófilos migram pela corrente sanguínea para o local de infecção no tecido pulmonar. Uma vez no tecido, os monócitos se diferenciam em macrófagos e auxiliam no processo de controle da infecção. Macrófagos alveolares reconhecem o *M. tuberculosis* através dos receptores do tipo *toll* (TLR), a exemplo dos TLR-2 e TLR-4 e assim, fagocitam o bacilo. Uma vez dentro do fagossoma, o bacilo é processado e antígenos bacterianos podem ser apresentados aos linfócitos T através do complexo principal de histocompatibilidade (MHC). A partir desta interação, inicia-se a resposta imune inata pró-inflamatória com produção de citocinas inflamatórias.

A apresentação antigênica ao receptor dos linfócitos T (TCR) ocorre através do MHC que irá necessitar de um segundo sinal através do complexo de receptores e co-receptores (CD4 e CD8) e da molécula co-estimulatória (CD28) expressos nas células T para ser efetiva (SCHWARTZ, 1990). O controle imunológico da TB é mediado principalmente por linfócitos T $CD4^+$ que se diferenciam em células T *helper* do tipo 1 (Th1) através da interleucina-12 (IL-12), e nesse caso a apresentação dos antígenos acontece através do MHC classe II (HENDERSON; WATKINS; FLYNN, 1997). Essa interação celular é crucial no controle inicial da doença, pois os linfócitos T $CD4^+$ através da liberação do IFN-γ promove a ativação dos macrófagos infectados induzindo-os a produzir reativos de nitrogênio e oxigênio capazes de inibir ou eliminar os bacilos (BRIGHENTI; ANDERSSON, 2012a; LERNER; BOREL; GUTIERREZ, 2015; SMITH, 2003).

A resposta imune mediada por células é o principal mecanismo de controle da TB em que participam células da resposta imune inata e adaptativa através da apresentação antigênica. O papel dos linfócitos T, principalmente das suas subpopulações, assim como da resposta imune adaptativa, ainda é pouco compreendida na TB. O papel destas células parece não estar apenas associados a ativação de macrófagos e formação do granuloma, mas também com a ação efetora da resposta através da produção de citocinas pró-inflamatórias. Os antígenos proteicos do *M. tuberculosis* serão apresentados via MHC de classe II aos linfócitos T $CD4^+$ e assim, inicia-se a resposta clássica conhecida na TB (BRIGHENTI; ANDERSSON, 2012b; LERNER; BOREL; GUTIERREZ, 2015;

SMITH, 2003). Já os antígenos não proteicos, como os lipídios, podem ser apresentados aos linfócitos T $CD4^{-}CD8^{-}$ (células T duplo negativas) através da molécula CD1. Os linfócitos duplo negativos desempenham um papel similar aos linfócitos T $CD8^{+}$ uma vez que liberam granzimas e perforinas destruindo o *M. tuberculosis* dentro dos macrófagos (KAUFMANN, 2002).

Os monócitos/macrófagos, são células importantes, pois regulam e influenciam a natureza da resposta imune adaptativa através da apresentação de antígeno e secreções de citocinas e quimiocinas, além de participarem da formação do granuloma, em que células fagocíticas penetram no parênquima pulmonar, iniciando um foco inflamatório onde monócitos e outras células são atraídas, acumulando-se ao redor dos bacilos e dando início a formação de um granuloma coordenada principalmente por linfócitos T (BRIGHENTI; ANDERSSON, 2012a). Apesar do granuloma ser estudado na TB a mais de 200 anos, os seus mecanismos de formação, assim como o que acontece no seu interior, continuam não totalmente esclarecidos. Muitos estudiosos entendem que o granuloma é uma resposta essencial para a contenção da infecção tuberculosa, já que forma uma barreira delimitando o sítio da infecção (TUFARIELLO; CHAN; FLYNN, 2003).

Granulomas são caracterizados por um acúmulo local de células que foram atraídos para o sítio da infecção, como macrófagos que se tornaram células gigantes multinucleadas, monócitos, macrófagos epitelióides, macrófagos espumosos e neutrófilos para formar um halo envolto nos macrófagos infectados pelo *M. tuberculosis* (LUGO-VILLARINO et al., 2012; ORME; BASARABA, 2014; PEYRON et al., 2008; RAMAKRISHNAN, 2012; RUSSELL, 2007). Todas essas células estão envoltas por um conjunto de linfócitos T $CD4^{+}$ e $TCD8^{+}$ e linfócitos B, sendo o fator de necrose tumoral (TNF) produzida pelos linfócitos T e macrófagos responsáveis por manter a formação e estrutura desse granuloma (LUGO-VILLARINO et al., 2012; VAN ALTENA et al., 2011). TNF é capaz de regular a resposta imune por ativação, migração, proliferação e indução da expressão de receptores em outras células do sistema imune. De uma forma ainda não compreendida, no centro do granuloma surge uma necrose caseosa, que é resultado da destruição tecidual irreversível.

Alguns autores sugerem que os lipídios da parede do *M. tuberculosis* possam estar envolvidos nessa destruição tecidual, uma vez que lipídios foram encontrados no sítio caseoso, assim como uma grande quantidade de proteínas envolvidas no metabolismo de lipídios dentro de diversas células envoltas deste sítio (KIM et al., 2010; RUSSELL et al., 2009). As necroses podem ser observadas através da radiografia do tórax na forma de cavernas pulmonares. A necrose

possibilita que os bacilos, antes contidos pelo granuloma, sejam liberados para os bronquíolos e brônquios, facilitando a contaminação aérea e infectando novos indivíduos sadios e, desta forma, perpetuando a doença (FLÓRIDO; COOPER; APPELBERG, 2002). Diante disso, a habilidade do bacilo em persistir no hospedeiro e levar a doença para a fase crônica, parece estar estreitamente relacionada com as características do patógeno.

2.3 AGENTE ETIOLÓGICO DA TUBERCULOSE PULMONAR

O *M. tuberculosis* é do gênero Mycobacterium que pertence à família Mycobacteriaceae. O *M. tuberculosis* é um bacilo delgado, aeróbico obrigatório, intracelular facultativo, sem flagelos e não formador de esporos, com dimensões de 0,2 a 0,7 x 1,0 a 4 μm, que parasita principalmente os macrófagos alveolares (SCHOREY; CARROLL; BROWN, 1997). Não são produtores de toxinas e possuem longos períodos de duplicação (16 a 20 horas) (CHRISTINE L. CASE; BERDELL R. FUNKE; GERARD J. TORTORA., 2000).

As micobactérias são relativamente resistentes aos procedimentos padrões de coloração, e quando coradas com carbol-fucsina não podem ser descoradas com ácido ou álcool, e assim, são classificadas como bacilos álcool-ácido resistentes (BAAR). Esta característica deve-se a composição peculiar da parede celular, que contém grandes quantidades de lipídios (ácidos micólicos, ceras e fosfolipídios) (LEE; BRENNAN; BESRA, 1996; SINGH et al., 2018). Cerca de 40% do peso seco da parede celular do *M. tuberculosis* é composta por lipídios (ANDERSON, 1943), grande parte da capacidade codificante do genoma do *M. tuberculosis* é dedicado a biossíntese e degradação de lipídios (COLE et al., 1998). Uma análise de lipidômica (MycoMap) revelou que o *M. tuberculosis* contém mais de 5000 tipos de lipídios (LAYRE et al., 2011) e o efeito bioativo dos produtos lipídicos do *M. tuberculosis* nos hospedeiros têm sido reconhecidos desde as décadas de 50 e 60 (KARAKOUSIS; BISHAI; DORMAN, 2004a).

2.4 LIPÍDIOS DA PAREDE CELULAR DO *M. TUBERCULOSIS*.

Devido a composição da parede celular, assim como suas localizações que favorecem a interação com células no sistema imune (Figura 2), os lipídios desempenham um importante papel na resposta imune do hospedeiro que tem sido descrito e estudado nos últimos anos (CONVERSE et al., 2003; COX et al., 1999; KARAKOUSIS; BISHAI; DORMAN, 2004; REED et al., 2004). Os principais lipídios bem descritos são dimicolato de trealose (TDM), também conhecida como fator corda, descrito por induzir a formação do granuloma, indutor da migração defeituosa de leucócitos e a perda de peso em camundongo (BEKIERKUNST et al., 1969; BLOCH, 1950), lipoarabinomanana (LAM), sulfolipídios (SL), glicolipídio fenólico (PGL), Dimicocerato de fitilcerol (PDIM), e a lipoproteína 19kDa que exercem uma variedade de efeitos nos sistema imunes inato e adaptativo, tais como migração de células imunes, fagocitose e maturação do fagossomo, função macrofágica, apresentação antigênica, produção de anticorpos e função das células T (CAMACHO et al., 2001; KARAKOUSIS; BISHAI; DORMAN, 2004a).

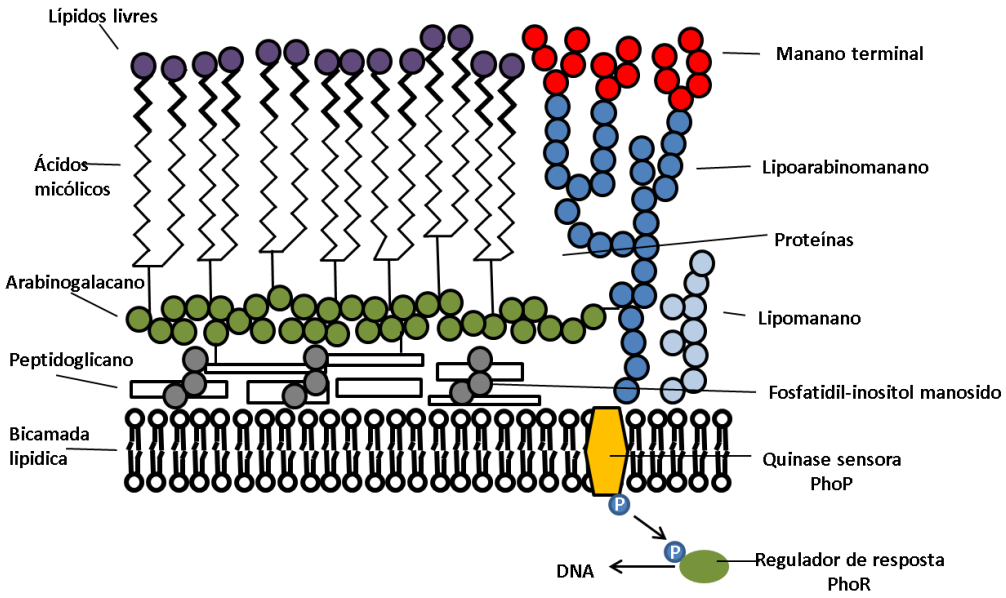

Figura 2. Representação esquemática do envelope celular do *M. tuberculosis*. Fonte: Adaptado de Park & Bendelac, 2000.

2.4.1 Lipídios envolvidos na resposta inicial do hospedeiro ao M. tuberculosis

Na resposta imune inata, a primeira linha de defesa contra o *M. tuberculosis*, está os macrófagos e células dendríticas, reconhecem os padrões moleculares associados a patógenos (PAMPs) através dos receptores de reconhecimento padrão (PRRs). Na TB, os macrófagos alveolares e células dendríticas reconhecem o bacilo principalmente pelos TLR2 e TLR4. Os glicolipídios, como dimicolato de trealose (TDM), e os lipoglicans, como o liboarabinomanana manosilado (ManLAM), são exemplos de PAMPs reconhecidos pela resposta imune inicial (CAMACHO et al., 2001). O TDM é um glicolipídio, que na cepa virulenta do *M. tuberculosis*, em meio de cultura com detergente, está associada com o crescimento da cepa em forma de corda de serpentina. O fato de a cepa virulenta crescer desta forma em cultura, associou-se este lipídio a um fator de virulência do bacilo. De fato, a inoculação desta cepa em camundongos demonstrou uma capacidade rápida e progressiva do bacilo em estabelecer infecção, multiplicando-se mais cedo e mais rapidamente nos pulmões murinos (MIDDLEBROOK; DUBOS; PIERCE, 1947). Além disso, o TDM demonstrou-se tóxico e inibiu a migração de células do sistema imune para o sítio de infecção, que na época que foi avaliado ajudou no esclarecimento da resposta granulomatosa em camundongos (BLOCH, 1950).

Macrófagos possuem um receptor para o TDM, a lectina tipo C (Mincle), o qual ativado induz a produção de óxido nítrico em macrófagos, TNF, CXCL2 e por consequência formação do granuloma (ISHIKAWA et al., 2009). Outra via de ativação dos macrófagos é da proteína MyD88. Macrófagos com interrupção no gene *myD88* não foram capazes de produzir citocinas pró-inflamatórias em resposta a microesfera revestidas com TDM. As metaloproteases (MMPs) 8 ,9, 12, 13, 14 e 19 são reguladas positivamente na forma dependente de MyD88 após estimulação *in vitro* com TDM (SAKAMOTO et al., 2013). Essas MMPs estão relacionadas com o processo de remodelação de tecido e inflamação crônica, assim como na formação do granuloma, cavitação e no rompimento do granuloma (QUIDING-JÄRBRINK; SMITH; BANCROFT, 2001). O monomicolato de trealose (TMM) é o precursor do TDM, que através da transferência da cadeia de ácido micólico produz micolato arabinogalactano e TDM. O TDM também serve como fonte

para síntese de outro lipídio importante do *M. tuberculosis*, os ácidos micólicos livres (AML), através da sua hidrólise pela proteína cut 3 (OJHA et al., 2010; YANG et al., 2014)

O fosfatidilinositol manosídeo (PIM) e os glicolipídios lipomanana (LM), Lipoarabinomanana (LAM) e Lipoarabinomanana manosilada (ManLAM) desempenham um importante papel na modulação da resposta imune do hospedeiro a partir da interação com diferentes receptores de macrófagos e células dendríticas (SCHLESINGER; HULL; KAUFMAN, 1994). A maioria dos relatos desses lipídios estão associados com a manutenção da integridade da parece celular (FUKUDA et al., 2013) e o crescimento intracelular do bacilo (HAITES et al., 2005). No entanto, essas moléculas também desempenhem um importante papel na resposta imune do hospedeiro. Enquanto os LM são indutores de IL-12 e apopstose (DAO et al., 2004), LAM pode inibir a produção de IL-12 através das DCs e modular a apoptose de macrófagos induzidos com *M. tuberculosis* (NIGOU et al., 2002). Já a ManLAM tem a capacidade de imitar compostos endógenos, como o receptor de manose de células de mamíferos, o que permite o *M. tuberculosis* em evadir os mecanismos de defesa do organismo, como por exemplo, a maturação do fagossoma pela supressão de IL-12 e TNF, além do aumento da produção de IL-10 por DCs (JÓZEFOWSKI; SOBOTA; KWIATKOWSKA, 2008).

A taxa de LM e LAM expressos na parede celular do bacilo pode influenciar a virulência, assim como o desfecho da infecção pelo *M. tuberculosis* (DAO et al., 2004). LM é reconhecido por ativar a resposta inflamatória do hospedeiro, sendo um potente indutor de citocinas inflamatórias como TNF e IL12p40 por macrófagos espumosos. LM, mas não LAM, ativa macrófagos através da expressão de moléculas co-estimulatórias como CD40 e CD86, dependente de TLR2 e MyD88 (QUESNIAUX et al., 2004). Estes dados sugerem que a adição de arabinoman no LAM mascara a atividade da manana em LM, prevenindo-a de induzir apoptose e expressão de IL-12. O que torna LAM e ManLAM menos inflamatórios.

O *M. tuberculosis* consegue muitas vezes sobreviver a resposta imune inicial do hospedeiro e então persistir no organismo, levando a doença para fase crônica. A transição para a fase crônica envolve outros grupos de lipídios (BARRAL; BRENNER, 2007). Nesta fase da infecção, o hospedeiro, através da resposta imune adaptativa, formará o granuloma na tentativa de controlar a replicação bacteriana e permitir a eliminação do bacilo. Os macrófagos apresentam antígenos proteicos às células T $CD4^+$ através do MHC classe II, entretanto, para estimulação das células T $CD8^+$, é necessário a apresentação dos antígenos citosólicos pelo MHC classe I dependente de

CD1(VAN DER WEL et al., 2007). Uma revisão feita por Barral e Brenner (2007) sobre o mecanismo de apresentação de antígeno via CD1 mostrou que muitas espécies de lipídios do *M. tuberculosis* podem ser apresentados via MHC-I dependente de CD1, incluindo AM, lipoglicans, PIM, lipopeptídios e sulfolipídios. Outros lipídios como dimicocerato de fitiocerol (PDIM), diaciltrealose (DAT) e poliaciltrealose (PAT) foram descritos por ativar e modular a resposta imune (KARAKOUSIS; BISHAI; DORMAN, 2004b).

2.4.2 Lipídios envolvidos na resposta tardia do hospedeiro ao M. tuberculosis.

A resposta imune ao extrato total de lipídios das seis maiores cepas do *M. tuberculosis*, realizada por Krishman e colaboradores (2012), sugerem que os diferentes perfis lipídicos influenciam a virulência dos bacilos e a resposta imune inata (KRISHNAN et al., 2011). Portanto, os lipídios da parede celular do *M. tuberculosis* são fatores determinantes no desfecho da fase inicial da infecção. A partir da perspectiva que para o *M. tuberculosis* se manter dentro do hospedeiro por um período prolongado de tempo, é necessário um fornecimento estável de energia, o que pode ser obtida a partir de carbonos derivados de lipídios. O *M. tuberculosis* parece utilizar a via da β-oxidação para sintetizar lipídios com papel putativo na proteção do bacilo contra as condições de estresse impostas pelo hospedeiro (MENDUM et al., 2015). Esta via metabólica tem como produtos o diaciltrealose (DAT) e o poliaciltrealose (PAT), assim como o sulfolipídio-I e o Dimicocerato de fitiocerol (PDIM).

DAT e o PAT são glicolipídios localizados na superfície externa da parede das espécies de micobactérias patogênicas e está associado com a entrada do bacilo na célula hospedeira (KARAKOUSIS, P.C; BISHAI, W.R; DORMAN, S.E, 2004). Além disso, o DAT está relacionado com a inibição da proliferação de células T de camundongos, o que sugere que este lipídio pode estar envolvido na resposta diminuída das células T e a imunossupressão associada a infecção pelo *M. tuberculosis* (SAAVEDRA et al., 2001). Embora esses lipídios estejam associados com a entrada do bacilo na célula, eles não parecem estar envolvidos com a sobrevivência do mesmo dentro dos macrófagos. Um estudo com a cepa com interrupção em um gene (*msl3)* associado a produção de DAT e PAT demonstrou que o crescimento intracelular macrofágico do *M.*

tuberculosis não apresenta diferença entre a cepa mutante para o *msl3* e a cepa selvagem H37Rv (ROUSSEAU et al., 2003a). Outro grupo importante associado com a virulência do bacilo é o grupo de sulfolipídios (SL).

O SL-1 é o lipídio mais abundante da superfície do *M. tuberculosis* estando presente apenas nas cepas patogênicas e, portanto, sendo alvo de estudos de biomarcadores. Experimentos com macrófagos peritoniais de camundongos demonstraram que o SL-1, extraído da cepa H37Rv do *M. tuberculosis*, é um potente inibidor da fusão fagolisossoma por causar disfunção na membrana do fagossomo e do lisossomo (GOREN, 1972). Além disso, o SL-1 inibe diretamente a ação macrófagica, reduzindo a fagocitose, secreção de IL-1 e superóxidos (PABST et al., 1988), assim como induz múltiplos efeitos na sinalização e ativação de neutrófilos (ZHANG; ENGLISH; ANDERSEN, 1991). Apesar dessas evidências, o papel dos SL-1 na infecção pelo *M. tuberculosis* é controverso (ROUSSEAU et al., 2003b).

O primeiro relato da possível relação do PDIM na imunopatogênese *do M. tuberculosis* foi observado quando a cepa H37Rv mutante, com deficiência na produção de PDIM, mostrou-se menos capaz de replicar e de formar granuloma do que a cepa selvagem (GOREN; BROKL; SCHAEFER, 1974). Da mesma forma, em estudos realizados em camundongos, demonstraram que a cepa com deficiência na produção de PDIM se mantem atenuada, e apresenta um crescimento bacteriano reduzido nos pulmões dos camundongos (COX et al., 1999). Outro estudo *in vivo* avaliou a resposta da cepa mutante, com deficiência na síntese de PDIM, e demonstrou que os pulmões e baços dos camundongos possuíam diminuição da carga bacteriana ao longo de um período de quatro meses. Estes resultados sugeriram que a diferença de carga bacteriana durante a fase aguda da doença é provavelmente a causa da formação tardia do granuloma observado em camundongos infectados pela cepa mutante. Apesar do PDIM não estar envolvido na inibição da fusão do fagolisossoma, o lipídio contribui com a proteção bactericida do hospedeiro, pois o PDIM não só interferiu na fagocitose como bloqueou a acidificação do fagolissoma (ASTARIE-DEQUEKER et al., 2009; ROUSSEAU et al., 2004).

O PDIM parece estar envolvido também na permeabilidade da parede celular (CAMACHO et al., 2001). Cepas mutantes, com inserções que impedem a síntese de PDIM, são mais permeáveis e têm maior sensibilidade a detergentes quando comparadas com a cepa selvagem. Além disso, essas cepas apresentaram maiores taxas de captação de sondas hidrofóbicas (CAMACHO et al., 2001), que são utilizadas para avaliar a fluidez da parece celular lipídica do *M. tuberculosis* (LIU

et al., 1996). De forma geral, o *M. tuberculosis* parece utilizar o PDIM a fim de reduzir a atividade bactericida dos macrófagos e na modulação da resposta imune inicial. Desta forma, o PDIM parece estar envolvido tanto no desfecho da infecção inicial, como no desenvolvimento da fase crônica.

Os ácidos micólicos (AM) são os lipídios mais abundantes da camada protetora de lipídios da parede celular do *M. tuberculosis* podem servir como esqueleto para antígenos lipídicos do *M. tuberculosis* na estimulação de células T restritas ao CD1 (LAYRE et al., 2009). Existem três classes de AM no *M. tuberculosis*: AM-alfa, keto e methoxy (GEORGE et al., 1995; YUAN et al., 1995). O AM-alfa tem dois anéis de ciclopropano, enquanto que o AM-keto e o AM-methoxy têm um anel cada (BARRY et al., 1998). Os AM são, normalmente, ligados covalentemente ao arabinogalactano e ao TDM ou TMM (BRENNAN; NIKAIDO, 1995). A diferença relativa nas quantidades de AM-keto e methoxy influencia a taxa de crescimento intra-macrófago do *M. tuberculosis* (YUAN et al., 1998). A ausência de AM-keto e methoxy está associada com a atenuação bacteriana em camundongos (DUBNAU et al., 2000) e, por consequência, está relacionada com a taxa de crescimento e atenuação bacteriana intracelular. Além disso, os AM estão associados com a hiperinflamação e morte mais rápida dos camundongos infectados pelo *M. tuberculosis*. A forma de AM não aderido ao substrato, AML, tem sido reconhecido por demonstrar um papel importante na regulação da persistência do patógeno no hospedeiro. Não está descrito que o AM possui um papel na fase inicial da resposta imune, entretanto uma vez que os AML acumulados na parede celular do bacilo são derivados da hidrólise de TDM, estes lipídios podem interagir com células do hospedeiro e induzir alguma resposta imune inata.

Evidências demonstram que a via da β-oxidação tem como produtos lipídios que estão relacionados com a persistência do bacilo na fase crônica (MENDUM et al., 2015). De forma geral, os lipídios DAT/PAT, SL-1 e PDIM, que são gerados pela via da β-oxidação, contribuem para uma diminuição da resposta imune induzida pelo *M. tuberculosis.* A produção de PDIM e SL-1 acontece através de um mesmo fluxo metabólico e possuem um precursor comum, o metilmalonil- coenzima A (CoA). A expressão aumentada da enzima metilmalonil-CoA-mutase, que catalisa metilmalonil-CoA em succinil-CoA, através do ciclo dos ácidos tricarboxílicos, leva a diminuição de PDIM e SL-1 *in vivo*, o que sugere que durante a fase crônica da infecção, o *M. tuberculosis* usa preferencialmente a via da β-oxidação ao invés da via dos ácidos tricarboxílicos como fonte de metilmalonil-CoA (JAIN et al., 2007).

Muñoz-Elias e colaboradores (2006) demonstraram que a via do ácido tricarboxílico, que tem como produto a propionil-CoA, não é importante para a virulência do bacilo em camundongos. O propionil-CoA e o acetil-CoA são produtos da β-oxidação de ácidos graxos de cadeia ímpar (MUÑOZ-ELÍAS et al., 2006). Assim, na fase crônica da doença e após a ativação da resposta imune, o bacilo utiliza a via da β-oxidação para metabolizar os lipídios acumulados intracelularmente como substrato para produção de DAT/PAT, SL-1 e PDIM. Estes lipídios induzem uma imunossupressão local que pode contribuir para o estabelecimento da infecção pelo *M. tuberculosis* a longo prazo (MENDUM et al., 2015).

A maneira que *M. tuberculosis* regula a composição lipídica da sua parede celular durante a infecção e como este perfil lipídico determina o desfecho da infecção pelo bacilo ainda não está bem esclarecido. Entretanto, a proteína transcripcional PhoP, que forma um sistema regulatório de dois componentes denominado PhoP/PhoR, é responsável pela resposta imune adaptativa a diversos estímulos e pode estar relacionado com a regulação da síntese dos lipídios da parede celular do *M. tuberculosis*. Isso foi evidenciado através de ensaios *in vitro* e *in vivo* com a cepa com interrupção no *PhoP* e *PhoR* que apresentou habilidade reduzida de se replicar em modelos animais e celulares (PÉREZ et al., 2001). Um outro estudo também observou que o *PhoP* regula de forma positiva a síntese de SL, DAT e PAT (GONZALO ASENSIO et al., 2006). Um modelo recente de rede de regulação, desenvolvido em condição de hipóxia por Galan e colaboradors (2013), propôs que o *PhoP* regula o *whiB3* e que tanto o PhoP quanto o *whiB3* regulam a síntese de PAT/DAT e SL (GALAGAN et al., 2013). O gene *whiB3* está envolvido na manutenção da homeostasia *redox* através da regulação do metabolismo dos ácidos graxos e modulação da biossíntese de PAT, DAT, SL-1, PDIM e triacilglicerol (TAT) (SINGH et al., 2009). Outra sugestão para regulação homeostática da parede celular do *M. tuberculosis* é o *operon mce1*, relatados em estudos como um transportador putativo de lipídios.

2.5 OPERON MCE1

O gene promotor da Mce1A (*mce1*), constituinte do *operon mce1*, assim como seu produto a proteína Mce1A, foram descritos pela primeira vez em 1993 por Arruda e colaboradores

(ARRUDA et al, 1993). A proteína é expressa na superfície celular do complexo *M. tuberculosis* e está associada a entrada e a sobrevivência do bacilo dentro da célula hospedeira, o que evidencia um papel importante do gene *mce1* na ação virulenta do bacilo (CASALI et al., 2002; CHITALE et al., 2001; EL-SHAZLY et al., 2007; LU et al., 2006).

Estudos em modelo animal demonstraram que a interrupção do *operon mce1* (Δ1), tornou o bacilo mais virulento. Camundongos infectados pela cepa Δ1 da estirpe H37Rv foram a óbito de forma precoce quando comparado com a cepa selvagem (WT), que expressa o *operon mce1* (SHIMONO et al., 2003). Além de uma maior capacidade virulenta, a repressão do *operon mce1* por uma proteína reguladora (Mce1R), em condição de hipóxia (como no granuloma, por exemplo) promove a manutenção do estágio de latência do bacilo (HAILE; BJUNE; WIKER, 2002). O *operon mce1* na cepa WT do *M. tuberculosis* não é expresso durante as quatro primeiras semanas de infecção em camundongo, o qual o torna funcionalmente equivalente à cepa mutante no *operon mce1* (UCHIDA et al., 2007). Diante disso, o *M. tuberculosis* deve usar a expressão do *operon mce1* como um dos mecanismos para modificar o conteúdo lipídico da parede celular e, com isso, controlar a resposta imune do hospedeiro. Esse fenômeno permite ao bacilo estabelecer uma infecção persistente e o aumento de ácidos micólicos da sua parede parece estar associado a esse fenômeno.

Estudos em modelo murino demonstraram que a cepa com interrupção no *operon mce1*(Δ1) do *M. tuberculosis* produz 10 vezes mais AML e maior ação virulenta comparados com a cepa selvagem (WT) do *M. tuberculosis* (SHIMONO et al., 2003). Uma vez que o *operon mce1* na cepa WT do *M. tuberculosis* não é expresso durante as quatro primeiras semanas de infecção, a cepa WT deve expressar AML na sua superfície, *in vivo*, durante o período inicial da infecção e após quatro semanas, o operon volta a ser expresso e o AML na superfície é reciclado pelo *M. tuberculosis* como fonte de energia (DUNPHY et al., 2010; UCHIDA et al., 2007).

Um estudo de metaboloma com as duas cepas do *M. tuberculosis* (WT e Δ1) mostrou que não só os ácidos micólicos livres (AML) são alterados na parede celular do bacilo. Cerca de três mil espécies de lipídios apresentaram alterações comparando as duas cepas. O trabalho de Queiroz e colaboradores demonstrou que a cepa Δ1 causou supressão nos níveis de diaciltrealose (DAT), dimicocerato de fitiocerol (PDIM) e do sulfoglicolipídio diacilado (Ac_2SGL), lipídios estes que estão relacionados com a imunogenicidade do bacilo (QUEIROZ et al., 2015). Desde que DAT, PDIM, Ac_2SGL, e AM induzem respostas imunes distintas (CAMBIER et al., 2014; SEQUEIRA;

SENARATNE; RILEY, 2014), os dados sugerem que o *M. tuberculosis* controla o conteúdo lipídico de sua parede celular a partir da expressão/repressão do *operon mce1*. Os fatores e a razão pelo qual o indivíduo com TBL desenvolve a forma ativa ainda não estão bem elucidados. Entretanto, o *operon mce1* pode ter um importante papel na transição da doença, sendo alvo para estudos nessa área.

Outro aspecto característico na cepa Δ1 é sua habilidade de induzir um maior acúmulo de macrófagos espumosos, que contém gotículas de lipídios constituídos de colesterol, triacilglicerol, e fosfolipídios, se comparado com a cepa WT (PEYRON et al., 2008). A formação de corpos lipídicos está associada ao aumento da expressão do *peroxisome proliferator-activated receptor gamma* (PPARγ) e do *testicular receptor 4* (TR4), os quais são membros dos receptores nucleares ativados por lipídios (RNAL).

2.6 RECEPTORES NUCLEARES ATIVADOS POR LIPÍDIOS

Receptores nucleares (RN) pertencem a uma superfamília de proteínas intracelulares responsáveis por regular a transcrição gênica, sendo os mais abundantes fatores de transcrição. Os RNs têm como ligantes hormônios esteroides, ácidos retinóicos, hormônios tireoides, leucotrienos, prostaglandinas, ácidos graxos, vitamina D, entre outros (ROBINSON-RECHAVI; ESCRIVA GARCIA; LAUDET, 2003). São responsáveis pela reprodução, homeostasia, metabolismo e desenvolvimento do organismo. E o que diferencia os RN dos demais receptores celulares é a capacidade de se ligar ao DNA e regular diretamente os processos biológicos celulares (ROBINSON-RECHAVI; ESCRIVA GARCIA; LAUDET, 2003; VACCA et al., 2011). Diante dessa importância, o papel desses receptores vendo sendo estudado a fim de compreender a patogênese de diversas doenças, como câncer e diabetes, por exemplo (CONZEN, 2008; LONG; J. CAMPBELL, 2015; MEINKE; WOOD; SZEWCZYK, 2006; VACCA et al., 2011). No caso da TB, o estudo desses receptores é importante uma vez que existe um grupo de RN que são ativados por lipídios, um dos componentes mais abundantes da parede celular do *M. tuberculosis* (BRENNAN; NIKAIDO, 1995; KARAKOUSIS, P.C; BISHAI, W.R; DORMAN, S.E, 2004).

A infecção pelo *M. tuberculosis* está associada ao aumento da expressão do *peroxisome* PPARγ e do TR4, os quais são membros dos receptores nucleares ativados por lipídeos (RNAL) (ALMEIDA et al., 2012b; MAHAJAN et al., 2012). O *M. tuberculosis* interage com estes receptores, os quais induzem a produção de IL-10 e o bloqueio da maturação do fagolisossomo. Esta interação também aumenta a expressão do CD36, um receptor envolvido na captura do colesterol LDL oxidado. Este processo contribui para a formação de macrófagos espumosos e aumenta a sobrevivência bacteriana (MAHAJAN et al., 2012). Existem ainda outros RNALs descritos, como o *retinoic acid receptor* (RAR) que se liga ao colesterol; *hepatocyte nuclear factor* (LXRα,β) que reconhece ácidos graxos; e o *liver receptor homlogue1* (LRH1) que reconhece fosfatidilinositols (OLEFSKY, 2001; RANHOTRA, 2013; ROBINSON-RECHAVI; ESCRIVA GARCIA; LAUDET, 2003; VACCA et al., 2011). Esses ligantes lipídicos são todos componentes da parede celular do *M. tuberculosis*, uma estrutura dinâmica que constantemente se remodela e que pode induzir diversas respostas no hospedeiro.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar a resposta induzida *in vitro* pelo *Mycobacterium tuberculosis* (*M. tuberculosis*) com interrupção no *operon mce1* e pelo extrato apolar de lipídios da sua parede celular.

3.2 OBJETIVOS ESPECÍFICOS

Avaliar a resposta induzida pela cepa do *M. tuberculosis* selvagem e com interrupção no *operon mce1* em um modelo de tecido humano *in vitro*.

Avaliar a expressão gênica de macrófagos estimulados *in vitro* com o extrato apolar de lipídios da cepa selvagem e com interrupção no *operon mce1* do *M. tuberculosis.*

Avaliar a expressão gênica e a produção de citocinas das células mononucleares do sangue periférico estimuladas *in vitro* com o extrato apolar de lipídios com o extrato apolar de lipídios da cepa selvagem e com interrupção no *operon mce1* do *M. tuberculosis.*

Avaliar os níveis de expressão gênica dos receptores nucleares ativados por lipídios, em sangue total, como possíveis biomarcadores para tuberculose pulmonar.

4 RESULTADOS

Os resultados obtidos são apresentados em quatro capítulos que contemplam os objetivos da tese, sendo os manuscritos submetidos à publicação por revistas indexadas. O primeiro capítulo apresenta o manuscrito referente ao estudo realizado durante o doutorado sanduíche realizado na Universidade da California, Berkeley, USA, em que aborda os resultados obtidos sobre a resposta induzida por *M. tuberculosis* selvagem e com interrupção no *operon mce1* em um modelo humano de granuloma *in vitro,* intitulado " *In vitro tissue model of human granuloma formation after mutant mce1 operon of Mycobacterium tuberculosis infection"*, que contempla o primeiro objetivo específico da tese. O segundo capítulo, apresenta os dados obtidos a partir do trabalho experimental com células murinas e humanas, intitulado *"Mce1-dependent cell wall lipid reorganization in Mycobacterium tuberculosis dampen the host cell inflammatory responses",* atendendo aos objetivos específicos 2 e 3. O capítulo 3 e 4, contempla o objetivo específico 4 em que avalia a expressão gênica, em sangue total, de potenciais biomarcadores para a TB pulmonar.

4.1 CAPÍTULO 1: IN VITRO TISSUE MODEL OF HUMAN GRANULOMA FORMATION AFTER MUTANT MCE1 OPERON OF MYCOBACTERIUM TUBERCULOSIS INFECTION.

Manuscrito em processo de revisão para submissão: JÉSSICA PETRILLI, MELAINE DELCROIX , SERGIO ARRUDA AND LEE W. RILEY. In vitro tissue model of human granuloma formation after mutant *mce1 operon* of *mycobacterium tuberculosis* infection.

In vitro tissue model of human granuloma formation after _Mycobacterium tuberculosis_-interrupted _mce1 operon_ infection

Jéssica Petrilli 1*, Melaine Delcroix 2, Sergio Arruda 1 and Lee W. Riley 2.

1 Laboratório Avançado de Saúde Pública, Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Bahia, Salvador, Brasil.

2 Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, CA 94720, USA

* Corresponding author: E-mail: jessicadpetrilli@gmail.com

ABSTRACT

The development of granuloma is the hallmark of *Mycobacterium tuberculosis (M. tuberculosis)* infection. The host builds the granuloma to contain the spread of the bacillus and the success of this operation will characterize the outcome of the infection. The failure to control the dissemination of the bacillus is still poorly understood. *M. tuberculosis mce1 operon* has been associated with granuloma formation. However, there is no study demonstrating how *operon mce1* is associated with granuloma formation. Based on this, a human lung tissue model was developed to evaluate granuloma formation after infection with *M. tuberculosis* with interruption in *operon mce1* (*Δmce1*). Data showed that *Δmce1* strain can induce granuloma formation but cannot maintain structure after 11 days of infection as wild strain can do. Granuloma with scattered cells and necrotic center was observed by confocal microscopy in cultures with *Δmce1* strain. Further studies are needed to understand how the Δ*mce1* strain interferes with granuloma formation. However, this model provides a new perspective for further pathogenesis studies with *M. tuberculosis* and others microorganisms that can induce granuloma formation.

Keywords: Mycobacterium tuberculosis, operon *mce1*, granuloma, tissue model

1. **INTRODUCTION**

After inhalation of *Mycobacterium tuberculosis (M. tuberculosis)*, ethological agent of tuberculosis (TB), alveolar macrophage and alveolar epithelial cells are infected and leading an intensity proinflammatory response started. Immune cells are recruited to infection site by cytokines and a structure called granuloma is formed by the cells in attempt to control *M. tuberculosis* dissemination (1). TB granuloma is characterized by a local accumulation of cells such as multinucleated giant cells, monocytes, epithelioid macrophages, foamy macrophages and neutrophils forming a halo that involves *M. tuberculosis*-infected macrophages. All these cells are surrounded by a set of $CD4^+$ and $TCD8^+$ T lymphocytes and B lymphocytes, being the tumor necrosis factor (TNF) produced by T lymphocytes and macrophages responsible for maintaining the formation and structure of the granuloma (2,3).

The host built the granuloma to control bacillus dissemination. However, the granuloma also becomes important for the bacillus persistence. Within the granuloma, bacteria that have not been destroyed enter a dormancy stage and thus persist within their host. One of the factors that lead to persistence of *M. tuberculosis* is the operon called *mce1* that are a putative lipid transporter (4). Disruption of *mce1 operon* (*Δmce1*) undergoes remodeling of *M. tuberculosis* cell wall that induce immunomodulation of host response and resulting a granuloma formation with aberrant inflammatory cell migration (5–7). The formation of a disorganized granuloma may happen because *Δmce1* strain accumulates on its cell wall mycolic acids and decreases the expression of dimycolate (TDM), an important lipid to induce granuloma formation (8,9). Repression of *M. tuberculosis* operon *mce1* was observe in mice's macrophages by *Mce1*R regulatory protein in an environment granuloma-like, when the bacillus is in a hypoxic environment with low nutrient availability (10). Many studies have been performed to understand the role of *mce1 operon* in TB pathogenesis and the mechanisms of granuloma formation. However, what is happens inside TB granuloma remain not fully understood.

Several animal model studies have been performed to study the response induced by *M. tuberculosis* with interruption in the *mce1* operon. There are no studies demonstrating how operon *mce1*disruption influences granuloma formation. Delcroix et al (2018) established an *in vitro* model of peripheral blood mononuclear cells (PBMCs) leading to granuloma formation by

Mycobacterium bovis BCG infection. They built a granuloma onto a collagen matrix to analyze the monocytes infectivity in the granuloma formation context by confocal microscopy (11).

Therefore, in this study an established model of human lung tissue has been adapted to construct granulomas in environments that recreate complex cell-cell interactions and thus assess the response induced by *M. tuberculosis-mce1* disrupted strain in a granulomatous environment.

2. MATERIAL AND METHODS

2.1. Ethics

Human peripheral blood was obtained from healthy donors at the University of California, Berkeley. The study was approved by the Institutional Review Board of the University of California, Berkeley. All blood donors provided written informed consent.

2.2. Bacterial strain and growth conditions

The following *M. tuberculosis (Erdmann)* strains were wild-type and *mce1* operon mutant. *Mce1 operon* mutant construction was previously described (7). The strains were grown at 37°C in Middlebrook 7H9 medium (Difco) supplemented with 10% ADC (Beckton-Dickinson) and 0.2% glycerol (Fisher Scientific, NJ).

2.3. Maintenance and infection of alveolar epithelial cells

The human alveolar epithelial cell line A549 (ATCC CCL-185) was cultured and maintained in RPMI 1640 medium with 2mM L-glutamine, supplemented with 10% heat-inactivated FBS, in a 5% CO_2 humidified incubator at 37°C. Cell concentration was determined with a hemocytometer. Cells were seeded in tissue culture flask and incubated overnight until the cells were approximately 80% confluent. Immediately before infection, the medium was replaced with fresh and pre-warmed RPMI 1640 medium with 10% FBS. In a Biosafety level three laboratory (BSL3) bacterial cultures with an OD600 of 0.5-0.6 were spun down at 50 x g for five minutes to remove clumps. Supernatant was centrifuged at 1,200 x g for five minutes. Bacterial pellets were washed once and resuspended in PBS. Required strain of *M. tuberculosis* was added to A549 cells at a multiplicity of infection (MOI) of 0.1 in cRPMI. Infected cells and uninfected cells were kept at 37°C in 5% CO^2 incubator. Following four hours of infection, extracellular bacteria were removed by washing cells 2 times with warm PBS. A549 cells were detached with 0.5% trypsin and then seeded onto an extracellular matrix (ECM).

2.4. **Isolation and staining of PBMCs.**

Whole blood of donors was collected in Vacutainer tubes with sodium heparin anticoagulant reagent (BD Biosciences) and processed immediately for PBMC isolation. Blood was diluted 1:1 with phosphate-buffered saline (PBS), layered onto Ficoll-Paque PREMIUM (GE Healthcare) and centrifuged at 1,200 rpm for 30 min at room temperature (RT). Isolated PBMCs were frozen in fetal bovine serum (FBS, Mediatech, VA) with 10% dimethyl sulfoxide. For assays, PBMCs were thawed, washed and resuspended in complete RPMI (cRPMI) containing 10% FBS and 2 mM L-glutamine. Cells were rested for one hour at 37°C with 5% CO2 before cell count (trypan blue dye exclusion method). PBMC were stained with PKH26 dye stock (Sigma) according to manufacturer's instructions.

2.5. **Formation of *in vitro* granuloma**

Following infection with *M. tuberculosis*, A549 cells were washed with PBS and seeded onto an ECM, pH 7.0, containing 0.75 mg/mL type I bovine collagen (Purecol, Advanced Biomatrix) and 1.25μg/mL bovine fibronectin (Sigma) in cRPMI. Before adding cells, 250 μL ECM were pipetted onto each 12-well Costar Transwell insert (Corning). Plates were incubated for two hours at 37°C to solidify ECM and $1x10^5$ A549 cells were seeded per insert. cRPMI was added in the outer well and seeded A549 cells were incubated at 37°C in 5% CO^2 incubator. After 24 hours, medium in lower chamber was changed and 300 μL with $6x10^5$ PBMC were seeded per insert.

2.6. **Granuloma staining for confocal imaging**

After 4, 7 or 11 days of infection, PBMCs in Transwells were fixed overnight at 4°C in 4% formaldehyde. Following two PBS washes, membranes of inserts were cut out. Cells were then stained for lipid bodies for one hour in 1X Bodipy 493/503 (Invitrogen); or incubated with α-CD3 (T-cell marker) polyclonal rabbit and α-CD68 (KP1; macrophage marker) mouse monoclonal anti-human antibodies (Fisher) overnight followed by one-hour incubation with secondary antibodies goat anti-rabbit IgG, Alexa Fluor 488 and goat anti-mouse IgG1, Alexa Fluor 633, respectively. Secondary-antibody-only controls were run in parallel. After two PBS washes, membranes were mounted onto microscope slides in Prolong Gold antifade reagent with DAPI (Molecular Probes).

2.7. **Granuloma counting and measurement**

Following staining PKH26 dye stock, each membrane was scanned on the widefield DeltaVision Elite microscope (GE Healthcare) under TRITC filter. Images were then analyzed on

Bitplane Imaris (Oxford Instruments) with the Surfaces function. To count and measure aggregates with an area of at least 6,000 μm2, the following parameters were used: source channel at 523 nm; Smooth option selected with Surface Detail at 10 μm; Background Subtraction with Sphere Diameter at 100 μm; threshold was adjusted manually to cover individual granulomas; surfaces were filtered to include areas larger than 1200 voxels. The Surfaces function rendered 3D objects with two sides and thickness of 1 voxel, so the 2D cross-sectional area is the total surface area divided by two.

2.8. **Confocal imaging**

Cells were imaged on a Zeiss LSM710 confocal microscope. DAPI fluorescence was excited with a 405-430 nm diode laser line and collected with a 410-490 nm emission filter. For *M. tuberculosis* staining, lipids droplets with 1X Bodipy 493/503 (Invitrogen) and for Alexa Fluor 488, we used the 488 nm line of an Argon laser and fluorescence emission was collected between 500 and 550 nm. For Alexa Fluor 633, we used the 633 nm line of a Helium-Neon laser and emission above 650 nm was collected. Images were acquired on a Zeiss LSM710 confocal microscope and processed on ImageJ.

2.9. **Statistical analysis**

Scatter plots were generated with using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA). We performed One-way ANOVA followed turkey’s multiple comparison test to compare the numbers and the areas of aggregates in different conditions of infection.

3. RESULTS

3.1. In vitro granuloma formation after WT and *Δmce1* infection

First, to stablish the best conditions for granuloma formation, we infected the A549 cells with WT and Δ*mce1* strains at different MOI (0.1, 1, 1:10). Mtb was able to induce greater numbers of granulomas and most stable granuloma formation with at a 0.1 MOI (data not show), based on that we performed the assays on this condition. From two-independent experiments the mean number of aggregates were 9, 21 and 23 in non-infected, WT and Δ*mce1* mutant strain culture, respectively. At day 7 of infection we found a solid aggregate for all donors with mean area between 18,000 and 21,000 μm^2 in WT cultures and 16,000 and 21,000 μm^2 in *Δmce1* culture. When we observed the granuloma formation in different infection conditions (Figure 1), the aggregates of non-infected had an mean area of 20,195 μm^2 and WT and *Δmce1* showed aggregates with 19,950 μm^2 and 18,739 μm^2 respectively.

3.2. Cultures with A549-*Δmce1* infection have the number of aggregates decreased with 11 days post-infection

Once we stablished and observed the aggregates formation after seven days of after A549-WT and *Δmce1* infection, when the aggregate were supposed to be mature, we followed four days more (11 days) to assess the granuloma-like structure. Figure 2A shows the number of aggregates counted on day four, day seven and day 11 after A549-WT or *Δmce1* infection. WT (9.9 ± 2.4) and *Δmce1* (8.7 ± 0.4) strains produced similar number of aggregates, but they greater as compared with non-infection culture (1.4 ± 0.6; $p<0.01$). At day 7, a similar figure was observed. A counted of 7.2 ± 1.3 aggregates in WT culture and 9.8 ± 1.3 aggregates in *Δmce1* culture were performed ($p>0.05$). Followed up to 11 days post-infection the aggregates started breaking down in all infection's conditions, but *Δmce1 seemed to disrupt more*. WT and *Δmce1* culture had mean number of 6.7 ± 4.6 and 4 ± 3.3 aggregates, respectively. In cultures with the A549-*Δmce1*

infection, the number of aggregates decreased between days four and day 11 (8.7 ± 0.4 and 6.7 ± 4.6, respectively; p=0.01) and day seven and day 11 (9.8 ± 1.3 and 6.7 ± 4.6, respectively; p= 0.001).

3.3. *Δmce1* strain not maintain aggregate formation seven days after postinfection.

As we observe the structure of the granuloma-induced by WT and *Δmce1*strains we not only evaluated the number but the area of granulomas formation over 11 days (Figure 2B). The mean area of aggregates at day 4 were similar among all the culture conditions (p> 0.05). WT strain induced aggregate with a mean area of 20,083 μm^2 and *Δmce1* strains an mean area of 21,140 μm^2. Seven days after the aggregates of all culture's conditions maintained their area with size of 20,618, 19,231 and 18,851μm^2 in non-infected, WT and *Δmce1* infection, respectively. With 11 days post-infection, aggregates induced by *Δmce1* strain decreased theirs mean area (4,315μm^2) comparing with day seven (18,851μm^2; p= 0.02) and day 4 (21,140 μm^2; p= 0.004). Different from *Δmce1* strain, aggregates induced by WT infection maintain the size with 11 days post-infection (18,242 μm^2) comparing with day four (20.083 μm^2) and day seven (19.231 μm^2).

3.4. **Aggregate morphology of WT and *Δmce1* infection**

In addition to evaluating the number and size of aggregates formed after WT and *Δmce1* infection, we evaluated by microscopic the structure of aggregates. Figure 3A illustrate the granulomas structure at day four, seven and 11 after A549-WT and *Δmce1* infection at an MOI of 0.1. Granuloma Imaging at day four showed an early granuloma with similar size between both strains. Tight aggregate was observed at day seven and *Δmce1*-induced granuloma reveled be slightly bigger with a several PBMCs around the aggregate. On day 11, showed loose aggregates with scattered cells and even more scattered cells in *Δmce1*-induced granuloma. Figure 3B showed the hallmark of granulomas. In a 20x lens we can observe *M. tuberculosis* intracellular (figure

3B.1), recruitment of $CD68^+$ macrophage and $CD3^+$ lymphocytes, and multinucleated giant cells formation (figure 3B.2).

4. **DISCUSSION**

Pulmonary alveolar epithelium cells can play an important role after *M. tuberculosis* enter in alveolar space of the lungs, since the *M. tuberculosis* infected these cells (12,13). Once the bacillus enters the pulmonary cavity, *M. tuberculosis* infects the epithelial cells and pulmonary macrophages phagocytose the bacteria. At the same time, cells are recruited to site of infection and leukocytes surround the infected cells in an attempt to contain the infection. As a result, an organized cellular structure is formed to help to control de infection (14,15). According to this, we developed a tissue model to provide the granuloma formation using *M. tuberculosis*-A549 infected cells and PBMC from health individuals. Here we described a confocal microscopy assay to characterize the *M. tuberculosis*-induced granuloma with two strains of *M. tuberculosis* that have differences mainly on their cell wall envelope.

For our knowledge is the first study that describe granuloma formation by *mce1* mutant strain of *M. tuberculosis* in n in vitro tissue model. Many studies *in vitro* and *in vivo* showed that Δ*mce1* strain can induce granuloma formation but is the first time that we can describe the granuloma an *in vitro* tissue model that mimics the pulmonary environment (7,16–18). First, we defined the conditions of A549 cells infection to stablish a stable granuloma where we can see the hallmarks of granuloma and no presence of extracellular bacteria in the first days resulted of cell death (data not shown). Our results showed a stable granuloma at day seven post-infection in all culture conditions revealing that even without stimulus the cells can aggregate spontaneously. Although aggregates in non-infected culture have same mean size, the number counted is lower than the aggregates counted in *M. tuberculosis* infection's cultures which demonstrated that the bacillus is the great inducer aggregate formation. There are not many studies that show sizes characteristics of *granuloma* formation during *M. tuberculosis* infection. However, Delcroix et al characterized BCG-induced granulomas in a similar model and *M. tuberculosis* induced larger

aggregates than BCG (11). We expected found less and smaller aggregates formation in cultures with *Δmce1* strain infection, thus we evaluated the aggregates development for 11 days.

We followed the aggregate formation over 11 days to characterize the structure in different stage. A previously study with BCG showed that the granuloma starts to breaking down from day 15 (11) and we wanted to observe how the granuloma behaves in different times following *M. tuberculosis* infection. At the first time point we can observe similar mean number and size of granulomas induced by WT and *Δmce1* strain showing that the both strains are able to recruit cells to the site of infection an set up an organized structure trying to control *M. tuberculosis* dissemination. However, over the infection course of the days, changes can be quantified. Both strains have similar number and size aggregates. However, we can observe a slightly increase of aggregates number induced by *Δmce1* strain while WT strain have a slight decrease. The mean area of the aggregates induced by WT and Δ*mce1* strain were similar. Although WT strain have a slight decrease in numbers, the granulomas keeping their size between seven and 11 days, maintaining control of the infection. In contrast, in this model with 11 days post-infection we started to see the granuloma breaking down mainly followed *Δmce1* strain infection. Not only the number as well as the size of aggregates decrease dramatically, thus these data reveled that *Δmce1* strain cannot maintain the structure of granuloma after 11 days postinfection. These results corroborate with studies in vivo which demonstrated that *Δmce1* strain is not able to control the infection for a long time (7,17,18). The morphology and characteristic of the aggregates that confirm these data are illustrated in figure 3. Shimono et al showed that mice infected with *Δmce1* strain died faster than mice infected with WT and mice infected by *Δmce1* strain presented lungs with looser aggregates as the same we can observe in figure 3A at day 11 (7). During all time points, WT strain was able to maintain the granuloma structure according to the size of aggregates.

The hallmark of granuloma is the presence of multinucleated cell, intracellular *M. tuberculosis* and foam macrophages (19–21). We were able to observe multinucleated giant cells in granulomas-induced for both strains with presence of macrophages and lymphocytes compound and surround the cells infected. We did not do a robust analyses of foam macrophage but we believe that *Δmce1* strain induced granuloma formation with accumulation of lipids droplets inside the macrophage since foam macrophage have been associated with persistence of intracellular bacteria (20,22). These approaches are important since the mainly difference between the strains are the cell wall lipids composition. Understand the role of these lipids can be the key for realize the behave

of the bacillus once they are inside de cells. More studies are necessary to elucidate why *Δmce1* strain cannot maintain stable granuloma structure and how the lipids can be participating in this phenomenon. This model of induced granuloma formation brings a new perspective for *M. tuberculosis*'s studies but also, it's an opportunity to work with other diseases in attempt to better understand their pathogenesis.

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Figures

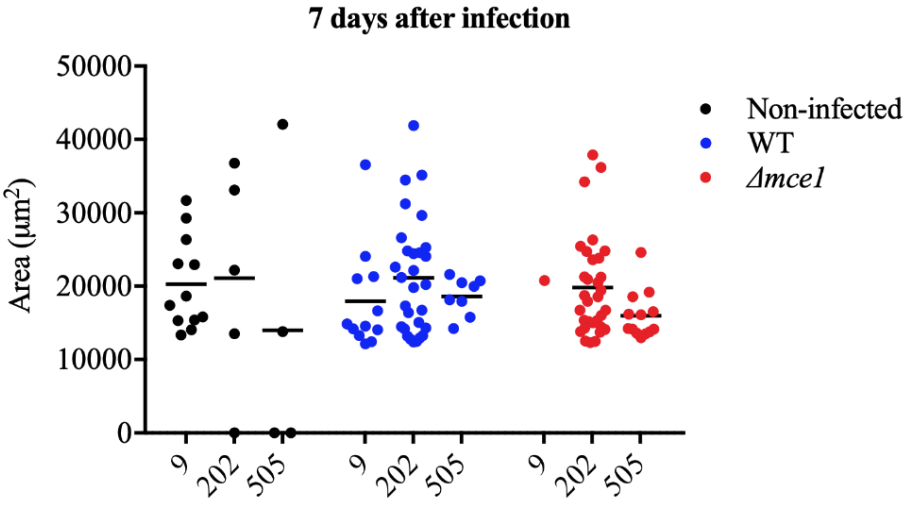

Figure 1. Number and size of aggregates formed at day 7 post-infection with WT and *Δmce1* strains. Each dot representes an aggregate counted. Mean area of aggregates is shown for each donor corresponding a two-independent assay (except for donor 9 in *Δmce1's* infection).

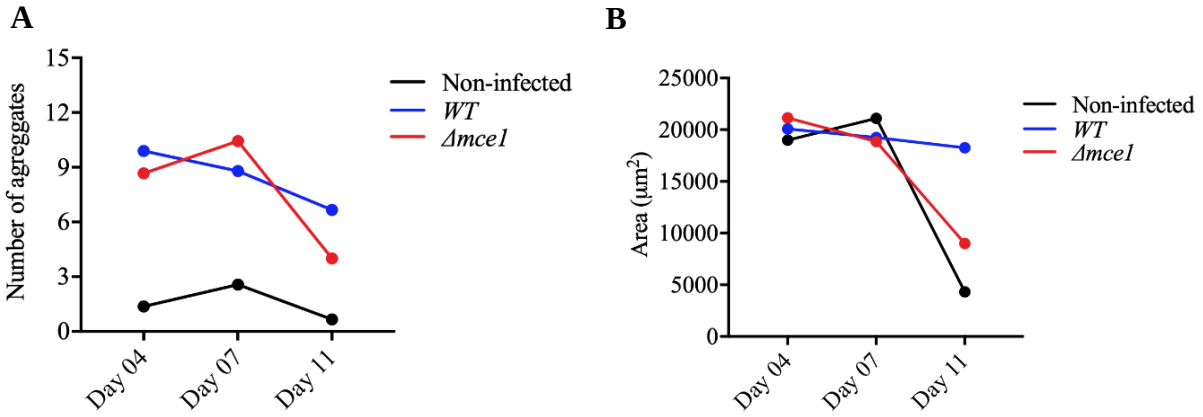

Figure 2. Number (A) and size (B) of aggregates formed at day 4, 7 and 11 post-infection with WT-Erdman (black line) and Δmce1-Erdman (red line). Mean area and numbers of aggregates corresponding a three (number of aggregate) or two-independent assay (area of aggregates) with 3 donors.

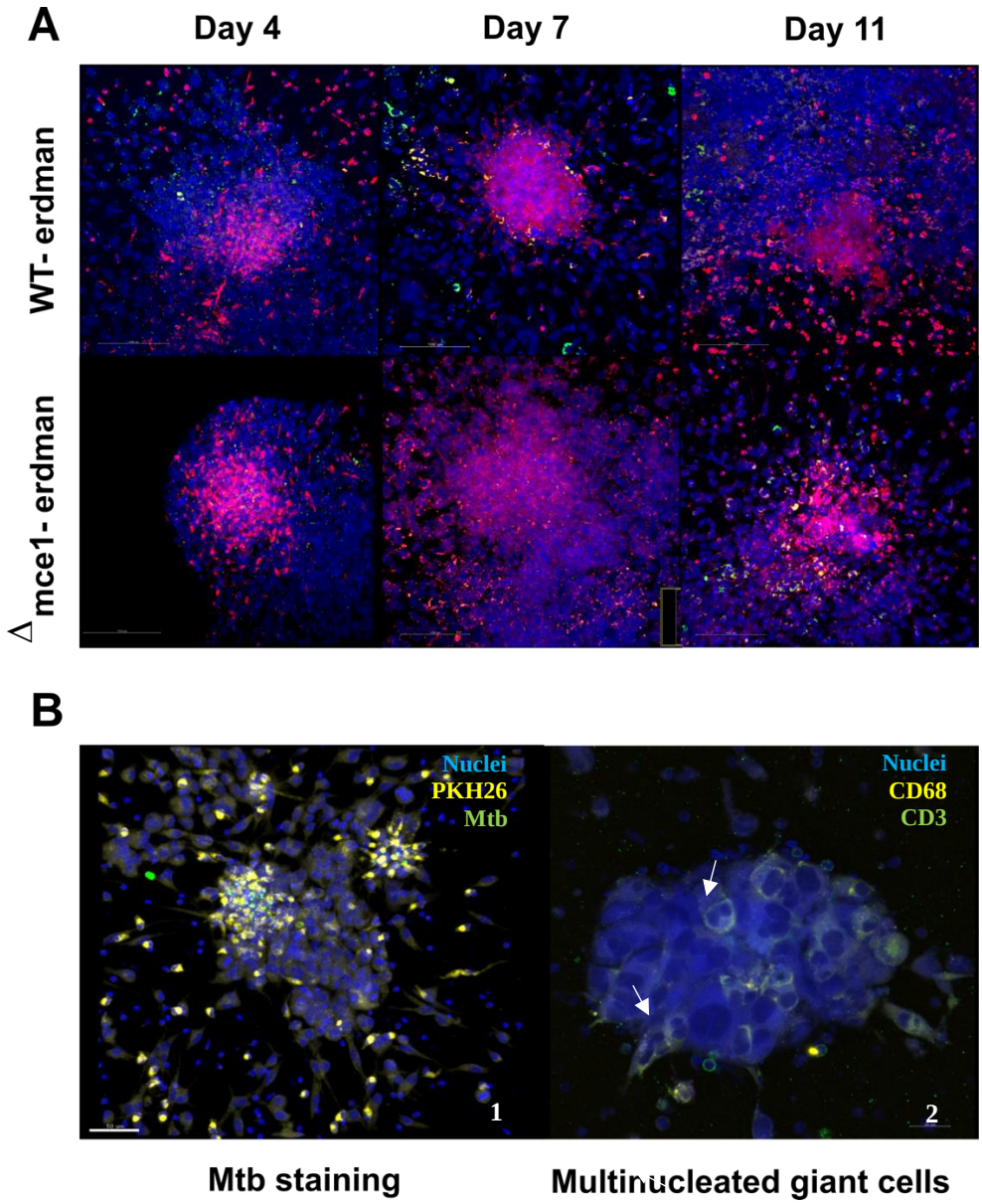

Figure 3. Infection of A549 cells with WT and Δmce1-erdman strain led to formation of granuloma-like structure. A, Aggregates formation at day 4, 7 and 11 after four hours of infection at an MOI of 0.1 of WT and Δmce1-erdman strain. Nuclei were stained with DAPI (blue) and PBMC cells with PKH26 (red). Some lipids droplets can be seen on green (Bodipy 493/503) or yellow (overlays). B, Granuloma hallmarks include intracellular bacteria (1, green), multinucleated giant cells (2, white arrows) and presence of $CD68^{+}$ macrophage and $CD3^{+}$ lymphocytes. Nuclei were stained with blue (DAPI) and $CD68^{+}$ macrophages (yellow) and $CD3^{+}$ lymphocytes (green). Scale bar: 500μm

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4.2 CAPÍTULO 2: DIFFERENTIAL HOST PRO-INFLAMMATORY RESPONSE TO MYCOBACTERIAL CELL WALL LIPIDS REGULATED BY THE MCE1 OPERON

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Differential Host Pro-Inflammatory Response to Mycobacterial Cell Wall Lipids Regulated by the *Mce1* Operon

Jéssica D. Petrilli,[1] Igor Müller,[1] Luana E. Araújo,[1] Thiago M. Cardoso,[2] Lucas P. Carvalho,[2] Bruna C. Barros,[1] Maurício Teixeira,[2] Sérgio Arruda,[1] Lee W. Riley,[3] Adriano Queiroz,[1#].

[1]Laboratorio Avançado de Saúde Pública, Instituto Gonçalo Moniz, Salvador, Bahia, Brazil;

[2]Laboratório de Pesquisa Clínica, Instituto Gonçalo Moniz, Salvador, Bahia, Brazil;

[3]Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, USA.

#Corresponding Author:

Prof Dr Adriano Queiroz, Gonçalo Moniz Institute – Fiocruz. Bahia, Brazil.

Phone: +55 71 3176-2232, e-mail: adrianoqs@gmail.com.

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Abstract

The cell wall of wild-type (WT) *Mycobacterium tuberculosis* (Mtb), an etiologic agent of tuberculosis (TB) and a Mtb strain disrupted in a 13-gene operon *mce1* (Δmce1) varies by more than 400 lipid species. Here, we examined Mtb lipid-induced response in murine macrophage, as well as in human T-cell subpopulations in order to gain an insight into how changes in cell wall lipid composition may modulate host immune response. Relative to WT Mtb cell wall lipids, the non-polar lipid extracts from Δmce1 enhanced the mRNA expression of lipid-sense nuclear receptors TR4 and PPAR-γ and dampened the macrophage expression of genes encoding TNF-α, IL-6 and IL-1β. Relative to untreated control, WT lipid-pre-stimulated macrophages from healthy individuals induced a higher level of $CD4^{-}CD8^{-}$ double negative T-cells (DN T-cells) producing TNF-α. Conversely, compared to WT, stimulation with Δmce1 lipids induced higher mean fluorescence intensity (MFI) in IL-10-producing DN T cells. Mononuclear cells from TB patients stimulated with WT Mtb lipids induced an increased production of TNF-α by $CD8^{+}$ lymphocytes. Taken together, these observations suggest that changes in *mce1* operon expression during a course of infection may serve as a strategy by Mtb to evade the host proinflammatory responses detrimental to its in vivo persistence.

Introduction

Despite the availability of effective treatment regimens against *Mycobacterium tuberculosis* (Mtb), an etiologic agent of tuberculosis (TB), TB remains a major public health challenge, which has surpassed AIDS as the most common infectious disease cause of death in adults (Tornheim and Dooley, 2017). A large proportion of the world's population latently infected with Mtb serves as the main reservoir of reactivation TB (WHO, 2018). The mechanisms by which Mtb remains persistent in a host and how a subset of the infected hosts reactivates to develop TB are still not fully understood.

Mtb contains four homologous copies of an operon called *mce*1-4, which resemble ATP-binding cassette transporters possibly involved in lipid important across its cell wall (Cole *et al.*, 1998; Casali and Riley, 2007; Pandey and Sassetti, 2008). Disruption of one of these operons (*mce1*) precludes Mtb to induce a strong Th1 type T-cell immune response and to form organized granuloma in mouse lungs (Shimono *et al.*, 2003). The expression of *mce1* operon is repressed in the WT strain during the first eight weeks of mouse infection (Uchida *et al.*, 2007) and its expression decreases 4 hours post infection in macrophages (Casali *et al.*, 2006). Thus, at some point during in vitro macrophage or mouse infection, the WT strain displays the same phenotype shown by the *mce1* operon mutant (Δmce1).

The cell wall lipid content of Mtb is highly affected by the repression of the *mce1* operon. The mutant strain accumulates several-fold greater amount of free mycolic acids on its surface (Cantrell *et al.*, 2013; Forrellad *et al.*, 2014). As revealed by lipid metabolomics analysis, the mutant also contains diminished amounts of diacyltrehalose, diacylated sulfoglycolipid,

phosphatidylethanolamine, phthienoic acid and phthioceranic acid in its cell wall compared to its parent wild-type (WT) strain. In fact, a lipidomics analysis has shown differential expression of more than 400 lipid species between the mutant and WT Mtb strains (Queiroz *et al.*, 2015).

The cell wall lipid rearrangement during infection could be a determining factor for bacterial persistence, as previously suggested (Queiroz *et al.*, 2017). Individually, such lipids have been shown to induce granuloma formation (Bekierkunst *et al.*, 1969) , leukocyte migration (Bloch, 1950) and inflammatory cytokines expression (Gilleron *et al.*, 2004), or to inhibit macrophage responses (Pabst *et al.*, 1988; Zhang *et al.*, 1991; Sequeira *et al.*, 2014). However, the evaluation of host cell behavior in response to Mtb cell wall lipid rearrangement has never been accessed. Here, we hypothesized that by comparing responses of host immune cells challenged in vitro with lipids extracted from the *mce1* operon mutant or WT Mtb, we may be able to demonstrate how differences in cell wall lipid composition affect host cell responses that would be advantageous to Mtb.

RESULTS

Δmce1 lipid extracts dampen the pro-inflammatory response in murine macrophages

The transcriptional expression of the proinflammatory genes encoding *TNF-α*, *IL-6* and *IL-1β* in RAW cells, stimulated by either Δmce1 or WT lipid extracts, was evaluated (FigurFig 1A). The expression of these genes was, respectively, 9.7-, 1,250- and 199-fold higher in macrophages stimulated with WT lipid extract than in untreated control cells. Conversely, Δmce1 lipid stimulation dampened the expression of *TNF-α*, *IL-6* and *IL-1β* genes by 3.4-, 47- and 26-fold respectively. The WT/Δmce1 expression ratios of these markers were, respectively, 2.8, 27 and

7.6 ($p < 0.05$, 0.01 and 0.05). *IL-12* was increased 3.6- and 2.4-fold in macrophages stimulated by WT and Δmce1 lipids, respectively.

Differential induction of lipid sense nuclear receptors (LSNR) by Mtb lipids.

Mtb interacts with LSNR to modulate macrophage immune and metabolic functions (Almeida *et al.*, 2012; Mahajan *et al.*, 2012). Since mycobacterial lipid extracts modulated proinflammatory response in macrophages, we attempted to determine whether LSNR activation was involved in this process. Here, we studied the expression by macrophages of LSNR PPAR-γ, TR4, RAR, and LXR-α genes. As depicted in Figure 1B, while the WT lipid extracts consistently repressed *PPAR-γ*, *TR4* and *RAR* expression by 0.6-, 0.8- and 0.7-fold, respectively, the lipid extracts of the mutant strain enhanced the expression of these same genes by 1.4-, 1.2- and 1.2-fold, respectively, relative to untreated controls. The resulting Δmce1/WT expression ratios were, respectively, 2.3, 1.5 and 1.7. Fold-differences between groups were statically significant for *TR4* and *RAR* ($p < 0.05$). Both lipid extracts, WT and Δmce1, induced similar expression levels of another LSNR gene, *LXR-α* (1.3- and 1.5-fold, respectively).

Macrophages pre-stimulated by mycobacterial lipids activate both $CD4^+$ and double-negative ($CD4^-$ $CD8^-$) T cells.

A co-culture assay was performed with both non-adherent and lipid-pre-treated adherent cells isolated from the peripheral blood of healthy subjects. Following the activation of IFN-γ-, TNF-α- and IL-10-producing $CD4^+$ and $CD8^+$ T cells by lipid-stimulated macrophages, the proportion of IL-10-producing $CD4^+$ was found to be higher in Δmce1 lipid-induced cells than in those stimulated by WT ($p \leq 0.05$) (Figure 2A). Also, compared to untreated control, stimulation

with Δmce1 lipids induced lower mean fluorescent intensity (MFI) in TNF-α-producing $CD4^+$ T cells ($p \leq 0.05$) (Figure 2A). However, neither the frequency nor the MFI of $CD8^+$ T cells changed (Figure 2B).

This co-culture assay showed that pre-stimulation with both WT and Δmce1 lipids also increase the frequency and MFI of co-cultured DN T cells (Figure 3), which were defined as CD3 positive but CD4 and CD8 negative ($CD3^+CD4^-CD8^-$) (Figure 3A). The proportion of TNF-α-producing DN T cells was only found to be higher in WT lipid-induced cells than in non-stimulated controls ($p \leq 0.001$) (Figure 3B). Interestingly, compared to WT, stimulation with Δmce1 lipids induced higher MFI in IL-10-producing DN T cells ($p \leq 0.001$) (Figure 3C).

Cytokine production in cultures of lipid-induced PBMC.

The levels of cytokines IL-6, IFN-γ, TNF-α, IL-1β, and IL-10 were assessed in the supernatants of PBMCs stimulated with mycobacterial lipids (Figure 4). As expected, cells stimulated with WT lipids showed increased levels of proinflammatory cytokines IL-6 (1,319 pg/mL), IFN-γ (251 pg/mL), TNF-α (580 pg/mL) and IL-1β (222 pg/mL) relative to non-stimulated controls (91, 6, 5 and 0 pg/mL, respectively).

Characterization of lipid-induced response in PBMCs isolated from patients with active TB.

Next, to evaluate the effects of stimulation with WT and Δmce1 lipid extracts on the inflammatory response in PBMCs isolated from patients with active TB (Figure 5), the frequency of $CD4^+$, $CD8^+$,

$CD56^+$, NKT, $CD4^-/CD8^-$ DN, $CD38^+$ and $CD14^+$ subpopulations were assessed, in addition to the production of IFN-γ, TNF-α and IL-10 by these cells (see Supplementary Figure 1A and Supplementary Figure 1B). Compared to untreated control, stimulation with Δmce1 lipids induced lower MFI in IFN-γ-producing DN T cells ($p \leq 0.05$) (Supplementary Figure 1B). In contrast to the response seen in DN T cells from healthy subjects stimulated with Mtb lipids, no significant differences were observed in the Mtb lipid-stimulated TNF-α- and IL-10-producing DN T cells from patients with active TB compared to untreated cells.

The proportion of IFN-γ-producing $CD14^+$ cells stimulated by Δmce1 lipids was higher than that in WT lipid-induced cell cultures ($p < 0.05$) (Supplementary Figure 1A). Interestingly, although not statistically significant, the MFI of $CD8^+$ cells-producing TNF-α increased 3.4-fold in WT lipid-stimulated PBMC cultures relative to untreated controls ($p > 0.05$) (Figure 5A). The levels of IL-6, IL-1β, TNF-α and IFN-γ were increased in WT lipid-induced PBMCs relative to untreated controls ($p < 0.05$) (Fig 5B). Importantly, the production of TNF-α seen in supernatant of WT lipid-stimulated PBMCs was strongly correlated to MFI of $CD8^+$ T cells-producing TNF-α ($p < 0.05$; Pearson r=0.8) (Figure 5C).

DISCUSSION

Mice infected with a strain of *M. tuberculosis* disrupted in the *mce1* operon are unable to mount a strong proinflammatory response against WT Mtb, as evidenced by decreased levels of IL-6 and TNF-α; consequently, these animals do not form organized granulomas (Shimono *et al.*, 2003).

Recent studies have demonstrated that free mycolic acid accumulates on the surface of Mtb mutant cells (Cantrell *et al.*, 2013; Queiroz *et al.*, 2017), and that the cell wall contains decreased levels of some saccharolipids and glycerophospholipids species (Queiroz *et al.*, 2017). As the *mce1* operon of WT Mtb becomes repressed during the course of infection in a mouse model of TB (Uchida *et al.*, 2007), we reasoned that the lipids in the cell walls of this *mce1* mutant could be representative of the lipid composition of WT Mtb at some point during a course of *in vivo* infection. Thus, we hypothesized that the Mce1-mediated remodeling of lipids in the Mtb cell wall may contribute to the differential host proinflammatory response that could facilitate the persistence of Mtb in the infected host.

When stimulated with WT or Δmce1 lipid extracts, the RAW cells evaluated herein exhibited opposite expression responses in genes encoding *TNF-α*, *IL-6* and *IL-1β* (Figure 1A), as lipids extracted from the *mce1* operon mutant strain were found to virtually reverse the proinflammatory response triggered by stimulation with WT lipids. One mechanism for this modulation of inflammatory response may involve the mutant's lipids interacting with LSNRs PPAR-γ, TR4 and RAR (Figure 1B). Mtb lipids can serve as ligands of LSNRs, a large family of intracellular proteins responsible for regulating gene transcription (Alaynick, 2008). Previous studies have shown that Mtb infection is associated with increased expression of PPAR-γ and TR4 leading to Mtb survival through IL-10 production and prevention of phagolysosome maturation (Almeida *et al.*, 2012; Mahajan *et al.*, 2012). Also, PPAR-γ ligands inhibit monocyte production of TNF-α, IL-6 and IL-1β (Jiang *et al.*, 1998) and the transcriptional activation of the proinflammatory transcription factor NF-κB in murine macrophage cells (Ricote *et al.*, 1998). Also, TR4 is sensed by mycolic acids

(Dkhar *et al.*, 2014), which is greatly increased in amount in the cell wall of the mutant strain (Cantrell *et al.*, 2013; Queiroz *et al.*, 2017).

On the other hand, we found that lipid extracts from both Mtb strains induced a similar expression level (1.3- and 1.5-fold) of another LSNR LXR-α (Figure 1B). This transcriptional factor triggers a protective immune response against Mtb (Barral and Brenner, 2007; Almeida *et al.*, 2012) and it is activated by oxysterols, the oxygenated derivatives of cholesterol (Janowski *et al.*, 1999; Edwards *et al.*, 2002). As far as known, oxysterols are not included among those mycobacterial cell wall lipids that are altered by Mce1 repression (Queiroz *et al.*, 2015), which may explain why we did not see a difference in the expression of LXR-α by cells exposed to lipids from the WT or mutant Mtb (Korf *et al.*, 2009).

Macrophages can present mycobacterial lipids via CD1 (CD1a – CD1e) family of MHC-class-I-like glycoproteins to T cells (Barral and Brenner, 2007). Depending on their species, these lipids can be presented by CD1a, CD1b or CD1c to either: (A) $CD4^{-}CD8^{-}$ double negative or to $CD4^{+}$ and $CD8^{+}$ αβ TCR clonally diverse T-cells that mediate adaptive immunity, or (B) be presented by CD1d molecules to natural killer T (NKT) cells (Porcelli *et al.*, 1992; Gilleron *et al.*, 2004). Thus, since the lipid rearrangement on Mtb cell wall dampens the macrophage inflammatory response, we wondered if this modulation would determine the pattern of T cell activation. Here, compared to WT, Δmce1 lipid-pre-stimulated macrophages induced higher frequency and MFI level of, respectively, IL-10-producing $CD4^{+}$ and DN T cells (Figs. 2 and 3). Conversely, relative to untreated control, WT lipids induced a higher proportion of TNF-α-producing DN T-cells and Δmce1 lipids decreased the MFI level in TNF-α-producing $CD4^{+}$ T-cells. $CD8^{+}$ T-cells, isolated from healthy subjects, were not activated by lipid-pre-stimulated macrophages (Figure 2). Taken

together, these results suggest that the Mtb´s cell wall lipid-rearrangement modulates the macrophage's inflammatory response and, indirectly, determine the function of both $CD4^+$ and DN T-cells. In Figure 6, we propose a model of *mce1* operon-mediated differential Mtb lipid-induced host inflammatory control in macrophage.

We have not determined which DN T-cell population subsets respond to each lipid extract used here, but we can speculate that WT lipids would induce the production of TNF-α through αβ DN T-cells, while the Δmce1 lipids may activate the IL-10-producing γδ DN T-cells by lymphocyte-dependent immunoregulatory mechanisms, as previously suggested (Antonelli *et al.*, 2006).

In contrast to the results with healthy donors, in PBMC collected from TB patients, the MFI of TNF-α-producing $CD8^+$ T-cells induced by WT lipids was 3-fold higher than in non-stimulated or Δmce1 lipids-stimulated PBMC, suggesting distinct roles of Mtb lipids in activating T lymphocytes subpopulations in healthy and TB subjects. The TNF-α production by $CD8^+$ T-cells is supported by the increased level of this cytokine in culture supernatants of WT lipid-induced PBMC relative to untreated control, suggesting that $CD8^+$ T-cells stimulated by mycobacterial lipids maybe an important source of TNF-α during active disease.

We propose here that Mtb uses its cell wall lipid-remodeling capacity to adapt to host inflammatory response during the course of an infection. LSNR may serve as one component of this process to negatively dampen macrophage proinflammatory response, and perhaps indirectly influence T-cell-dependent proinflammatory responses. This lipid-mediated microbial defense strategy may contribute to Mtb's long-term survival in an infected host.

EXPERIMENTAL PROCEDURES

Bacterial strains, media and growth conditions

The following bacterial strains were used: *mce1* operon mutant *M. tuberculosis* (Δmce1) and its parent Erdman wild-type (WT) strain. The generation of Δmce1 was previously described by Shimono et al (Shimono *et al.*, 2003). Both Mtb strains were initially cultured in Middlebrook 7H9 broth (Difco, MD) containing 10% ADC (Beckton-Dickinson, MD) and 0.2% glycerol (Fisher Scientific, NJ) to obtain similar numbers of bacterial cells for growth in detergent-free minimal media. Bacteria were incubated at 37°C until stationary phase in Sauton's media (without Tween) as previously described (Ojha *et al.*, 2008). Briefly, bacteria were grown in 125 mL polycarbonate bottles containing 30 mL of Sauton's media with 300 μl of saturated planktonic culture (OD of each test strain was adjusted so that equal numbers of each bacteria strain were inoculated) and incubated without agitation at 37°C for 19 days.

Lipid extraction

The extraction of lipids from biofilm cultures was performed as previously described (Ojha *et al.*, 2008). Briefly, biofilm from 19-day *M. tuberculosis* cultures was harvested, and apolar lipids were extracted from using 5 mL of methanol: 0.3% NaCl (100:10) mixed with 2.5 mL of petroleum ether, incubated at room temperature for 30 minutes. The upper petroleum ether layer containing the apolar lipids was separated by centrifugation. After solvent evaporation, apolar lipids were weighed and resuspended at a concentration of 0.02 mg/mL in hexane/isopropanol. Lipid extracts (0.5 mL) were layered onto 24-well tissue culture plates (0.01 mg/well) and the solvent was

allowed to evaporate. Control wells were layered only with hexane/isopropanol in the absence of lipid extracts.

RAW macrophage assay

RAW 264.7 murine macrophage cultures: RAW 264.7 murine macrophage-like cells (ATCC TIB-71) were cultured and maintained in Dulbecco's modified Eagles medium (DMEM; Gibco) supplemented with 10% FBS at 37 °C, 5% CO_2, in a humidified incubator. Macrophage cell number and viability were assessed by staining in a trypan blue (Gibco) exclusion assay. Prior to experimentation, macrophages were first seeded in 75-cm^2 flasks overnight to achieve 70% confluency, and then seeded onto Mtb lipid-coated 24-well tissue culture plates at a concentration of 3.5 x 10^5 cells/well, and incubated at 37°C in a 5% CO_2 humidified atmosphere for 72h. Control macrophages were cultured on layered wells with hexane/isopropanol, and incubated in the absence of lipid extracts.

RNA extraction and purification: RNA was extracted from RAW cells according to a standard Trizol RNA extraction protocol supplied by Invitrogen (Invitrogen, Life Technologies). Extracted RNA was treated with DNase (Qiagen) to ensure that no DNA was present in the samples. DNA-free RNA (500 ng) was mixed with 50 ng of random hexamers and 50 μM of oligo (dT) (Invitrogen) at a final volume of 10 μl, then reverse transcribed to cDNA with Superscript III reverse transcriptase (Invitrogen) following the manufacturer's recommendations.

RT-qPCR: Eight genes encoding lipid-sensing nuclear receptors (LSNR) were selected as targets, in addition to genes related to proinflammatory response (complete list of genes available in Supplementary Table 1). Primers were designed to produce a 100–195 bp amplicon for each gene.

qPCR reactions were performed using 25 ng of previously generated cDNA and Maxima SYBR Green/ROX qPCR Master Mix (2X) (Fermentas) in accordance with the manufacturer's instructions. Relative changes in gene expression between lipid-induced and unstimulated RAW cells (controls) were analyzed by $2^{-\Delta\Delta CT}$ method according to a previously described method (Livak and Schmittgen, 2001). The expression of all tested genes was normalized to both *β-actin* and *glyceraldehyde 3-phosphate dehydrogenase* (GAPDH) gene expression.

Assays with peripheral blood mononuclear cells isolated from healthy subjects and TB patients

Study participants: Seventeen participants were enrolled in this study, divided into healthy subjects (n=11) and TB patients (n=6). All healthy volunteers, recruited from the Gonçalo Moniz Institute (IGM-Fiocruz), were tested for latent TB infection (LTBI) by interferon γ release assay (QuatiFERON® TB Gold); two tested positive for LTBI. Six TB patients were recruited at the Octavio Mangabeira Hospital (HEOM), a reference center for respiratory disease in Salvador, Bahia-Brazil. All patients were diagnosed with active TB based on GeneXpert MTB/RIF test results. The age of the study subjects ranged between 18-60 years.

PBMC isolation from study subjects and culture conditions: Peripheral blood mononuclear cells (PBMC) were obtained by the Ficoll-Paque (GE Healthcare) density gradient method. PBMC concentrations were adjusted to 1 x 10^6 cells/mL in 1 mL of complete RPMI 1640 (100 µl/mL gentamicin, 2mM L-glutamine, 30mM HEPES) containing 10% inactivated bovine fetal serum (FBS) (Life technologies GIBCO BRL, Gaithersburg, MD). PBMCs from TB patients were cryopreserved (FBS containing 10% DMSO) in liquid nitrogen prior to performing culture assays.

All PBMCs were dispensed into previously prepared 24-well plates and incubated at 37ºC under 5% CO_2.

Co-culturing of adherent and non-adherent cells: Adherent cells were isolated from six healthy subjects PBMC ($2x10^7$) on sterile 6-well tissue culture plates. After two hours of adherence, cells in suspension (non-adherent cells) were harvested and cryopreserved (FBS with 10% DMSO) at -80°C. Adherent cells were kept on 6-well plates for 5 days at 37ºC under 5% CO_2. After differentiation, macrophages were collected by washing the wells with 2mM EDTA saline at 4°C, with the aid of a cell-scraper. Cells ($1x10^6$) were then cultured on 24-well tissue culture plates in RPMI medium supplemented with 10% FBS in the presence or absence of Mtb non-polar lipids for 72 hours at 37ºC under 5% CO_2. Non-adherent cryopreserved cells were then added to pre-stimulated macrophage cultures at a ratio of 10:1 (non-adherent cells:macrophage), incubated for 72 hours at 37ºC under 5% CO_2, and finally stained to perform flow cytometry.

PBMC assay with TB patients: Cryopreserved PBMCs from TB patients ($1x10^6$) were thawed and incubated for 72 hours on 24-well tissue culture plates pre-treated with lipids at 37ºC under 5% CO_2 in the presence or absence of Mtb non-polar lipids (0.01mg/well) extracted from WT and Δmce1 strains. After incubation, cells were stained for flow cytometry analysis.

Cytokine analysis: Supernatants of PBMCs from healthy subjects and TB patients were collected and stored at -20ºC for cytokine analysis (TNF-α, IL-6, IL-1β, IFN-γ and IL-10) by either ELISA (R&D Systems, Minneapolis, MN) or Cytometric Bead Array (BD™ CBA Human TH1/TH2 cytokine kit) according to the manufacturer's instructions. Results are expressed as pg/mL.

Flow cytometry: Co-cultured cells were stained with CD3-FITC (clone SP34-2), CD4-APC-CY7 (clone RPA-T4) and CD8-PerceP-CY5.5 (clone RPA-T8). PBMCs from TB patients were stained with CD3-APC-CY7 (clone HIT3a), CD4-APC (clone RPA-T4), CD8-ALEXA700 (clone 53-6.7), CD56-Brilliant Violet 421 (clone HCD56), CD38-PE-CY5 (clone HIT2) and CD14-FITC (clone 61D3). All cells were then fixed with 4% PFA (BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit with GolgiPlug™). For intracellular staining, fixed cells were permeabilized using a cytofix/cytoperm kit (BD Biosciences) and stained intracellularly with anti-TNF, IFN-γ, and IL-10 antibodies. Cells were acquired (100,000 events) on a BD LSRFortessa® device. The frequencies of stained cells and MFI were estimated using FlowJo 7.10.1 software (Tree Star, Inc, Ashland, OR.).

Statistical analysis and data representation

Statistical analyses were performed with GraphPad Prism v.7.0 (GraphPad Inc., San Diego, CA, USA). Statistical significance between variables was assessed by Mann-Whitney U test or Friedman test, followed by Dunn's post-test. Continuous variables with normal distribution were expressed as mean ± standard deviation, while those with non-normal distributions were expressed as median and interquartile interval. Significance was considered when $p<0.05$.

Ethics Statement

The institutional review board at the Gonçalo Moniz Institute (IGM-Fiocruz, Bahia) approved the present study (CAAE: 76009417.9.0000.0040). All enrolled subjects provided written and informed consent prior to participation.

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Figures

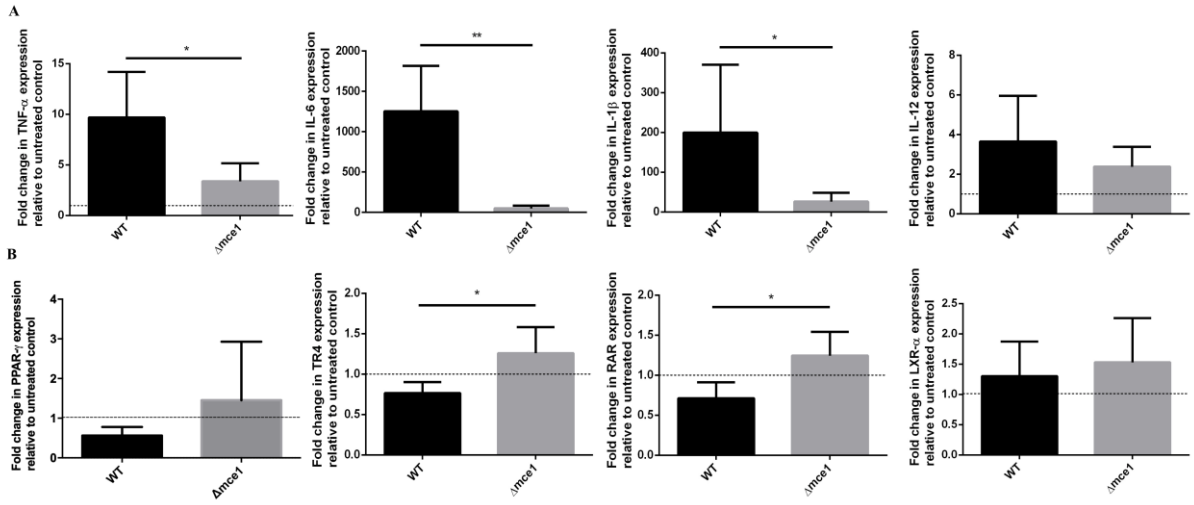


Figure 1. RT-PCR analysis of selected cytokine genes *(A)* and lipid-sense nuclear receptor (LSNR) genes *(B)* in murine macrophages exposed to Mtb apolar lipid extracts. Data are presented as fold-difference in gene expression levels in cells stimulated with apolar lipids extracted from wild type or *mce1* operon mutant *M. tuberculosis*, relative to untreated control, mean ± SD; gene expression was normalized to β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes; six replicates of each reaction were performed. Statistical significance was evaluated by Mann-Whitney $p < 0.05$. * $p < 0.05$; ** $p < 0.01$. WT = *Mycobacterium tuberculosis* wild type strain; Δmce1 = strain with disruption of *mce1* operon.

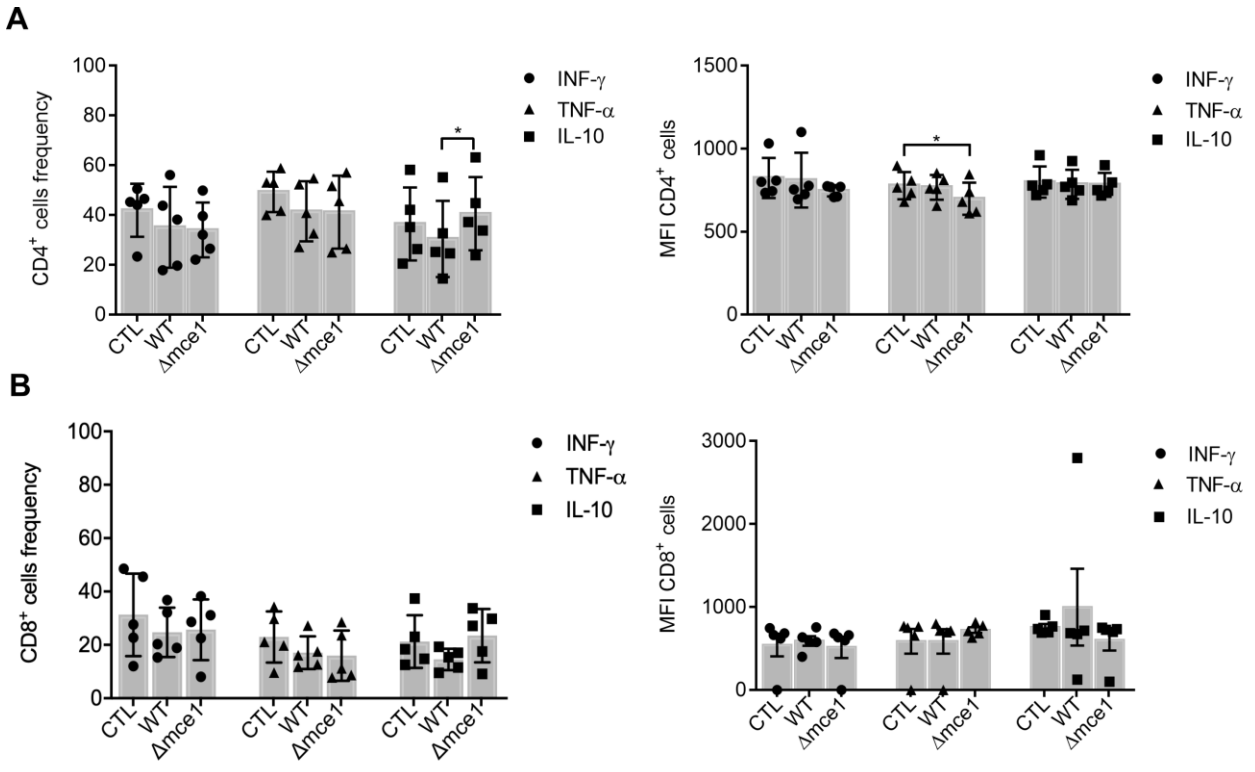


Figure 2 IFN-γ-, TNFα- and IL-10-producing by CD4$^+$ and CD8$^+$ T cells from six healthy donors in a co-culture assay. Non-adherent cells were added to a culture containing adherent cells exposed to apolar lipids extracted from wild type vs *mce1* operon mutant *M. tuberculosis*. (A) Frequency and quantification of the mean fluorescence intensity of IFN-γ-, TNFα- and IL-10 produced by CD4$^+$ T cells. (B) Frequency and quantification of the mean fluorescence intensity of IFN-γ-, TNFα- and IL-10 produced by CD8$^+$ T cells. Results are expressed as median ± IQR; n = 6/group. Statistical significance was determined by Friedman test followed by Dunn's post-test; significance was considered at * $p < 0.05$; ** $p<0.01$; *** $p< 0.001$. WT = Mtb wild type strain; Δmce1 = Mtb strain disrupted in *mce1 operon*; CTL = no stimulus.

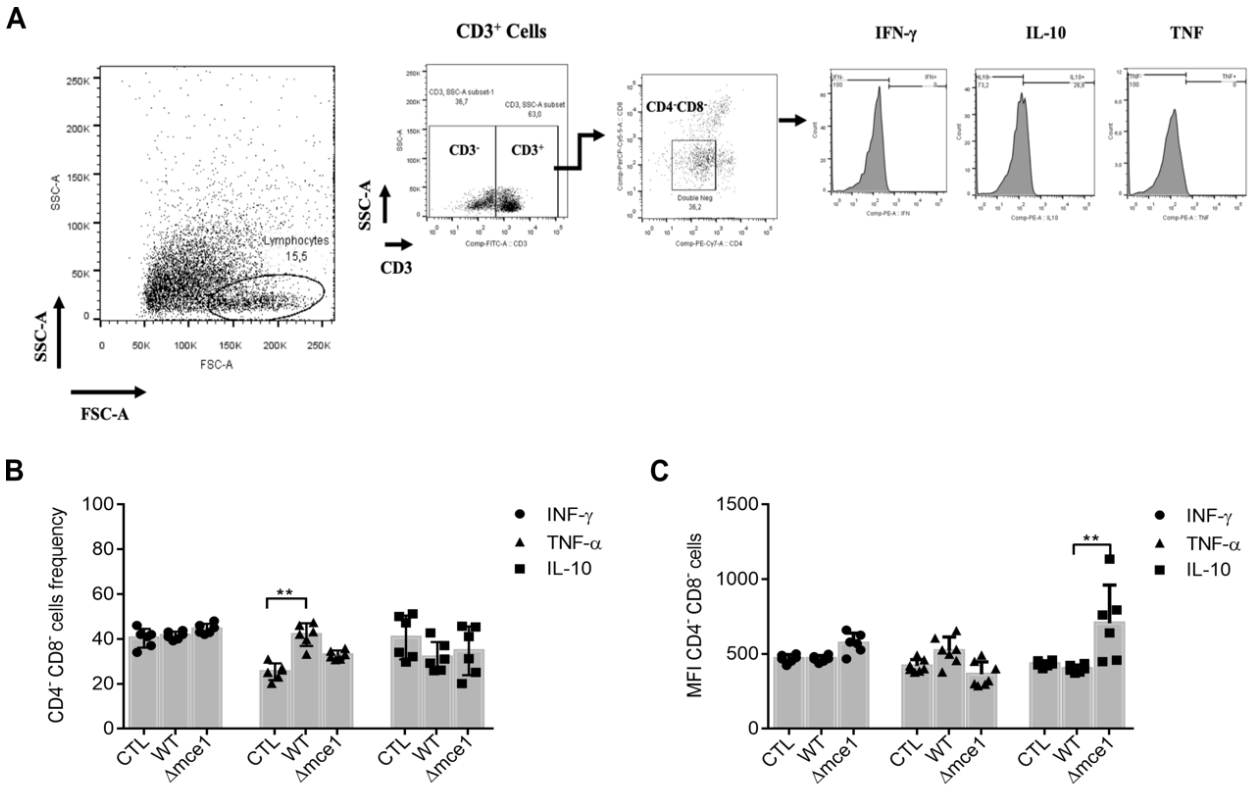


Figure 3 IFN-γ-, TNFα- and IL-10-producing by CD4$^-$CD8$^-$ double negative (DN) T cells from six healthy donors in a co-culture assay. Non-adherent cells were added to a culture containing adherent cells exposed to apolar lipids extracted from wild type vs *mce1* operon mutant *M. tuberculosis*. *(A)*, The gating strategy to assess the DN T cells. *(B)*, Frequency of IFN-γ-, TNFα- and IL-10 produced by DN T cells. *(C)*, Quantification of the mean fluorescence intensity of IFN-γ-, TNFα- and IL-10-producing DN T cells. Results are expressed as mean ± SD; n = 5/group. Statistical significance was evaluated by one-way ANOVA test and for non-parametric samples the Kruskal–Wallis test, followed by Dunn's post-test. * $p < 0.05$; ** $p<0.01$; *** $p< 0.001$. WT = Mtb wild type strain; Δmce1 = Mtb strain disrupted in *mce1 operon*; CTL = no stimulus.

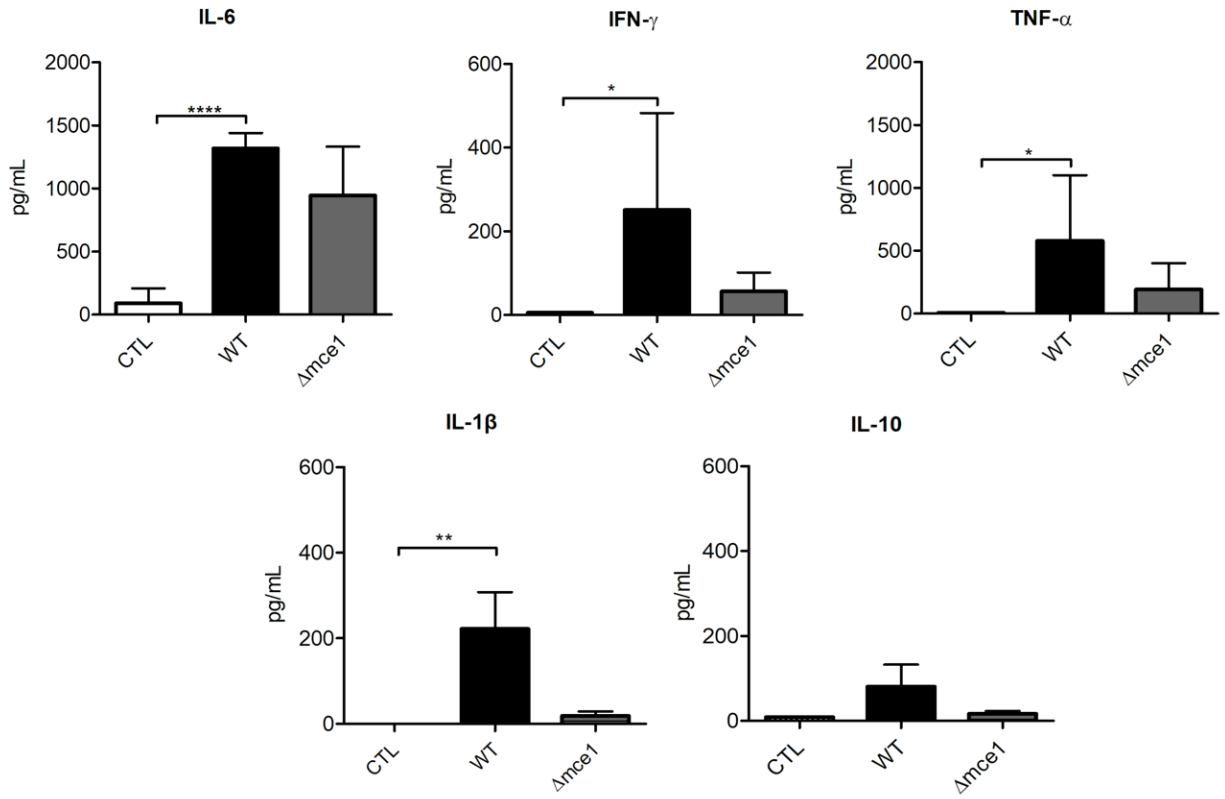


Figure 4 Cytokine production in peripheral blood mononuclear cells (PBMC) from six healthy donors exposed to apolar lipids extracted from wild type vs *mce1* operon mutant *M. tuberculosis*. Results are expressed as mean ± SD; n = 6/group. Statistical significance was evaluated by one-way ANOVA test and for non-parametric samples the Kruskal–Wallis test, followed by Dunn's post-test. * $p < 0.05$; ** $p<0.01$; *** $p< 0.00$; **** $p< 0.0001$. WT = Mtb wild type strain; Δmce1 = Mtb strain disrupted in *mce1 operon*; CTL = no stimulus.

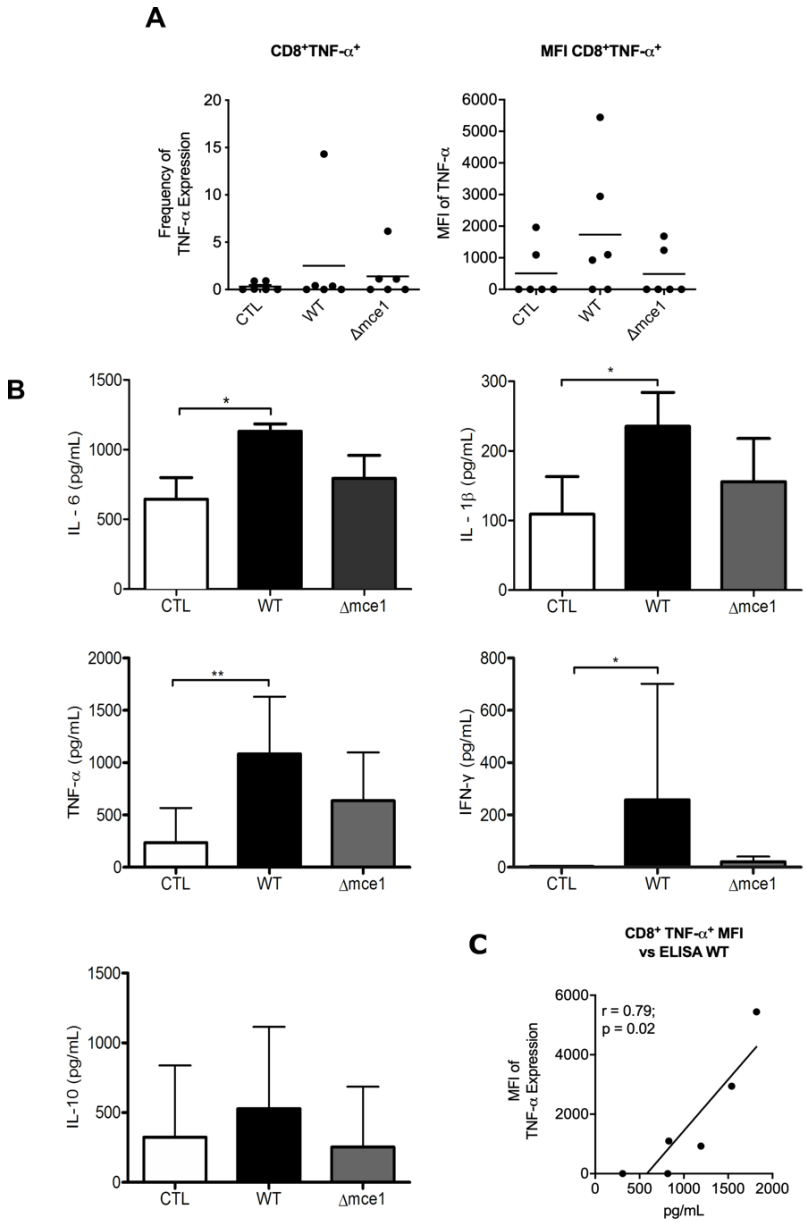


Figure 5 Peripheral blood mononuclear cell (PBMC) and cytokine production from five TB patients exposed to apolar lipids extracted from wild type vs *mce1* operon mutant *M. tuberculosis*. *(A)*, Frequency and quantification of the mean fluorescence intensity of $CD8^+$ T lymphocyte. *(B)*, Cytokines production from PBMC cultures. *(C)*, Analysis of correlation of cell cytokines production and cytokine release in PBMC supernatant. Results are expressed as mean ± SD; n = 5/group. Statistical significance was evaluated by one-way ANOVA test and for non-parametric samples the Kruskal–Wallis test, followed by Dunn's post-test. * $p < 0.05$; ** $p<0.01$. WT = Mtb wild type strain; Δmce1 = Mtb strain disrupted in *mce1 operon*; CTL = no stimulus.

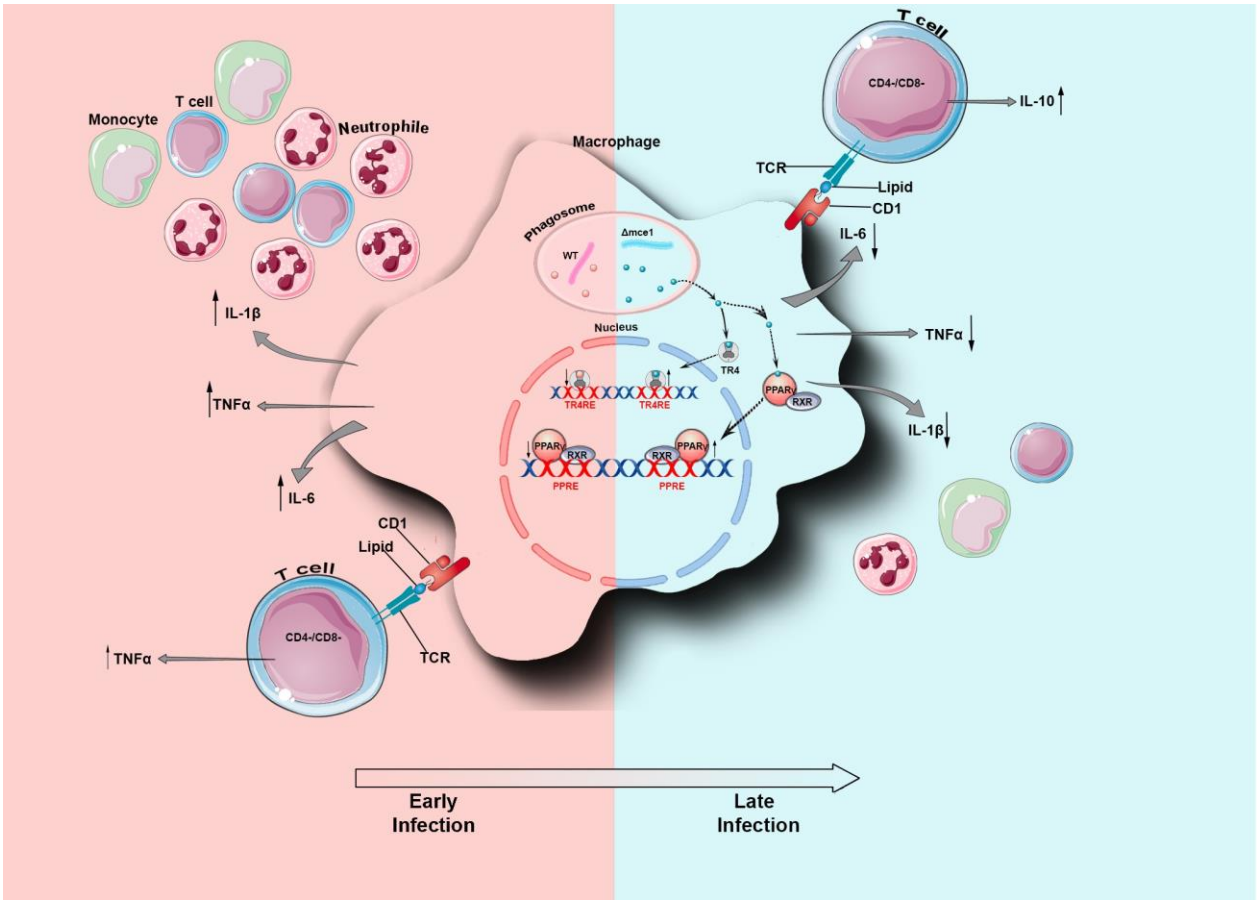


Figure 6 Schematic representation of *Mycobacterium tuberculosis* (Mtb) lipid-induced host inflammatory control in macrophage. During infection and four hours after phagocytosis by the macrophage, the *mce1* operon of Mtb is naturally repressed (12) and the bacterial cell undergoes cell wall lipid rearrangement (9). At the early phase of infection, the WT lipids induce the macrophage to secrete TNF-α, IL-1β and IL-6. Also, these lipids are presented by CD1 to TNF-α-producing $CD4^{-}CD8^{-}$ double negative (DN) T cells. Together, these secreted cytokines should recruit immune cells to the site of infection. Conversely, during the later phase of infection, the Δmce1 lipids interact with the LSNR PPAR-γ and TR4 which in turn may negatively modulate the secretion of proinflammatory cytokines. These lipids are also presented by CD1 to IL-10- and IFN-γ-producing DN T cells. WT = Mtb wild type strain; Δmce1 = Mtb strain disrupted in *mce1 operon*; TR4= testicular nuclear receptor 4; PPAR-γ= Peroxisome proliferator-activated receptor gamma.

SupSuporting Information Appendix for

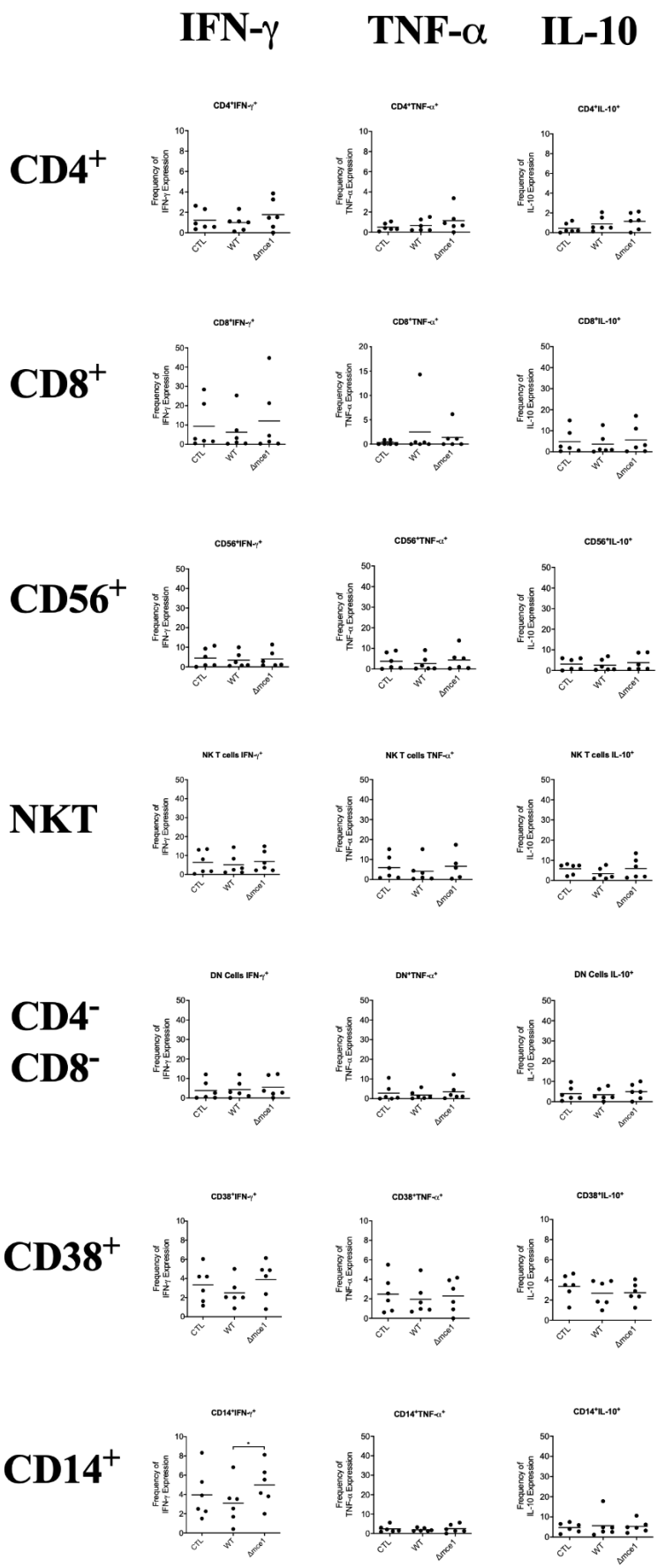
Differential host pro-inflammatory response to Mycobacterial cell wall lipids regulated by the mce1 operon

Jéssica D. Petrilli, Igor Müller, Luana E. Araújo, Thiago M. Cardoso, Lucas P. Carvalho, Bruna Barros, Maurício Teixeira, Sérgio Arruda, Lee W. Riley, Adriano Queiroz

This PDF file contains the Table S1, Figures S1 and S2.

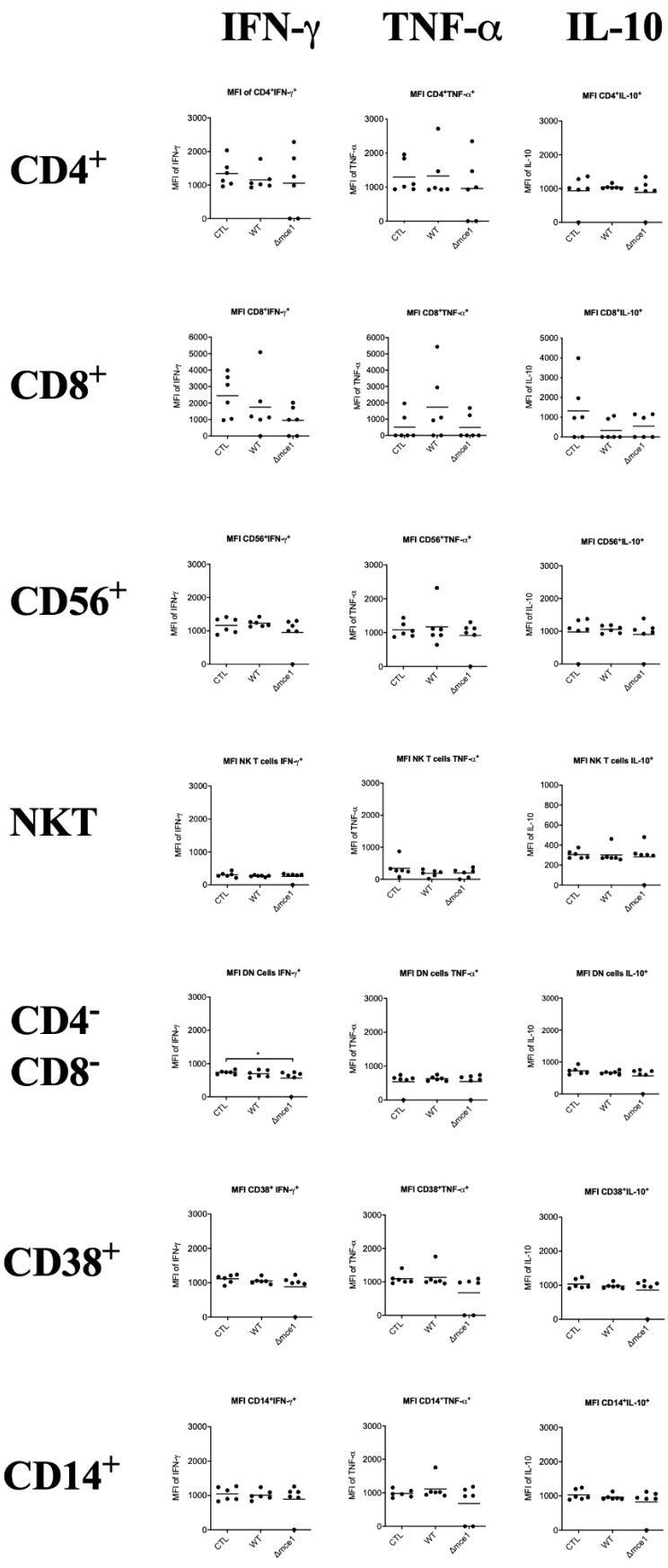
Table S1: List of primers sets used for qPCR screening.

Gene	**Nucleotide sequences (5' to 3')**
TNF-α Forward	CTACCTTGTTGCCTCCTCTTT
TNF-α Reverse	GAGCAGAGGTTCAGTGATGTAG
IL-6 Forward	CGAGAGTCCTTCAGAGAGATACA
IL-6 Reverse	CCTTCTGTGACTCCAGCTTATC
IL-1β Forward	TCGTGCTGTCGGACCCATAT
IL-1β Reverse	GTCGTTGCTTGGTTCTCCTTGT
IL-12 Forward	AAAGCAGGGCCTAGACATTC
IL-12 Reverse	CTCTGGGAAGGCCCATTTATTA
PPAR-γ Forward	CTGGCCTCCCTGATGAATAAAG
PPAR-γ Reverse	AGGCTCCATAAAGTCACCAAAG
TR4 Forward	CTGATAGCCACTCCCACATTT
TR4 Reverse	GAACTGTACCATCCTCACGTATC
RAR Forward	CTGGATCAATGCCACCTCTC
RAR Reverse	GGGACTGTGCTCTGCTATATTC
LXR-α Forward	GCTACAACCACGAGACAGAA
LXR-α Reverse	GAGAACTCGAAGATGGGATTGA
GAPDH Forward	TCAACGGCACAGTCAAGG
GAPDH Reverse	ACTCCACGACATACTCAGC
β-actin Forward	GAGGTATCCTGACCCTGAAGTA
β-actin Reverse	CACACGCAGCTCATTGTAGA


IFN-γ
TNF-α
IL-10
CD4+
CD8+
CD56+
NKT
CD4-
CD8-
CD38+
CD14+
CD4+IFN-γ+
CD4+TNF-α+
CD4+IL-10+
CD8+IFN-γ+
CD8+TNF-α+
CD8+IL-10+
CD56+IFN-γ+
CD56+TNF-α+
CD56+IL-10+
NK T cells IFN-γ+
NK T cells TNF-α+
NK T cells IL-10+
DN Cells IFN-γ+
DN+TNF-α+
DN Cells IL-10+
CD38+IFN-γ+
CD38+TNF-α+
CD38+IL-10+
CD14+IFN-γ+
CD14+TNF-α+
CD14+IL-10+
Frequency of IFN-γ Expression
Frequency of TNF-α Expression
Frequency of IL-10 Expression
CTL
WT
Δmce1

Supplementary Figure 1A: Mtb Lipid Stimulated Cell Panel.

Frequency of $CD4^+$, $CD8^+$, $CD56^+$, NKT, $CD4^-CD8^-$, $CD38^+$ and $CD14^+$ cells stimulated with WT, Δmce1, H37Rv lysate. Data were represented by mean ± SD (n = 5 for each group). Statistical significance was evaluated by one-way ANOVA followed by Dunn's post-test. (* $p < 0.05$; ** $p<0.01$). CTL = no stimulus; WT = wild-type strain; Δmce1 = Mtb strain with disruption on operon mce1; MFI= Median Fluorescence Intensity.


IFN-γ
TNF-α
IL-10
CD4+
CD8+
CD56+
NKT
CD4-
CD8-
CD38+
CD14+
MFI of CD4+IFN-γ+
MFI CD4+TNF-α+
MFI CD4+IL-10+
MFI CD8+IFN-γ+
MFI CD8+TNF-α+
MFI CD8+IL-10+
MFI CD56+IFN-γ+
MFI CD56+TNF-α+
MFI CD56+IL-10+
MFI NK T cells IFN-γ+
MFI NK T cells TNF-α+
MFI NK T cells IL-10+
MFI DN Cells IFN-γ+
MFI DN cells TNF-α+
MFI DN cells IL-10+
MFI CD38+ IFN-γ+
MFI CD38+TNF-α+
MFI CD38+IL-10+
MFI CD14+IFN-γ+
MFI CD14+TNF-α+
MFI CD14+IL-10+
MFI of IFN-γ
MFI of TNF-α
MFI of IL-10
CTL
WT
Δmce1

Supplementary Figure 1B: Mtb Lipid Stimulated Cells Panel.

MFI of $CD4^+$, $CD8^+$, $CD56^+$, NKT, $CD4^-CD8^-$, $CD38^+$ and $CD14^+$ cells stimulated with WT, Δmce1, H37Rv lysate. Data were represented by mean ± SD (n = 5 for each group). CTL = no stimulus; WT = wild-type strain; Δmce1 = Mtb strain with disruption on operon mce1; MFI= Median Fluorescence Intensity.

4.3 CAPÍTULO 3: AVALIAÇÃO DA EXPRESSÃO GÊNICA DE RECEPTORES NUCLEARES ATIVADOS POR LIPÍDIOS E SEUS ALVOS, EM SANGUE TOTAL, COMO POTENCIAIS BIOMARCADORES PARA A TB PULMONAR.

Indivíduos infectados pelo *M. tuberculosis* tem maior chance de desenvolver a forma ativa da doença nos primeiros dois anos pós infecção, mas predizer esse fenômeno é o grande desafio do controle da TB, já que 95% não o fazem nos 2 anos. Assim, diversos estudos vêm sendo realizados para avaliações de biomarcadores da TB, desde estudos descrevendo potenciais marcadores a partir de moléculas do próprio microrganismos como moléculas produzidas em resposta ao bacilo.

Estudos prévios demonstraram que o *M. tuberculosis Δmce1* apresenta composição lipídica da parede celular distinta do *M. tuberculosis* WT. Essa alteração estaria associada com o controle da resposta imunopatológica pelo *M. tuberculosis* conforme sugerido por Queiroz e colaboradores. (QUEIROZ et al., 2015, 2017). Quando os macrófagos estão infectados pelo *M. tuberculosis Δmce1* acumulam corpos lipídicos no citoplasma, resultado do acúmulo de colesterol, triacilglicerol e fosfolipídios (PEYRON et al., 2008). Esse acumulo intracelular de lipídios está associado ao aumento da expressão do *peroxisome proliferator-activated receptor gamma* (PPARγ) e do *testicular receptor 4* (TR4), os quais são membros dos receptores nucleares ativados por lipídios (RNAL). O *M. tuberculosis* interage com estes receptores, os quais induzem a produção de IL-10 e o bloqueio da maturação do fagolisossomo. Esta interação também aumenta a expressão do CD36, um receptor envolvido na captura do colesterol LDL oxidado. Este processo contribui para a formação de macrófagos espumosos e aumenta a sobrevivência bacteriana (MAHAJAN et al., 2012).

O papel desses receptores vendo sendo estudado a fim de compreender a patogênese de diversas doenças, como câncer e diabetes, por exemplo (CONZEN, 2008; LONG; J. CAMPBELL, 2015; MEINKE; WOOD; SZEWCZYK, 2006; VACCA et al., 2011). No caso da TB, alguns desses receptores são ativados por lipídios, que compõe a parede celular do *M. tuberculosis* (BRENNAN; NIKAIDO, 1995; KARAKOUSIS, P.C; BISHAI, W.R; DORMAN, S.E, 2004). A avaliação das respostas induzidas pelos lipídios da parede celular do *M. tuberculosis Δmce1* e selvagem sugerem que o *M. tuberculosis* usa os componentes lipídicos para imunomodular a resposta imunológica

orquestrada pelo hospedeiro. Portanto, os dados sugerem que os componentes lipídicos do *M. tuberculosis* está envolvido no desenvolvimento da TB ativa e latente. Se esse postulado estiver correto, podemos utilizar a expressão dos RNAL como marcadores para prever a reativação da TB. Diante disso, este estudo avaliou a expressão gênica dos RNAL e seus alvos em células de pacientes com TB ativa e latente, além de pacientes com outras doenças pulmonares com o intuito de identificar possíveis biomarcadores para TB.

Um total de 22 indivíduos foram selecionados com TB ativa, 14 com TB latente (LTBI) e 13 com outras doenças pulmonares (OPD). O estudo foi conduzido no Laboratório Avançado de Saúde Pública (LASP) do Centro de Pesquisas Gonçalo Moniz, Fiocruz (IGM/Fiocruz) e os pacientes foram identificados no Instituto Brasileiro de Investigação da Tuberculose (IBIT) da Fundação José Silveira (FJS) e no Centro de Saúde de Salvador Ramiro de Azevedo (2º Centro de Saúde). Nesses locais, contamos com uma equipe de médicos, enfermeiros e técnicos que auxiliaram na identificação, recrutamento e coleta de dados dos pacientes e de seus comunicantes.

Os pacientes incluídos no estudo com TB pulmonar tinham suspeita clínica de TB e um resultado positivo na pesquisa direta do bacilo álcool ácido resistente (BAAR) no escarro; e ou alterações sugestivas de TB na radiografia de tórax; ou ainda, teve diagnóstico presumido sem confirmação bacteriológica, mas com resposta ao tratamento, foi recrutado para o estudo. Os indivíduos com LTBI e controles saudáveis foram identificados e recrutados entre os comunicantes domiciliares dos pacientes com TB ativa. Todos aqueles que conviviam no mesmo domicílio ou que relataram, pelo menos, 100 horas de contato com o paciente bacilífero foram considerados como comunicantes domiciliares. Contudo, a presença da infecção pelo bacilo foi estabelecida pelo Ensaio de Liberação de IFN-γ (IGRA) através do kit comercial QuantiFERON® TB Gold in Tube. De acordo com dados anteriores de validação deste ensaio, um valor igual ou acima de 0,35 UI/mL de IFN- γ em resposta aos antígenos específicos presentes no kit foi considerado positivo, sugestivo de TBL e aqueles com resultado negativo para o IGRA foram considerados não infectados e alocados ao grupo HC. Foram excluídos de todos os grupos, indivíduos com evidência de TB extrapulmonar; indivíduos em uso de corticosteroides; coinfectados pelo vírus da imunodeficiência humana; menores de 18 anos e com resultados para detecção de TBL indeterminado.

Um volume de 2,5 mL de sangue total foi coletado por cada indivíduo em tubos *PAXgene blood RNA* (PreAnalytiX) e a extração do RNA foi feita com o *PAXgene blood RNA kit* (Qiagen). O cDNA foi sintetizado a partir do RNA extraído utilizando o Superscript III (Invitrogen-Invitrogen, Life

Technologies) de acordo com as instruções do fabricante. Inicialmente em 7 µl de RNA foi adicionado uma solução de 1 µl de DNTP e 1 µl de primers oligo (dt) e 1 µl de Random hexameres, totalizando uma solução de 10 µl. A solução de RNA foi incubada em um termociclador (Applied Biosystems®) a 65ºC por 5 minutos. Posteriormente foi adicionada nessa solução 2 µl de 10xRT buffer, 4 µlde 25mM MgCl2, 2 µl de 0,1 M dtt e 1 µl do Superscript III. Em seguida, esta reação foi incubada por10 minutos a 25°C, seguidos de 95 minutos a 50ºC para realização da transcrição e 5 minutos a 85°C. A Reação de RT-qPCR foi realizada baseado na incorporação do SYBR Green (Maxima SYBR Green/ROX qPCR Master Mix, 2, Thermo Fisher Scientif) de acordo com as instruções do fabricante. Para cada gene de interesse, foi utilizado 1,3 µl de cDNA em um mix de PCR contendo 2,1 µl do primer de interesse-*Foward* e 2,1 µl do primer de interesse-Reverse, 8,3 µl de água para PCR e 17,5 µl de SYBR Green, totalizando um mix de 30 µl. A reação foi incubada então no sistema de detecção Applied Biosystems® 7500 Real-Time PCR Systems, com a seguinte programação: 50ºC por 2 minutos (1 ciclo), seguido de 95ºC por 10 minutos (1 ciclo), 95ºC por 15 segundos (40 ciclos), 60ºC por 30 segundos (40 ciclos) e por fim 72ºC por 30 segundos (40 ciclos). Ao final da reação de amplificação, as amostras foram submetidas a uma nova incubação para geração da curva de dissociação, com a seguinte programação: 95ºC por 15 segundos, 60ºC por 1 minuto, 95ºC por 30 segundos e 60ºC por 15 segundos. A expressão dos genes foi normalizada pelo gene de referência *glyceraldehyde 3- phosphate dehydrogenase* (GAPDH). Foi realizada uma análise de PCR em tempo real (RT-qPCR) de genes de células sanguíneas conhecidos por serem induzidos por moléculas de lipídios, como PPAR-γ, TR4, LXR-α e os genes ativados pelos RNALs e relacionados com a expressão de citocinas inflamatórias como CD36, TNF-α, INF-γ, IL-1β e IL-6.

A identificação de biomarcadores que pudessem predizer a chance de um indivíduo infectado pelo *M. tuberculosis* adoecer poderia ser a chave para romper o ciclo de transmissão da TB. Além disso, um teste diagnóstico rápido e simples que pudessem identificar o indivíduo com TB ativa dentre aqueles que apresentam um quadro clínico similar por outras doenças poderia acelerar o diagnóstico correto da TB. Desta forma, a análise da expressão gênica de marcadores associados aos lipídios foram avaliadas entre os grupos com TB ativa, OPD e indivíduos saudáveis na tentativa de identificar um biomarcadores de diagnóstico (Figura 1) e entre indivíduos com TB ativa, TB latente e HC para identificar marcadores de reativação da TB (Figura 2).

A figura 1 mostra os níveis de expressão gênica dos RNAL e seus alvos entre os indivíduos com TB ativa, OPD e HC. Indivíduos com ODP apresentaram maior expressão de PPAR-γ (10,9 ± 1,1) do que indivíduos com TB ativa (10 ± 0.8; p< 0.05) e saudáveis (9.9 ± 0.7; p=0.02). A expressão gênica diferenciada entre TB ativa e ODP não foi observada em nenhum outro marcador. A expressão gênica de IL-6 (p = 0.0002), TNF-α (p = 0.0001) e INF-γ (p= 0.008) foram maiores em indivíduos com TB ativa em relação a indivíduos saudáveis.

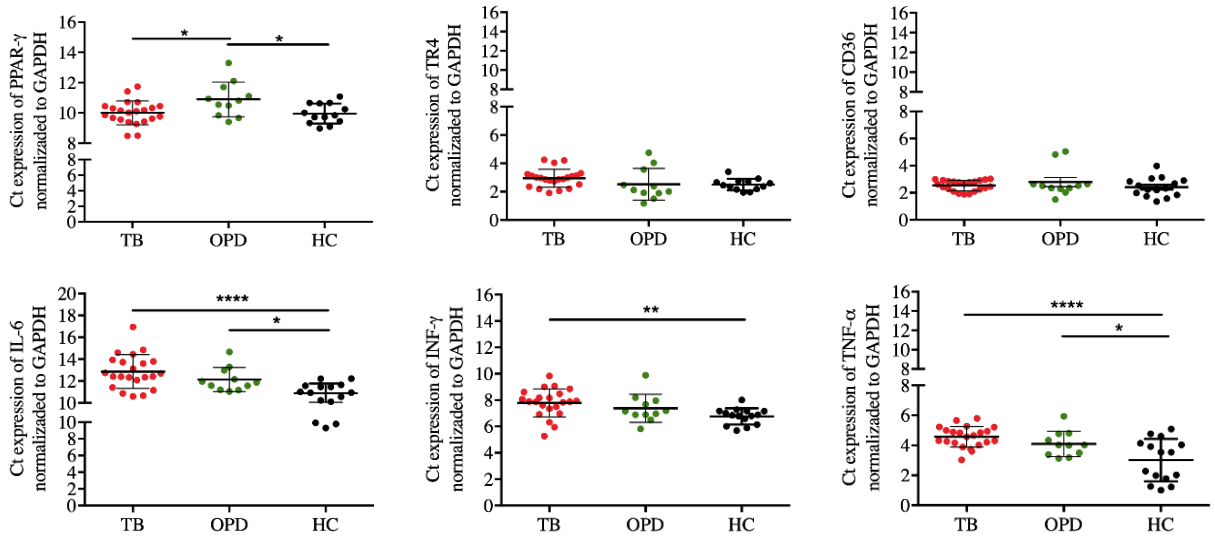


Figura1. Níveis de expressão dos RNALs e seus alvos analisados por RT-qPCR em células do sangue total de pacientes com TB ativa, OPD e HC. Os valores foram normalizados pelo gene de referência GAPDH e expresso em valores de Ct. Análise de variância, ANOVA, seguida do teste para múltiplas comparações de Turkey, foi aplicada para comparação entre os grupos e os valores estão representados em média e desvio padrão. TB= tuberculose ativa, OPD = outras doenças pulmonares; HC = indivíduos saudáveis; CT= ciclo de *threshold; GAPDH= glyceraldehyde 3-phosphate dehydrogenase.*

Quando avaliados a expressão gênica dos marcadores de predição da doença e diagnóstico de infecção pelo *M. tuberculosis* em indivíduos infectados pelo bacilo, com a forma ativa da doença

e indivíduos saudáveis, observa-se que as células de indivíduos LTBI expressam maiores níveis de CD36 (3,2 ± 1,1) quando comparados com indivíduos com TB ativa (2,5 ± 0.4) e saudáveis (2,4 + 0,7), p= 0.01. Os níveis de expressão de IL-6 foram aumentados em TB em relação ao LTBI (p< 0.001) e HC (p< 0.0001).

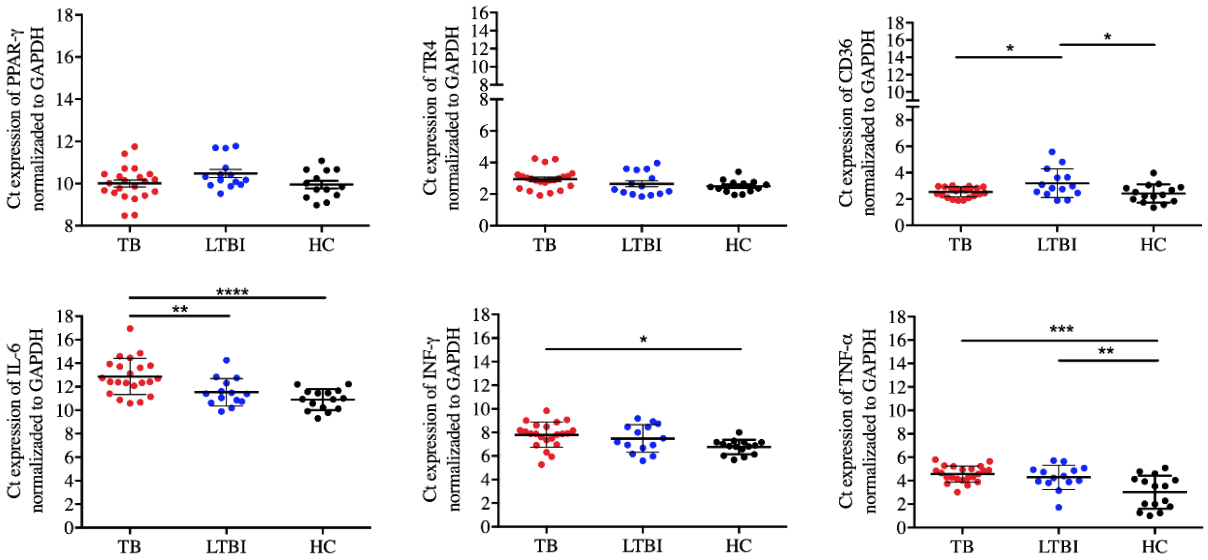


Figura 2. Níveis de expressão dos RNALs e seus alvos analisados por RT-qPCR em células do sangue total de pacientes com TB ativa, LTBI e HC. Os valores foram normalizados pelo gene de referência GAPDH e expresso em valores de Ct. Análise de variância, ANOVA, seguida do teste para múltiplas comparações de Turkey, foi aplicada para comparação entre os grupos e os valores estão representados em média e desvio padrão. TB= tuberculose ativa, LTBI = tuberculose latente; HC = indivíduos saudáveis; CT= ciclos de *threshold; GAPDH= glyceraldehyde 3- phosphate dehydrogenase.*

4.4 CAPÍTULO 4: WHOLE BLOOD mRNA EXPRESSION-BASED TARGETS TO DISCRIMINATE TUBERCULOSIS FROM OTHER PULMONARY DISEASES AND FROM HEALTHY LATENTLY INFECTED SUBJECTS

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Whole blood mRNA expression-based targets to discriminate tuberculosis from other pulmonary diseases and from healthy latently infected subjects

Jéssica D. Petrilli,[1] Luana E. Araújo,[1] Igor Müller,[1] , Luciane Sussuchi, [2] Carolina Laus, [2], Rui Manuel Reis [2], Sérgio Arruda,[1] Lee W. Riley,[3] Adriano Queiroz,[1].

[1] Laboratorio Avançado de Saúde Pública, Instituto Gonçalo Moniz, R. Waldemar Falcão, 121 - Candeal, Salvador, Brazil;

[2] Hospital de Amor, R. Antenor Duarte Viléla, 1331 - Dr. Paulo Prata, Barretos - SP, 14784-400

[3] Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, USA.

Introduction

Tuberculosis (TB), an aerosol-borne disease caused by *Mycobacteria tuberculosis* (Mtb), is one of the top 10 causes of death worldwide and the leading cause of death from a single infectious agent. About a quarter of the world's population is latently infected with Mtb and about 10% of these individuals will progress to have active TB disease during their lifetime [1]. Despite longstanding intense efforts to control this disease, tuberculosis remains an expanding global health crisis that mandates new diagnostics and preventative strategies.

Predict when the individuals infected by Mtb will develop active TB it is still a challenge. The identification of biomarkers that are differentially expressed between latent individuals and those with have the active disease becomes an important strategy to predict active TB and consequently reducing the risk of disease activation. In addition to predicting the activation of the disease, the development of tests to detect symptomatic individuals is extremely necessary since tuberculosis is a disease that presents symptoms that are similar and have association with others inflammatory chronic lung diseases [2,3]. A test can distinguish, quickly and efficiently, symptomatic infected individuals from those symptomatic with non-TB it is important during the initial screening.

The use of biomarkers as a tool for tuberculosis diagnosis has been widely used in several studies. An 86-gene whole-blood transcriptional Mtb signature - predominantly neutrophil-driven type 1 interferon was reported [4]. Fc gamma receptor 1B (FCGRIB) was identified as the most differentially expressed gene, and, in combination with four other markers, produced a high degree of accuracy in discriminating TB patients and latently infected donors, using the whole-blood microarray gene expression analyses [5]. A mechanism by which Mtb can limit macrophage responses to IFN-γ by altering host miRNA expression, using the NanoString technology was identified [6].

Recognizing the importance of identifying genes as possible biomarkers that can be used as signatures for disease progression and diagnosis, in this paper we report the results of an immune profile experiment using the NanoString technology in an cohort of individuals with active disease, latent infection, other pulmonary disease and healthy subjects. The choose of the nCounter gene expression system is because this tool allows a gene expression analysis system that is sensitive,

reproducible and simple to use [7]. Here, we identified 23 genes associated with inflammatory mechanisms that distinguish patients with active tuberculosis and others inflammatory diseases, seven genes that distinguish asymptomatic patients from those with active disease, and identify a possible marker (IL23R) that is differentially expressed in the population of latent individuals compared with healthy donors

Methods

Study participants:

Subjects were recruited between November, 2015 to December, 2016. Written informed consent was obtained from all participants. Our study included 17 patients with active tuberculosis (TB), seven healthy donors with latent *M. tuberculosis* infection (LTB), six healthy subjects (HC), and five patients suffering from other pulmonary diseases (OPD). All subjects were older than 18, and responded to a standardized questionnaire. All participants were recruited at the Brazilian Institute for Tuberculosis Research (IBIT), Bahia, Brazil. TB patients were confirmed to have pulmonary TB by chest x-ray, sputum smear microscopy and /or culture for BK positive. These samples were collected prior to TB treatment from patients without prior TB infection. LTB subjects were household contacts of TB patients and were tested for latent infection by the Interferon Gamma Release Assay (IGRA) test. Patients who were negative for tuberculosis but had similar symptoms were included in the OPD group.

RNA extraction: We collected 2.5 mL peripheral whole blood in a PAXgene blood RNA tube (PreAnalytiX) for every donor in each group. Total RNA was extracted with the PAXgene Blood RNA kit (Qiagen) for gene expression analysis using NanoString technology.

NanoString: Gene expression assay was carried out using NanoString technology, with nCounter Immunology Panel, which account to 594 targets and 15 internal reference genes. Up to 100ng of total RNA per sample was used and protocol was performed according to manufacture's recommendations. Briefly, RNA was hybridized with Reporter and Capture probes (NanoString Technologies) and incubated at 67°C for 21 hours. Samples were then loaded to automated nCounter Prep Station (NanoString, Technologies), to sample purification and immobilization in cartridge. Finally, cartridges were transferred to nCounter Digital Analyzer (NanoString Technologies), to image capture in 280 fields of view (FOVs) providing all gene counts.

Statistical Analysis: For Nanostring analysis, initially, the files corresponding to each cartridge were analyzed in nSolver Software (NanoString Technologies) to quality control assessment. Following, analyses were performed in R statistical environment 1. Distributions of raw counts were evaluated in quantro package 2. Normalization and differential expression were carried out with NanoStringNorm package 3. Raw data were normalized using the geometric mean of positive control and housekeeping genes. Hierarchical clustering with Pearson correlation coefficient distance of differentially expressed genes were performed on ComplexHeatmap package 4. The ability of genes discriminates the study groups was evaluated using receiver operating characteristic (ROC) curves. Chi-square test was used to assess associations among categorical variables. The level of statistical significance was set at $p < 0.05$.

Ethical statement: The study was approved by the Research Ethics Council (CEP) of the José Silveira Foundation (FJS), Brazilian Institute for Tuberculosis Research (IBIT), CAAE:

48844315.8.0000.5543. Following the basic norms of CEP, Resolution 466/12, all study participants were verbally and in writing informed about the objectives of the study, their form of participation and ways of contacting the study coordinator. All participants confirmed their agreement with the study by signing the Free and Informed Consent Form. The participation of all individuals was totally voluntary and confidential.

Results

Identification of differentially expressed genes between TB and Non-TB individuals

Here, we evaluated 594 inflammatory markers in the whole blood samples from 15 individuals diagnosed with active tuberculosis (TB), seven with latent tuberculosis infection (LTBI), five with other pulmonary lung diseases (OPD) and six healthy individuals. Of all genes, 46 genes were distinct among individuals with active TB, LTBI, OPD, and HC ($p < 0.001$). The levels of gene expression in the groups varied widely, but a homogeneous pattern of gene expression can be observed among individuals with active TB. Of all selected genes, only GZMK gene had a negative modulation on TB patients when compared to LTBI, OPD and HC individual. Therefore, this gene was able to distinguish individuals with active TB from other groups

Twenty-three genes are able to distinguish TB from ODP patients

Looking for targets for TB diagnosis we evaluated proinflammatory markers that could distinguish individuals with active TB and OPD. OPD patients generally show respiratory symptoms like TB patients but they are TB test negative. Thus, a marker that can distinguish symptomatic respiratory

individuals can be very useful for new TB diagnosis. The heatmap on figure 2A show 23 genes that differentiate TB and OPD patients. ($p<0.001$). The analyses of components (figure 2C) show that all 23 genes can be used for distinguish TB and OPD patients. Volcano plot analyses identify 10 top genes that can be used as a marker for TB diagnosis based on p value and fold change ratio (figure 2D).

IL23R are repressed on LTBI individual compared with healthy subjects.

Of all 594 inflammatory genes assessed, IL23R was identified as a putative marker for LTBI diagnosis (figure 2B). Relative to HC donors, LTBI donors had *IL23R gene* repressed ($p<0.001$). Also, the volcano plot showed based on p value and fold change ratio, 10 top genes that had different expression between both group and may be used for LTBI diagnosis (figure 2D).

Proinflammatory genes can be used to predict TB development.

Predicting the chances of an LTBI individual developing TB is one of the major challenges in TB control. Here, we evaluated proinflammatory markers that could undergo modulation during TB developing. Of all genes tested, the heatmap on figure 3A shows 7 genes that differentiate LTBI individuals and TB patients ($p<0.001$). The analyses of components (figure 3B) show the potential of these 7 genes to separate both groups and the Volcano plot analyses identify the top 10 genes that can be used as a marker for TB prediction based on p value and fold change ratio (figure 3C).

Analysis of Sensibility and Specificity for genes identified as targets for TB diagnoses and TB prediction.

ROC analysis was used to evaluated the individual discriminatory performance of all genes that showed p value less than 0.001 on heatmap for the study group's comparison. The values of area under the curve (AUC), sensibility, specificity and the optimal cut-off points are shown in Table 2. *CD274, CEACAM1, CR1, FCGR1A/B, IFITM1, IRAK3, LILRA6, MAPK14, PDCD1LG2* expression were the best markers to differentiate TB and OPD donors with an AUC of 1.00 *and CCR2 expression was the best marker to TB progression with* an AUC of 1.00.

Discussion

Transcriptomic has been a promise for TB diagnosis and mostly to TB predict monitoring. Predicting the chance that a LTBI individual for developing TB may be the key to broking the TB transmission cycle. Tests that diagnose TB by serology are not recommended by the WHO, thus gene expression analysis becomes an attractive option [1]. Studies have identified genes that can discriminate TB patient from Non-TB patients and TB risk [4,5,8–10]. However, here we identified inflammation targets that can distinguish TB and OPD individuals and possibly indicate a reactivated infection. Blood transcriptomic immune biomarkers were identified through NanoString, a multiplex gene expression that performed more than 200 inflammation genes analysis.

TB and OPD patients present similar symptom when arrived to health care system and mostly of them start TB treatment even with negative tests for TB [1]. Thus, find markers for TB detection becomes important in this scenario. In this study, 23 genes associated with inflammatory mechanisms that discriminate patients with TB and OPD were identify. The genes showed a higher

sensibility and specificity after validation test which showed the potential of these genes as targets for TB diagnosis. Other authors have already highlighted some of these genes [4]. The genes that had a AUC of 1.00 in the ROC analysis to discriminate TB and OPD donors are related mainly with adaptive immune response and lymphocyte activation. Further analysis may show the potential of these genes together serving as a signature for the development of TB diagnostic tests.

In addition to the demand for TB diagnosis biomarkers, we also screened a set of inflammatory genes that be able to discriminate LTBI individuals from health subjects. IL23R were repressed in blood cells of LTBI individuals and the LTBI 1 subject was the only one diagnosed with LTBI that have IL23R expressed in his cells. These data demonstrate that this patient may have been misdiagnosed through the QTF test, a test that quantify the INF-y production by blood cells after stimulation with specific proteins from Mtb. In addition, it is possible to speculate the importance of $TCD4^{+}$ cells polarization in Th17 profile during the course of infection and possibly assisting in defense against the bacillus since IL23R are associated with cytokine signaling, lymphocytes activation and IL-17 differentiation.

A major problem that hinders the interruption of the TB transmission cycle is the difficulty in predicting when an individual with LTBI will develop active TB. For this purpose, seven genes were suppressed in LTBI donors being able to discriminate TB from LTBI individual. These genes are associate with inflammatory mechanisms and showed to be a good target for TB predicting after analysis of sensibility and specificity. We can observe the potential of these genes to predict TB development in Table 5, once they are associated with innate immune response and activation of adaptive immune response thought T cell activation. Only one donor with TB have five genes repressed differently of other TB patients. It is possible that this donor has become TB recently and

beyond that may be a indicative that the first genes that become positive of this set of genes are MSR1 and MAPK14 in TB patients. Therefore, it is necessary the evaluation of these gene as signature to predict which of those with LTBI have better chances to development active TB.

The current TB diagnostic test used in the medical routine is a milestone for TB, however the test has significant limitation. Although, the GeneXpert MTB/RIF test offers a fast result for active TB with bacterial resistance screened, the test can be a challenge for TB diagnoses of children and the elderly due to difficulties of these groups to spitting. Also, The GeneXpert MTB/RIF requires a sophisticated technology and a well-trained staff, thus not being a democratic alternative to all health systems. Therefore, a simpler and cheaper test for TB diagnosis is necessary. The search for targets has grown greatly in an attempt to find a biomarker that can be used to diagnose active TB and even predict TB development. Currently, there is no test that can predict the development of TB in those who are infected with Mtb, which makes this searching for biomarker for TB prediction very important when thinking about the eradication of TB.

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Tables

Table 1. Validation targets to TB diagnosis

Gene	ROC AUC (95% CI)	ROC p-value	Sensitivity % (95% CI)	Specificity % (95% CI)	Cut-off
C1QA	0.98 (0.95-1.02)	< 0.0001	88.24 (63.56-98.54)	100 (66.37-100)	> 3.74
CD274	1.00	<0.0001	100 (80.49-100)	100 (66.37-100)	> 5.71
CD59	0.98 (0.95-1.02)	< 0.0001	94.12 (71.31-99.85)	100 (66.37-100	> 8.63
CEACAM1	1.00	< 0.0001	100 (80.49-100)	100 (66.37-100)	< 6.02
CR1	1.00	< 0.0001	100 (80.49-100)	100 (66.37-100)	> 10.02
FCGR1A/B	1.00	< 0.0001	100 (80.49-100)	100 (66.37-100)	> 7.83
ICAM1	0.98 (0.95-1.02)	< 0.0001	88.24 (63.56-98.54)	100 (66.37-100)	> 7.54
IFITM1	1.00	< 0.0001	100 (80.49-100)	100 (66.37-100)	> 12.86
IL18RAP	0.98 (0.95-1.02)	< 0.0001	88.24 (63.56-98.54)	100 (66.37-100)	> 8.97
IL4R	0.98 (0.95-1.02)	< 0.0001	94.12 (71.31-99.85)	100 (66.37-100	> 9.08
IRAK3	1.00	< 0.0001	100 (80.49-100)	100 (66.37-100)	> 6.86
JAK2	0.99 (0.97-1.01)	< 0.0001	94.12 (71.31-99.85)	100 (66.37-100)	> 9.86
JAK3	0.99 (0.97-1.01)	< 0.0001	94.12 (71.31-99.85)	100 (66.37-100	> 8.59
LILRA5	0.97 (0.92-1.02)	< 0.0001	88.24 (63.56-98.54)	100 (66.37-100)	> 9.87
LILRA6	1.00	< 0.0001	100 (80.49-100)	100 (66.37-100)	> 7.79
LY96	0.99 (0.97-1.01)	< 0.0001	94.12 (71.31-99.85)	100 (66.37-100)	> 7.69
MAPK14	1.00	0.0008757	100 (80.49-100)	100 (47.82-100)	> 10.03
NOD2	0.92 (0.81-1.04)	0.004259	82.35 (56.57-96.20)	100 (47.82-100)	> 8.79
PDCD1LG2	1.00	0.0008757	100 (80.49-100)	100 (66.37-100)	> 4.13
PML	0.98 (0.95-1.02)	0.001156	94.12 (71.31-99.85)	100 (47.82-100)	> 7.72
SOCS3	0.96 (0.88-1.04)	0.001981	94.12 (71.31-99.85)	100 (47.82-100)	> 7.40
TAP1	0.97 (0.92-1.03)	0.001518	88.24 (63.56-98.54)	100 (47.82-100)	> 8.63
TNFAIP6	0.98 (0.95-1.02)	0,001156	94.12 (71.31-99.85)	100 (47.82-100)	> 6.02

Table 2. Validation targets to TB progression

Gene	ROC AUC (95% CI)	ROC p-value	Sensitivity % (95% CI)	Specificity % (95% CI)	Cut-off
C1QB	0.97 (0.92-1.02)	0.0003360	88.24 (63.56-98.54)	100 (59.04-100)	> 4.15
C2	0.92 (0.82-1.02)	0.001348	82.35(56.57-100)	100 (59.04-100)	> 4.16
CCR2	1.00	0.0001594	100 (80.49-100)	100 (59.04-100)	> 8.61
CCRL2	0.86 (0.71-1.01)	0.005753	82.35 (56.57-96.20)	85.71 (42.13-99.64)	> 5.31
LILRB4	0.94 (0.82-1.05)	0.0008613	94.12 (71.31-99.85)	100 (59.04-100)	> 6.56
MAPK14	0.95 (0.88-1.03)	0.0005420	88.24 (63.56-98.54)	100 (59.04-100)	> 10.54
MSR1	0.99 (0.96-1.01)	0.0002051	94.12 (71.31-99.85)	100 (59.04-100)	> 4.72

Table 3. Targets information for important genes based on ROC curve analysis

Gene Symbol	Gene Name	Annotation gene	Target for
C1QA	Complement C1q A chain	Complement system, Host pathogen interaction, Innate immune system.	TB diagnosis
C1QB	Complement C1q B chain	Complement system, Host pathogen interaction, Innate immune system.	TB progression
C2	Complement component 2	Complement system, Host pathogen interaction, Innate immune system.	TB progression
CCR2	C-C chemokine receptor type 2	Chemokine signaling, Cytokine signaling, Innate immune system, Lymphocyte activation	TB progression
CCRL2	C-C chemokine ligand type 2	Chemokine signaling	TB progression
CD59	CD59 molecule	Complement system, Innate immune system, Lymphocyte activation.	TB diagnosis
CD274	CD274 molecule	Adaptive immune system, Cell Adhesion, Lymphocyte Activation,	TB diagnosis
CEACAM1	CEA Cell Adhesion Molecule 1	Hemostasis, Innate immune system and Lymphocyte activation.	TB diagnosis
CR1	Complement Receptor Type 1	Complement system, Host pathogen interaction, Innate immune system.	TB diagnosis
FCGR1A/B	Fc Fragment of IgG Receptor Ia	Adaptive immune system, Cytokine signaling, Host pathogen interaction, Innate immune system, MHC class I Antigen presentation, Phagocytosis and degradation and Type II interferon signaling.	TB diagnosis
ICAM1	Intercellular Adhesion Molecule 1	Adaptive immune system, Cell Adhesion, Innate immune system, Lymphocyte Activation.	TB diagnosis
IFITM1	Interferon Induced Transmembrane Protein 1	Adaptive immune system, B cell receptor signaling, Cytokine signaling and Type I interferon signaling.	TB diagnosis
IL18RAP	Interleukin 18 Receptor Accessory Protein	Cytokine signaling, Oxidative stress,	TB diagnosis
IL23R	Interleukin 23 Receptor	Cytokine signaling, Lymphocyte activation and Th17 differentiation.	LTBI diagnosis

IL4R	Interleukin 4 Receptor	Cytokine signaling, Lymphocyte activation and Th2 differentiation	TB diagnosis
IRAK3	Interleukin 1 Receptor Associated Kinase 3	Cytokine signaling, Innate immune system and TLR signaling.	TB diagnosis
JAK2	Janus kinase 2	Chemokine signaling, Cytokine signaling, Host pathogen interaction, Hemostasis, Oxidative stress, Th1 and Th17 differentiation, Type II interferon signaling.	TB diagnosis
JAK3	Janus Kinase 3	Chemokine signaling, Cytokine signaling, Host pathogen interaction, Hemostasis, Lymphocyte activation, Th2 and Th17 differentiation.	TB diagnosis
LILRA5	Leukocyte immunoglobulin like receptor A5	Adaptive immune system	TB diagnosis
LILRA6	Leukocyte immunoglobulin like receptor A6	Adaptive immune system and MHC class I antigen presentation	TB diagnosis
LILRB4	Leukocyte immunoglobulin like receptor B4	Adaptive immune system	TB progression
LY96	Lymphocyte Antigen 96	Adaptive immune system, Apoptosis, Host pathogen interaction, Innate immune system, MHC class I Antigen presentation, NF-κB signaling, TLR signaling.	TB diagnosis
MAPK14	Mitogen-Activated Protein Kinase 14	Cytokine signaling, Hemostasis, Host pathogen interaction, Innate immune system, Lymphocyte trafficking, NLR signaling, T cell receptor signaling, Th17 differentiation, TNF family signaling and TLR signaling.	TB diagnosis and TB progression
NOD 2	Nucleotide Binding Oligomerization Domain Containing 2	Cytokine signaling, Host pathogen interaction, Innate immune system, Lymphocyte activation, NLR signaling, TNF family signaling, TLR signaling.	TB diagnosis
MSR1	Macrophage Scavenger Receptor 1	Phagocytosis and degradation	TB progression
PDCD1LG2	Programmed Cell Death 1 Ligand 2	Adaptive immune system, Cell adhesion and Lymphocyte activation.	TB diagnosis
PML	Promyelocytic Leukemia	Cytokine signaling, Host pathogen interaction, Oxidative stress, Type II interferon signaling.	TB diagnosis

SOCS3	Suppressor of Cytokine Signaling 3	Adaptive immune system, Cytokine signaling, Host pathogen interaction, MHC class I Antigen presentation, TNF family signaling, Type I interferon signaling and Type II interferon signaling.	TB diagnosis
TAP1	Transporter 1, ATP Binding Cassette Subfamily B Member	Adaptive immune system, Host pathogen interaction, MHC class I Antigen presentation, Phagocytosis and degradation.	TB diagnosis
TNFAIP6	Tumor Necrosis Factor Alpha Induced Protein 6	Innate Immune system.	TB diagnosis

TB: Tuberculosis, LTBI: Latent tuberculosis infection

Figures

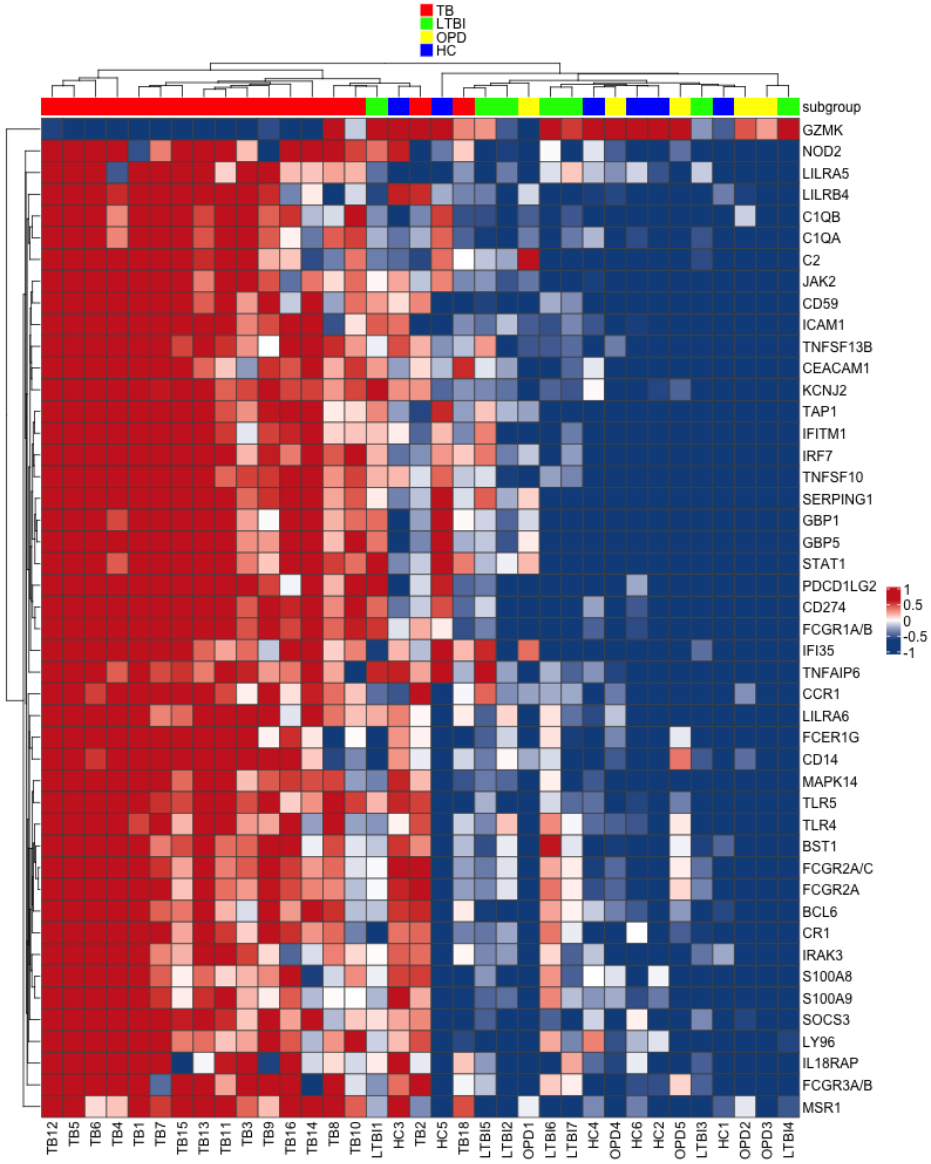

Figure 1. Heatmap showing different expression pattern of 46 proinflammatory genes out of 549 genes. Heatmap of gene expression levels in PBMC of individuals diagnosed with active TB (red), LTBI (green), OPD (yellow) and HC (blue) subjects. Dark red (+1) indicates 2-fold upregulation from the mean (white) and dark blue (−1) indicates a 2-fold downregulation from the mean.

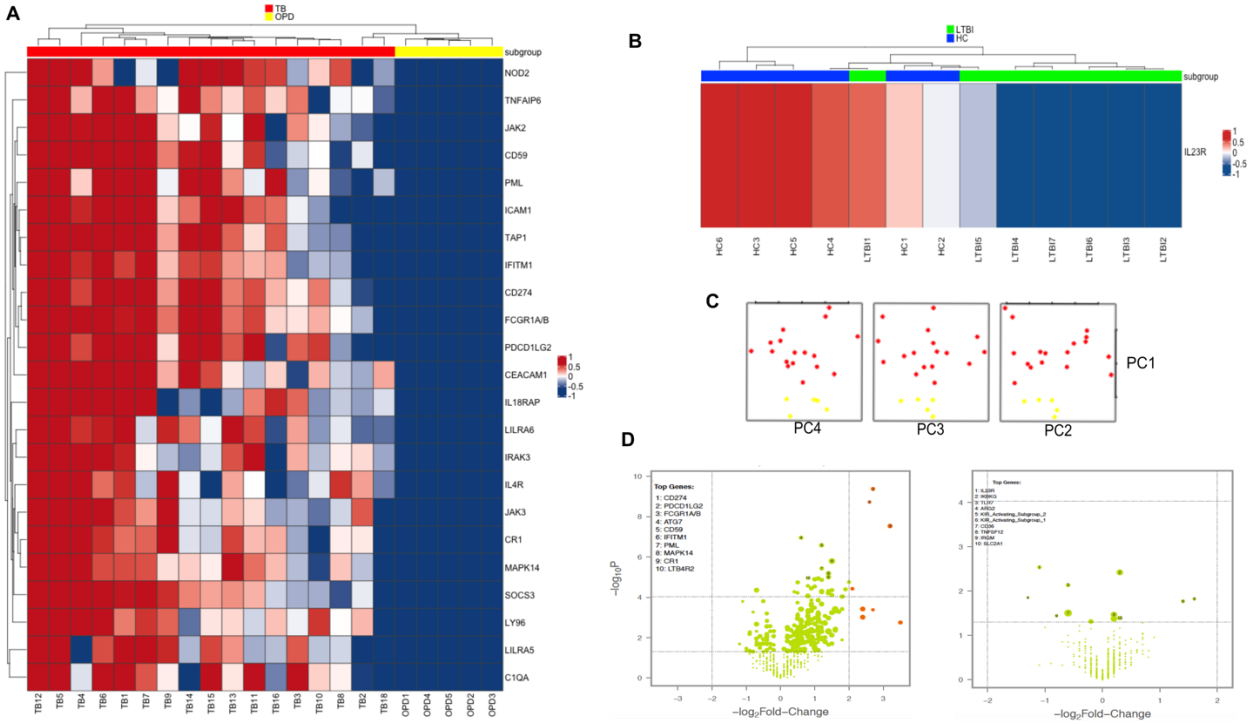

Figure 2. Identification of markers for TB and LTBI diagnosis. *(A)* Heatmap of 23 genes expression levels of TB (red) and OPD (yellow) patients. *(B)* Heatmap of IL23R expression level of LTBI (green) and HC (blue) individuals. Dark red (+1) indicates 2-fold upregulation from the mean (white) and dark blue (−1) indicates a 2-fold downregulation from the mean. *(C)* PCA score plot of TB and OPD patients. *(D)* Volcano plots showing the distribution of the gene expression fold changes in TB patients relative to OPD patients. *(E)* Volcano plots showing the distribution of the gene expression fold changes in LTBI subjects relative to HC subjects. Genes with absolute fold change ≥2 and p-value ≤ 0.05 are indicated in orange.

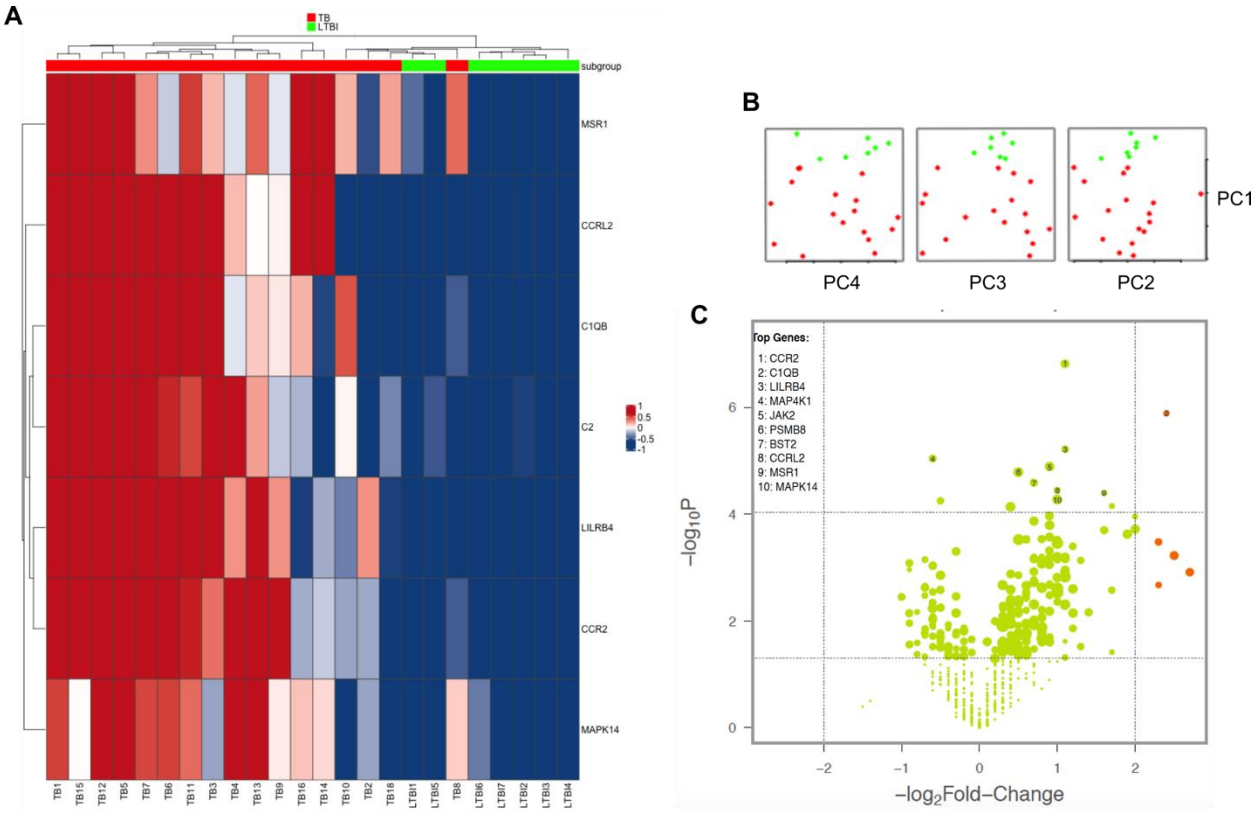

Figure 3. Identification of markers for TB and LTBI diagnosis. *(A)* Heatmap of seven genes expression levels of TB (red) and LTBI (green) subjects. Dark red (+1) indicates 2-fold upregulation from the mean (white) and dark blue (−1) indicates a 2-fold downregulation from the mean. *(B)* PCA score plot of TB and LTBI subjects. *(C)* Volcano plot showing the distribution of the gene expression fold changes in TB patients relative to LTBI. Genes with absolute fold change ≥2 and p-value ≤ 0.05 are indicated in orange.

5 DISCUSSÃO

Os estudos envolvendo a cepa do *M. tuberculosis Δmce1* datam desde 1993 quando Arruda e colaboradores identificaram o gene mce1 (ARRUDA et al., 1993). Após o sequenciamento do gene completo da cepa selvagem H37Rv do *M. tuberculosis* (COLE et al., 1998), o *operon mce1* virou alvo de estudos sobre a patogênese da TB (CASALI; WHITE; RILEY, 2006; CHEIGH et al., 2010, p. 1; EL-SHAZLY et al., 2007; LIMA et al., 2007; SHIMONO et al., 2003; UCHIDA et al., 2007). Sendo o granuloma um fenômeno importante da TB, estudos revelaram que a cepa *Δmce1* forma granuloma desorganizado, o que sugere que o controle da expressão desse operon está associado com a persistência intracelular do bacilo (CASALI; WHITE; RILEY, 2006; CHEIGH et al., 2010; QUEIROZ et al., 2015). Dessa forma, este trabalho buscou avaliar a resposta induzida *in vitro* pelo *M. tuberculosis Δmce1* e pelo extrato apolar de lipídios da sua parede celular.

É a primeira vez, pelo nosso conhecimento, que um modelo de tecido humano in vitro é descrito, utilizando *M. tuberculosis* com interrupção no *operon mce1*, para avaliação da formação do granuloma. Nosso modelo demonstrou que o *M. tuberculosis* induz a formação de um granuloma estável no sétimo dia pós-infecção, em que se apresenta, compacto, organizado e sem a presença de bactérias extracelulares. Embora as culturas com células não infectadas apresentem formação de alguns agregados celulares, o número de agregados é menor comparados com as células infectadas pelo bacilo, o que demonstra que o microrganismo é o grande responsável pela formação dos granulomas. Estudo prévio realizado por Delcroix e colaboradores, com a cepa BCG, demonstraram que os granulomas começam a se romper com 15 dias pós infecção. Diante disso, nós acompanhamos a formação do granuloma após infeção com a cepa selvagem e a cepa com interrupção no *operon mce1* do *M. tuberculosis*. As duas cepas apresentaram número e tamanhos similares de granulomas com 4 dias de infeção o que demonstrou que as duas cepas, inicialmente, são capazes de estimular a resposta imunológica e induzir a formação do granuloma. No entanto, no decorrer dos dias, observa-se que os granulomas induzidos pela cepa selvagem são capazes de manter sua estrutura, diferente da cepa com interrupção no *operon mce1*, em que os granulomas diminuem não só em números como em tamanho. Estudos anteriores em modelo animal demonstram essa capacidade reduzida da cepa com interrupção no *operon mce1* em controlar a infecção (LIMA et al., 2007; UCHIDA et al., 2007). Camundongos infectados pela cepa mutante no *operon mce1* morriam

precocemente justamente pela incapacidade de controlar a infecção através da formação do granuloma evidenciada nas análises de tecido pulmonar. Nossos achados *in vitro* corroboram com os achados em modelo animal e foi possível observar que as culturas com *M. tuberculosis* com interrupção no *operon mce1*, após 11 dias de infecção, apresentam granulomas em processo de rompimento. Embora a reativação da TB seja um cenário ainda pouco explicado e sabe-se que esse fenômeno é um conjunto de múltiplos fatores, os dados sugerem que *operon mce1* está associado com a capacidade do *M. tuberculosis* em persistir no hospedeiro e, portanto, parece desempenhar um importante papel na fase de reativação da doença.

Outros estudos demonstram que a cepa com interrupção no *operon mce1* acumula ácidos micólicos na sua parede celular (CANTRELL et al., 2013; FORRELLAD et al., 2014) e além disso, a cepa apresenta níveis reduzidos de alguns lipídios que são reconhecidamente importantes na imunopatogênese da TB, como os sacarolipídios e os glicerofosfolipídios (QUEIROZ et al., 2015). Baseado nisso, investigamos se esses conjuntos de lipídios poderiam estar associados com a modulação da resposta inflamatória, o que reforçaria a nossa hipótese de que o *M. tuberculosis* está associado com o desenvolvimento da fase latência.

Macrófagos desempenham um importante papel no controle da TB, uma vez que são as primeiras células a entrarem em contato com o bacilo no pulmão. Assim, a forma que essas células são ativadas pode ser importante no curso da doença. Nossos achados revelaram que o conjunto de lipídios da cepa selvagem e da cepa mutante tem capacidade distintas de estimular essas células. Os lipídios da cepa *Δmce1* induzem uma resposta menos inflamatória através da expressão reduzida de IL-6 e TNF-α, ao contrário da cepa selvagem. Esses dados corroboram com Eoh e colaboradores que demonstrou que a hipóxia reduz a expressão de TDM e, portanto, os macrófagos infectados têm uma capacidade reduzida de secretar IL-12p40 e TNF. Em um estudo prévio, uma análise de cromatografia de camada fina revelou que a cepa com interrupção no *operon mce1* apresenta níveis reduzidos de TDM em relação a cepa selvagem (dados não publicados). Casali e colaboradores associaram o ambiente de hipóxia à repressão do *operon mce1* pelo seu regulador mce1R (CASALI; WHITE; RILEY, 2006). Nossos dados reforçam os achados desses estudos, já que foi demonstrado que o conjunto de lipídios da cepa mutante favorece a persistência do bacilo pela capacidade reduzida de estimular a resposta inflamatória do hospedeiro.

A associação dos lipídios da cepa com interrupção no *operon mce1* com a persistência intracelular do bacilo foi evidenciada através das análises de expressão dos RNAL. A cepa mutante, em relação a cepa selvagem, induziu a expressão dos genes PPAR-γ, TR4 e RAR que estão associados com a sobrevivência dos bacilos dentro de macrófagos através da produção de

IL-10 e inibição da maturação do fagolisossoma (CANTRELL et al., 2013; EOH et al., 2017). Além disso, os ligantes do PPAR-γ inibem a produção de TNF-α, IL-6 e IL-1β de monócitos e a atividade transcricional do fator de transcrição pró-inflamatório NF-kB no macrófago (ALMEIDA et al., 2012a), corroborando com nossos achados. Portanto, podemos especular que os lipídios da cepa mutante modulam a resposta inflamatória de macrófagos ao longo de sua interação com o PPAR-γ e, possivelmente, com TR4 e RAR. Importante lembrar que os ácidos micólicos são detectados pelo TR4 e isso pode explicar os níveis aumentados desse gene na cepa com interrupção no *operon mce1* que tem maiores níveis desse lipídio comparado com a cepa selvagem.

O comportamento pró-inflamatório apresentado pelos macrófagos induzidos por lipídios WT, em relação ao controle não tratado, levou a hipotetizar que esse lipídio aumentaria a expressão de ambos os subconjuntos de monócitos $CD16^+$ e HLA-DR II. No entanto, os dados demonstraram que os lípidos apolares micobacterianos reprimem a expressão do MHC de classe II nos monócitos $CD16^+$. Essa inibição foi documentada por Pancholi e colaboradores (1993), em que demonstraram que os monócitos expostos a *M. bovis* da BCG por setes dias (assemelhando-se a infecção crônica) expressaram quantidades anormais de HLA-DR de classe II sugerindo que que o bacilo não estaria apresentando antígenos bacterianos viáveis para células T, o que ocorreu com dois dias de cultura (infecção aguda) (PANCHOLI et al., 1993). Além disso, ambos os extratos lipídicos, WT e Δmce1, modularam o HLA-DR classe II de maneira similar, sugerindo que o rearranjo lipídico da parede celular não parece influenciar a expressão do MHC classe II. Em conjunto, todos esses dados sugerem que a modulação da apresentação de antígenos é um processo micobacteriano que não depende especificamente dos lipídios em si ou do rearranjo lipídico. Paradoxalmente, a BCG do M. bovis bloqueia a apresentação do antígeno apenas durante a infecção crônica (PANCHOLI et al., 1993), sugerindo que esta bactéria sofre alterações durante a infecção que seriam responsáveis por essa repressão. Novos estudos devem ser abordados para identificar o papel dos lipídios e outras características moleculares bacterianas no controle da apresentação de antígenos durante infecções agudas e crônicas.

Uma vez que ficou demonstrado que os lipídios do *M. tuberculosis* determina a função dos macrófagos, nos perguntamos se esse fenômeno iria ser extrapolado para a ativação das células T. Os lipídios podem ser apresentados as células T e células NK através da família CD1 (CD1a – CD1e) (BARRAL; BRENNER, 2007) presente nas células apresentadoras de antígeno (BECKMAN et al., 1994; CAMBIER et al., 2013; PORCELLI; MORITA; BRENNER, 1992). Os resultados demonstram que os lipídios do *M. tuberculosis* induziram a produção de INF-γ-,

TNF-α- and IL-10 pelas células T DN e não por células T $CD4^+CD8^+$. Esperava-se que os lipídios da cepa *Δmce1* induzissem níveis menores de citocinas inflamatórias, no entanto a cepa *Δmce1* induziu maiores níveis de INF-γ- and IL-10 e menores níveis de TNF-α em relação a cepa selvagem. Isso pode ser explicado através das subpopulações de células T que os lipídios estão estimulando. Autores demonstraram que as células T DN αβ apresentaram maiores frequências de células produtoras de IFN-γ e TNF-α, enquanto os linfócitos γδ DN T apresentaram aumento da frequência de IL-10 após indução com leishmania solúvel (ANTONELLI et al., 2006). Embora não tenhamos determinado quais subconjuntos de população de células T DN respondem a cada extrato lipídico utilizado, pode-se especular que os lipídios da cepa selvagem induziriam a produção de ambos IFN-γ e TNF-α através de células T αβ DN enquanto os lipídios Δmce1 ativa as células T γδ DN produtoras de IL-10 como mecanismos imunoreguladores dependentes de linfócitos.

Os estudos de caracterização da resposta imune permitem uma melhor compreensão dos mecanismos de patogênese do microrganismo e assim e da resposta orquestrada pelo hospedeiro. A partir destes estudos, o campo para identificação de biomarcadores é ampliado. Devido a composição rica de lipídios da parede celular do *M. tuberculosis*, os lipídios e as moléculas que reconhecem essas estruturas, se tornaram alvos na identificação de biomarcadores na TB. Os níveis de expressão de PPAR-γ, IL-6 e TNF se apresentaram distintos entre os indivíduos com TB, ODP e saudáveis sugerindo um papel importante como marcador que consiga distinguir aqueles indivíduos com TB ativa e ODP. Esses indivíduos chegam ao ambiente ambulatorial com sinais e sintomas semelhantes e muitas vezes pacientes com ODP acabam iniciando tratamento para TB devido aos sinais e sintomas, mesmo com testes diagnósticos para TB negativo, como preconizado pelo Ministério da saúde. Um teste que pudesse confirmar a exclusão de TB seria imprescindível para evitar o tratamento desnecessário que muitas vezes traz diversos efeitos colaterais devido a toxicidade das drogas e resistência bacteriana em infeções por *M. tuberculosis* no futuro.

A TB é um grave problema de saúde pública e por isso, um grande desafio para os órgãos de saúde na tentativa de interromper o ciclo de transmissão do bacilo. Esse desafio se torna ainda mais difícil por não ser possível predizer quando um indivíduo infectado pelo *M. tuberculosis* irá desenvolver a forma ativa da doença. A molécula CD36 e a IL-6 demonstraram ser um alvo importante como biomarcador para progressão da doença já que as expressões gênicas dessas moléculas se apresentaram distintas entre os indivíduos com TB ativa, LTBI e saudáveis. Embora a capacidade da resposta imunológica do hospedeiro contra o bacilo,

determinantes sociais e ambientais e doenças imunossupressoras, principalmente o HIV, possam estar associados a progressão da fase latente para forma ativa da TB, a maioria desses quadros ocorrem em indivíduos sem quaisquer condições claras associadas ao hospedeiro. O marco da TB é a capacidade do bacilo persistir no hospedeiro e cada vez mais esse fenômeno parece estar associado as características do próprio microrganismo. Portanto, mais estudos precisam ser realizados para uma melhor compreensão dos mecanismos de patogênese do bacilo e assim auxiliar na identificação de biomarcadores para TB, melhorando os diagnósticos e os alvos terapêuticos para o combate a doença.

6 CONCLUSÃO

O *M. tuberculosis Δmce1* e os seus lipídios da parede celular parecem estar envolvidos diretamente na resposta inflamatória orquestrada pelo hospedeiro, favorecendo desta forma a sua persistência. Uma vez que a cepa *Δmce1* apresenta o fenótipo do *M. tuberculosis* durante a fase mais tardia da doença, o conjunto de lipídios expresso na parede celular do bacilo com o fenótipo da cepa *Δmce1* parece desempenhar um importante papel na patogênese da TB. Esses dados auxiliam na compreensão dos mecanismos envolvidos na patogênese da doença e assim, servem de ferramenta para estudos que buscam identificar biomarcadores para melhorar as opções de diagnóstico, tratamento e prevenção da TB.

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APÊNDICE

Durante o desenvolvimento do doutorado, foi possível publicar um artigo científico na revista indexada The International Journal of Tuberculosis and Lung Diseases. O artigo científico intitulado "*Serum antiphospholipid antibody levels as biomarkers for diagnosis of pulmonary tuberculosis patients*" que avaliou as imunoglobulinas anti-fosfolipídios como biomarcadores para diagnóstico da TB pulmonar e monitoramento da eficácia do tratamento contra a TB.

Serum antiphospholipid antibody levels as biomarkers for diagnosis of pulmonary tuberculosis patients

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Serum antiphospholipid antibody levels as biomarkers for diagnosis of pulmonary tuberculosis patients

I. Takenami,* C. C. de Oliveira,* J. D. Petrilli,* A. Machado Jr,†‡ L. W. Riley,§ S. Arruda*†

*Laboratório Avançado de Saúde Pública, Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Bahia, †Departamiento de Ciências da Vida, Universidade Estadual de Bahia, Salvador, Bahia, ‡Hospital Especializado Octávio Mangabeira, Secretaria da Saúde do Estado da Bahia, Salvador, Bahia, Brazil; §Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, California, USA

SUMMARY

SETTING: Salvador, Bahia, Brazil.

OBJECTIVE: To evaluate the immunoglobulin (Ig)M and total IgG antibody response to cardiolipin (CL), phosphatidylcholine (PTC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and sulfatide (SL-I) as biosignatures that can be used to diagnose pulmonary tuberculosis (TB) and its applicability for monitoring the efficacy of anti-tuberculosis treatment.

DESIGN: Serum samples from 37 adult pulmonary TB patients and 48 controls (16 healthy household contacts, 19 household contacts with latent tuberculous infection [LTBI] and 13 non-TB patients with lung disease) were screened using enzyme-linked immunosorbent assays (ELISAs) for IgM and total IgG against phospholipids.

RESULTS: Levels of IgM response to CL, PE and PI, and IgG response to CL, PE, PI and PTC were significantly higher in TB patients than in control groups. Anti-CL IgG had the best performance characteristics, with a sensitivity and specificity of respectively 86.5% and 87.2%. This IgG anti-CL ELISA test detected 86.5% (32/37) of the TB patients, whereas the number detected using sputum smear was only 65.9% (24/37). After anti-tuberculosis treatment, the median value for all anti-phospholipid antibodies decreased significantly compared with baseline values (P , 0.05).

CONCLUSION: Our results suggest that the total IgG anti-CL level could be useful to complement conventional bacteriological tests for the rapid diagnosis of adult pulmonary TB.

KEYWORDS: cardiolipin; phosphatidylcholine; phosphatidylethanolamine; phosphatidylinositol; sulfatide

MYCOBACTERIUM TUBERCULOSIS kills approximately 2 million people each year and is estimated to latently infect one third of the world's population.[1] The early diagnosis and adequate treatment of pulmonary tuberculosis (PTB) patients are considered essential to reduce transmission of *M. tuberculosis* and to achieve the World Health Organization's (WHO's) End TB goals.[2] The development of a real-time polymerase chain reaction assay for the detection of *M. tuberculosis* DNA and the mutations associated with resistance to rifampicin has been the most important advance in TB diagnostics in the last few years.[3] However, the need for sophisticated laboratory infrastructure and highly skilled laboratory technicians remains a major barrier to the test's introduction in low- and middle-income countries.[4] Furthermore, the test does not eliminate the need for conventional tests, which are required to monitor response to treatment and relapse status.

Alternatives to traditional methods designed to directly detect *M. tuberculosis* are serological tests. Serological tests are simple, economical and minimally invasive, and can be used to diagnose TB smear-negative patients. However, the WHO has not endorsed serological testing based on targeting *M. tuberculosis* proteins due to their low sensitivity and specificity.[5,6]

The mycobacterial cell wall has unique features consisting of various lipids comprising ~40% of the cell wall dry weight. These lipids can behave as antigens capable of stimulating specific B-cells to produce immunoglobulins (Igs).[7] They have gained vital importance in recent years mainly due to modern approaches of lipidomic analysis. IgG- or IgM-mediated responses against mycobacterial phospholipids may therefore constitute a clinically useful tool for presumptive diagnosis and discrimination of PTB from other pulmonary diseases.[8,9] Lipids abundant in *M. tuberculosis* include lipoarabinomannan (LAM), mycolic acids, phenolic glycolipids, polyacyltrehalose and lipooligosaccharides.[10] Other components of the cell wall are cardiolipin (CL), phosphatidylglycerol, phosphatidylcholine (PTC), phosphatidylinositol (PI) and basic phospholipids such as phosphatidyletha-

Correspondence to: Iukary Takenami, Instituto Gonçalo Moniz (IGM), Rua Waldemar Falcão, P O Box 121, Candeal, Salvador, BA 40296 710, Brazil. e-mail: iukary@yahoo.com.br
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nolamine (PE) and sulfatide (SL-I). These phospholipids have a dynamic structure involved in regulation of the transport of nutrients, toxic host-cell effector molecules and anti-tuberculosis drugs.[11]

The aim of the present study was to evaluate IgM and total IgG antibody responses against CL, PE, PI, PTC and SL-I to assess their potential as biomarkers for the diagnosis of PTB and in monitoring treatment of patients with PTB.

METHODS

Subjects and setting

A total of 85 subjects from 68 Centro de Saúde Rodrigo Argolo, Salvador, BA, Brazil, were enrolled in a cross-sectional study conducted between January 2012 and October 2013. Participants were categorised as PTB patients or controls. The control groups comprised healthy subjects with no evidence of infection, healthy subjects with LTBI and non-TB patients with other pulmonary diseases. Patients and controls who tested positive for human immunodeficiency virus and patients taking immunosuppressive drugs were excluded. All patients and all controls provided written informed consent. The study protocol was approved by the Human Subject Ethics Committee of Instituto Gonc¸alo Moniz, Salvador, BA, Brazil (IGM; Fundação Oswaldo Cruz).

Pulmonary tuberculosis patients

Thirty-seven patients with clinical symptoms suggestive of TB and one or more of the following characteristics were selected: 1) chest radiography (CXR) suggestive of TB opacities, 2) sputum samples that contained acid-fast bacilli (AFB) on microscopy; and 3) response to anti-tuberculosis drugs. Sputum smear microscopy was performed using Ziehl-Neelsen staining; results were grouped as negative, 1þ, 2þ or 3þ.[12]

Household contacts

Thirty-five individuals who had been exposed to TB patients during the patients' symptomatic period were invited to participate in the study. Active PTB was excluded from household contacts (HHCs) using CXR and sputum smear. This group included only HHCs who underwent both the tuberculin skin test (TST) and interferon-gamma release assays (IGRAs). The TST was carried out using the Mantoux procedure with 2 tuberculin units of RT23 purified protein derivative (PPD) (Statens Serum Institute, Copenhagen, Denmark). Reading was performed after 72 h and categorised as: 0–5 mm, negative; 75 mm, positive and indicative of infection by *M. tuberculosis*. For IGRAs, we used QuantiFERON™-TB Gold In-Tube (QFT-GIT; Qiagen, Hilden, Germany). The test was performed according to the manufacturer's instructions.[13] The cut-off value for a positive response was 0.35 international units (IU)/ml. The HHCs were stratified into two groups: 16 were TST- and IGRA-negative (HHC—) and 19 were TST- and IGRA-positive (HHC with LTBI, HHCþ).

Non-tuberculosis patients

Thirteen patients with lung diseases other than TB, who declared that they did not have any contact with patients with PTB, were used as a control group. TB was excluded using sputum smear, CXR and culture. Among these patients, 5 had bacterial pneumonia, 2 had lung cancer, 1 had bronchial asthma and the remaining 5 subjects had other pulmonary infections.

Enzyme-linked immunosorbent assay

Serum specimens were obtained upon recruitment and stored at -808C until tested. Patients who had a confirmed diagnosis of TB received the standard treatment in Brazil and serum samples were prospectively collected at baseline as well as at 2 and 6 months after starting treatment.

Total IgG and IgM levels were measured using an indirect enzyme-linked immunosorbent assay (ELISA).[14] Lipids were diluted to 10 mg/ml (CL, PE, PI and PTC) or 1 mg/ml (SL-I) (Sigma-Aldrich, Saint Louis, MI, USA) using anhydrous ethanol; 50 ll of the solution was then added to each well of polystyrene ELISA plates (Greiner Bio-One, Kremsmünster, Austria). The plates were covered with a plate sealer (Costare; Corning, Wiesbaden, Germany) and incubated overnight (18–24 h) at room temperature. The coated wells were washed once with 13 phosphate-buffered saline (PBS), pH 7.4 (13 PBS; Invitrogen, San Diego, CA, USA), blocked with 100 ll of 3% low fatty acid bovine serum albumin (BSA) (blocking buffer), and incubated for 1 h at room temperature. Plates were washed twice using 300 ll 13 PBS, and 100 ll of the serum sample diluted 1:100 in 3% BSA was added. After incubation for 1 h at room temperature, the serum samples were removed and the plates were washed thrice with 13PBS. Then, respectively 100 ll of 1:10 000 and 1:50 000 goat-derived anti-human IgM and total IgG, labelled with horseradish peroxidase (Sigma-Aldrich) diluted in 3% BSA/PBS, was added. After 1 h of incubation, a new cycle of washes and 100 ll/well of the chromogenic substrate tetramethylbenzidine (Invitrogen) was added, and the reaction stopped with 100 ll of 2N sulphuric acid. Reactions were read within 10 min at 450 nm in a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A group of three wells in each ELISA plate did not receive any samples; these blank wells were used as negative controls. Results were read out as the average optical density (OD) of triplicate samples and were re-run if - 10% coefficient of variance was observed.

Table Comparison of receiver operating characteristic curve analysis for phospholipids in discriminating between active tuberculosis cases and controls

Isotype	Phospholipid	AUC (95%CI)	*P* value	Sensitivity % (95%CI)	Specificity % (95%CI)	LRþ
IgM	CL	0.742 (0.636–0.848)	0.0001	70.3 (53–84.1)	68.8 (53.8–81.3)	2.2
	PE	0.762 (0.658–0.865)	, 0.0001	75.7 (58.8–88.2)	70.8 (55.9–83)	2.6
	PI	0.836 (0.748–0.923)	, 0.0001	77.8 (60.8–89.9)	79.2 (65–89.5)	3.7
IgG	CL	0.940 (0.890–0.989)	, 0.0001	86.5 (71.2–95.5)	87.2 (74.26–95.2)	6.8
	PE	0.782 (0.671–0.894)	, 0.0001	72.2 (54.8–85.8)	78.7 (64.3–89.3)	3.4
	PI	0.880 (0.808–0.953)	, 0.0001	81.1 (64.8–92)	79.2 (65–89.5)	3.9
	PTC	0.829 (0.737–0.921)	, 0.0001	75 (57.8–87.9)	75 (60.4–86.4)	3.0

AUC ¼ area under the receiver operating characteristic curve; CI ¼ confidence interval; LR ¼ likelihood ratio; þ ¼ positive; CL ¼ cardiolipin; PE ¼ phosphatidylethanolamine; PI ¼ phosphatidylinositol; PTC ¼ phosphatidylcholine.

Statistical analysis

Statistical analyses were performed using Prism v7.0 (GraphPad, San Diego, CA, USA). For analysis of antibody levels, differences between the three participant groups were first assessed using the Kruskal–Wallis test, followed by the Dunn's post-test. If significance was found (P , 0.05), pairwise comparisons were analysed using the Mann–Whitney U-test. For longitudinal analysis of Ig levels on anti-tuberculosis treatment, differences between time points were assessed using the Friedman test. Receiver operating characteristic (ROC) curves were constructed for the two anti-phospholipid antibody isotypes to identify cut-offs according to the value resulting in the combination of the highest sensitivity and specificity based on samples from the patients, together with the controls. We defined HHCs and non-TB patients as the control group. All tests for statistical significance used a level of P , 0.05.

RESULTS

Characteristics of study subjects

A total of 85 subjects volunteered to participate in our study. Of 37 (43.5%) PTB patients, 24 (64.9%) had sputum samples that were smear-positive for AFB. From these TB patients' households, 16 healthy controls (HHC–) and 19 individuals with LTBI (HHCþ) were also selected. In addition to these groups, we included 13 subjects with lung disease other than TB. Characteristics of the study population have been described elsewhere.[15]

IgM and total IgG antibody responses to CL, PE, PI, PTC and SL-I phospholipids in active TB patients at baseline

Serum levels of total IgG and IgM specific to CL, PE, PI, PTC and SL-I phospholipids were evaluated in active TB patients at their first visit to the out-patient clinic before treatment. IgM response to CL, PE and PI was consistently higher in TB patients than in the control groups (P ¼ 0.0014, P ¼ 0.0002 and P , 0.0001, respectively, Figure 1 A1–3). No difference was observed in PTC (P ¼ 0.231) or SL-I levels (P ¼ 0.06; Figure 1 A4, A5). It should be noted that when TB patients were separated by degree of smear status, TB patients with negative sputum smear tests had statistically higher levels of anti-CL IgM (P ¼ 0.023) and anti-PI (P ¼ 0.012) than sputum smear-positive TB patients (data not shown).

Moreover, the IgG antibody response to CL, PE, PI and PTC was also increased (all phospholipids P , 0.0001) in TB patients compared with controls (Figure 1 B1–B4). However, the median difference in levels of total serum IgG (P ¼ 0.430), as well as IgM to SL-I, was not statistically significant between TB patients compared with controls (Figure 1 A5). There were no statistically significant differences in the median IgG levels by sputum smear status (data not shown). Furthermore, there was no significant difference in changes in antibody levels between patients with cavitary PTB and those with non-cavitary PTB (P . 0.05; data not shown).

IgM and total IgG test

The ROC analysis indicated that PI performed better diagnostically in measuring IgM than CL and PE (Figure 2, Table). Conversely, CL had a better diagnostic performance on measuring IgG than PE, PI and PTC (Figure 3, Table). Total IgG had the best overall performance; on optimal ROC analysis, the IgG anti-CL ELISA test detected 86.5% (32 of 37) of TB patients (Figure 4), whereas the number detected using sputum smear was only 64.9% (24/37). Of the 32 IgG test-positive patients, 87.5% (21/25) were positive among AFB smear-positive patients. Anti-CL IgG ELISA thus identified 11 (84.6%) additional cases among 13 who were sputum smear-negative. When anti-CL and anti-PI IgG test results were considered, the number of positive results was not higher in TB patients; thus, the combined results did not improve the sensitivity of any test.

Time-course changes in IgG and IgM antibody titres after initiation of anti-tuberculosis treatment

To assess whether determination of phospholipid antibody responses could be useful for monitoring the efficacy of anti-tuberculosis treatment, a series of serum samples was collected from active TB patients who had undergone anti-tuberculosis treatment. The median levels of IgM to CL and PI, IgG to CL, PE, PI

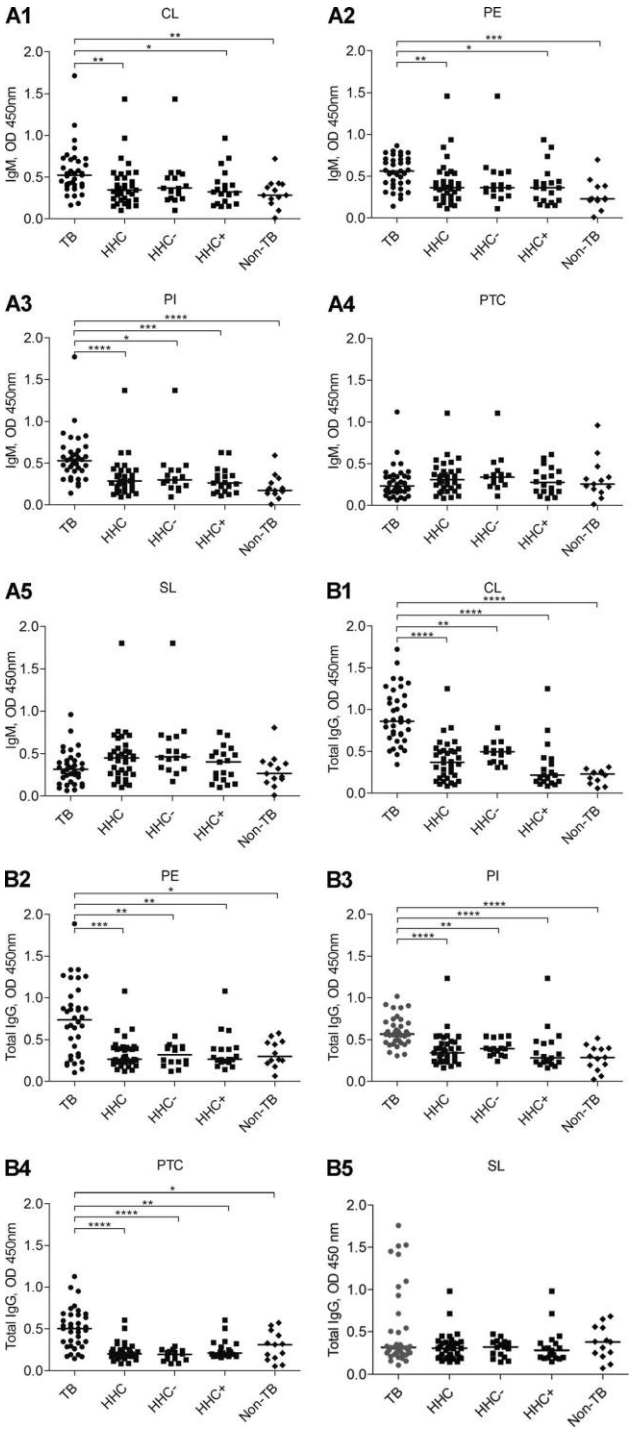

Figure 1 Serum IgM (A1–A5) and total IgG (B1–B5) levels against CL, PE, PI, PTC and SL-I phospholipids in TB patients (*n* 37), HHCs (*n* 35), HHC (*n* 16), HHC (*n* 19) and non-TB patients (*n* 43). Statistical significance was determined using the Kruskal–Wallis test, followed by the Dunn test if significance was found (P , 0.05); pairwise comparisons were analysed using the Mann–Whitney *U*-test. * P , 0.05; ** P , 0.01; *** P , 0.001 and **** P , 0.0001. Horizontal

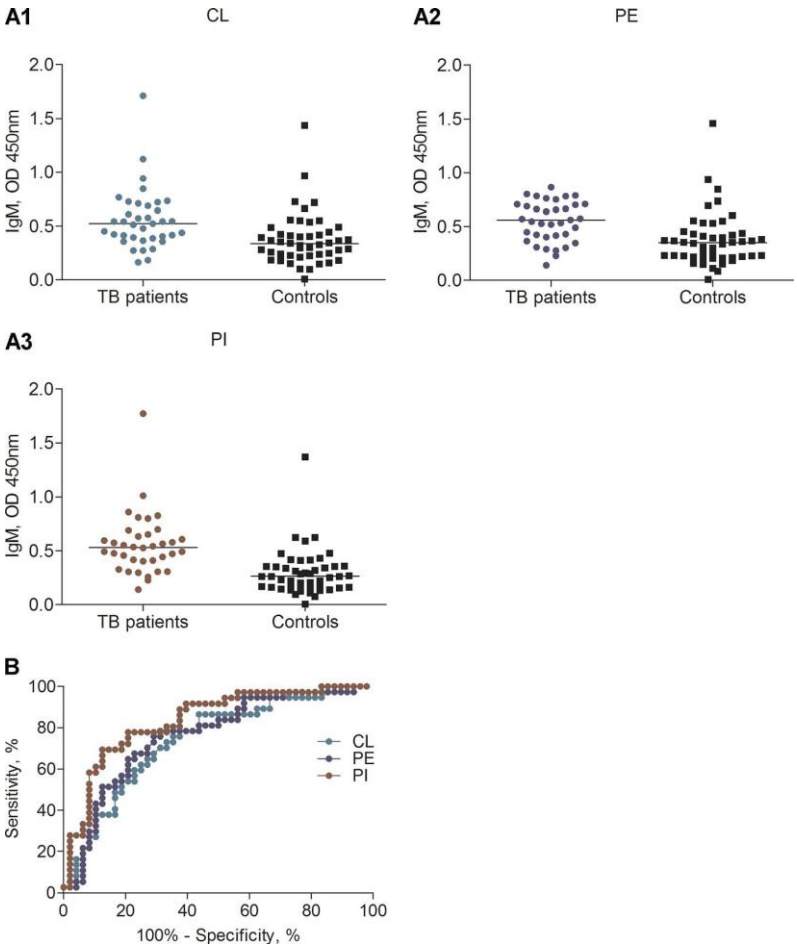

Figure 2 Serodiagnostic performance of IgM to A1) CL, A2) PE and A3) PI in active TB patients without treatment and a control group comprising HHCs and non-TB patients. B) ROC curves for CL, PE and PI based on measuring IgM levels. Ig¼ immunoglobulin; OD¼ optical density; TB¼ tuberculosis; CL¼cardiolipin; PE¼phosphatidylethanolamine; PI ¼phosphatidylinositol; HHCs ¼ household contacts of pulmonary TB patients. This image can be viewed online in colour at http://www.ingentaconnect.com/content/iuatld/ijtld/2018/00000022/00000009/art00016

and PTC decreased significantly during anti-tuberculosis treatment (Figure 5). In addition, there was no statistically significant difference between median levels of IgM to CL and HHCs or non-TB patients (P ¼0.059). With regard to the other phospholipids, even after treatment completion and despite reduc- tion in median antibody levels, TB patients continued to have higher levels of IgM to PE (P ¼0.0006) and PI (P ¼ 0.0001) or IgG to CL (P , 0.0001), PE (P¼ 0.038), PI (P ¼ 0.002) and PTC (P ¼ 0.019) than HHCs or non-TB patients.

DISCUSSION

We observed that antiphospholipid antibody levels, including anti-CL, anti-PE, anti-PI and anti-PTC, were significantly higher in new adult PTB patients than in adult control groups. The IgG test had the best performance. The prevalence of detection of TB patients using the total IgG test (CL, 86.5% and PI, 81.1%) was higher than that detected using sputum smear results (AFB 64.9%) or the IgM test (CL, 70.3% and PI, 77.8%). In post-primary PTB patients,

>
lines represent the median OD value. Ig¼ immunoglobulin; OD¼ optical density; TB¼ tuberculosis; HHCs¼household contacts (of pulmonary TB patients); HHC—¼healthy HHCs; HHCþ¼HHCs with latent tuberculous infection; CL ¼ cardiolipin; PE ¼ phosphatidylethanolamine; PI ¼ phosphatidylinositol; PTC¼phosphatidylcholine; SL-I ¼sulfatide.

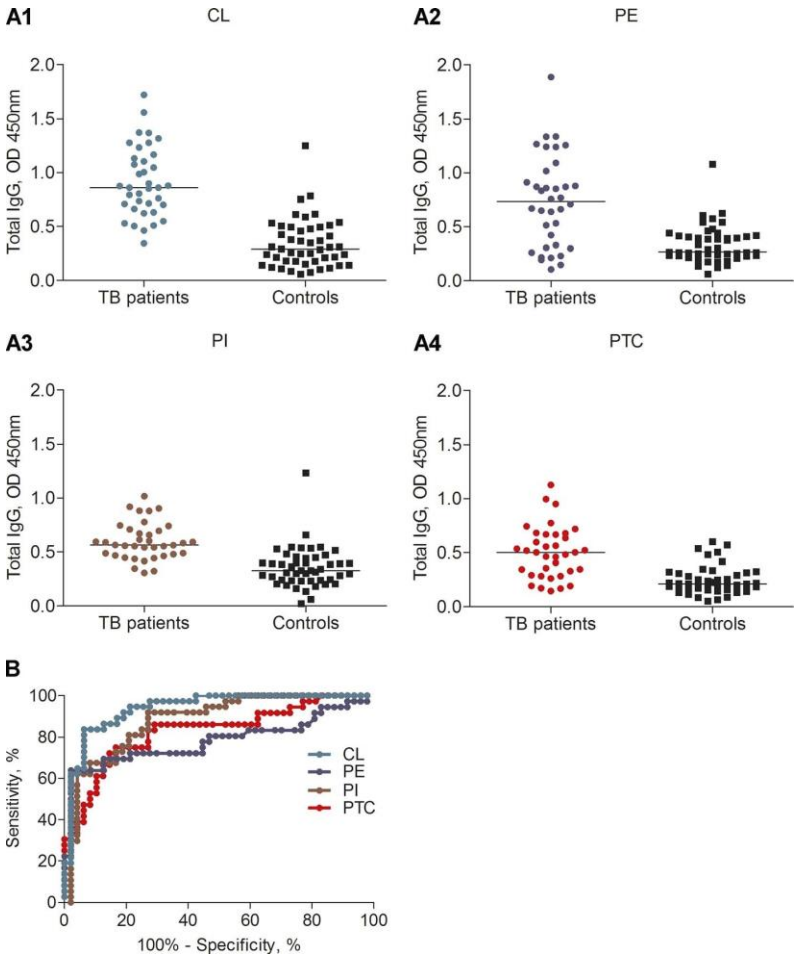

Figure 3 Serodiagnostic performance of IgG to A1) CL, A2) PE, A3) PI and A4) PTC in active TB patients without treatment and a control group comprising HHCs and non-TB patients. B) ROC curves of CL, PE, PI and PTC based on measuring IgG levels¼g immunoglobulin; OD¼ optical density; TB¼tuberculosis; CL¼cardiolipin; PE¼phosphatidylethanolamine; PI¼phosphatidylinositol; PTC¼phosphatidylcholine; HHCs¼household contacts (of pulmonary TB patients). This image can be viewed online in colour at http://www.ingentaconnect.com/content/iuatld/ijtld/2018/00000022/00000009/art00016

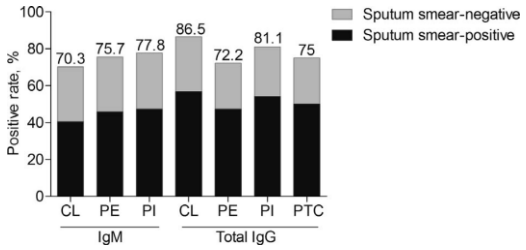

Figure 4 Positive rates of IgM and total IgG antibodies to phospholipids in serum samples from tuberculosis patients stratified by sputum smear status. CL ¼ cardiolipin; PE ¼ phosphatidylethanolamine; PI ¼ phosphatidylinositol; PTC ¼ phosphatidylcholine; Ig ¼immunoglobulin.

low titres of IgM and high titres of IgG could explain the high rates of IgG positivity.[16] Antiphospholipid IgG antibodies could thus be useful in complementing conventional bacteriological tests for rapid diagnosis and in discriminating PTB from other pulmonary diseases in patients presenting with similar symptomatology. Although the Xpert® MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) is not available at 68 Centro de Saúde Rodrigo Argolo, results should be discussed in the light of other diagnostic tests that are much more sensitive than sputum smear microscopy.

Although the role of *M. tuberculosis* cell wall phospholipids during infection is uncertain, CL is essential for *M. tuberculosis* growth in vitro.[17] Antibodies against *M. tuberculosis* CL have also been

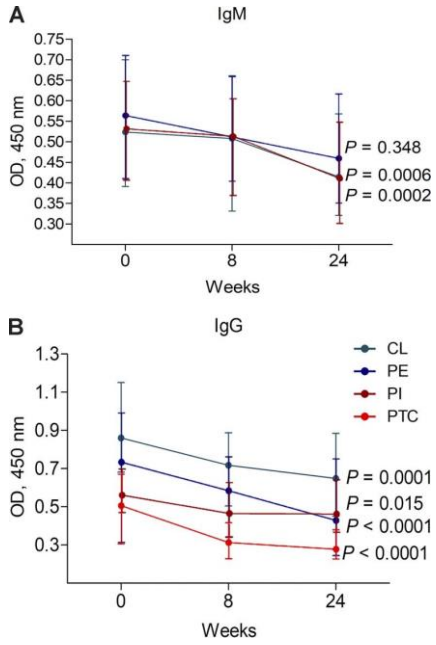

Figure 5 Plot of the median change and interquartile range in A) IgM antiphospholipid and B) IgG antiphospholipid antibody levels before, during and after anti-tuberculosis treatment. All 37 patients were followed up for 24 weeks, and blood samples were collected before treatment (baseline) as well as after 8 and 24 weeks of treatment. OD ¼ optical density; Ig ¼ immunoglobulin; CL ¼ cardiolipin; PE ¼ phosphatidylethanolamine; PI ¼ phosphatidylinositol; PTC ¼ phosphatidylcholine. This image can be viewed online in colour at http://www.ingentaconnect.com/content/iuatld/ijtld/2018/00000022/00000009/art00016

found in serum samples from TB patients in other studies.[18,19] In line with our results, higher levels of anti-CL antibodies were observed in TB patients than in other control groups before treatment.[19,20] However, anti-CL antibodies are also considered to be auto-antibodies produced by the immune system that non-specifically target the body's CLs, which are found almost exclusively in the inner mitochondrial membrane.[21]

As anti-CL antibody levels in TB patients have rarely been investigated, it is not clear if these antibodies represent auto-antibodies or result from polyclonal B cell activation after stimulation by mycobacterial CLs. In the present study, the phospholipids used in the assay were purchased from a commercial company and were therefore not extracted from *M. tuberculosis* cell wall lipids. Although the results do not reflect a 'real' response, the increase in levels of antiphospholipid antibodies in TB patients cannot be disregarded. If the anti-CL antibodies include auto-antibodies, these test results should be interpreted with caution based on the corresponding patient clinical symptoms and signs. No patients reported any symptoms that could be attributable to autoimmune disease, particularly those volunteers who had increased levels of anti-CL antibodies. Although the absence of symptoms does not exclude autoimmune disease, it is probable that antibody antiphospholipids are involved in TB disease. We therefore hypothesised that phospholipids released from lung cells during massive necrotic cell death induced by some strains of *M. tuberculosis* in a patient could be recognised by the immune system as antigens, contributing to the increased production of auto-antibodies. Furthermore, the presence of auto-antibodies to antigens that are not mycobacterial, such as anti-Ro, anti-b2-glycoprotein and anti-mitochondrial antibody, as negative controls could also be very helpful.

Goodridge et al. observed that in TB patients with cavitary pulmonary disease, the anti-lipid IgM response did not decrease with treatment.[14] In our study, 16/28 (57.1%) TB patients had cavitary disease. Anti-CL IgG levels decreased significantly after anti-tuberculosis treatment ($P = 0.003$) in patients with cavitary lung disease. In contrast, the IgM levels did not fall ($P = 0.144$), probably because large amounts of lipids from both dead lung cells and *M. tuberculosis* are released, inducing IgM antiphospholipid antibody production.

Our findings suggest that increased anti-CL antibody levels found in TB patients were not observed in other pulmonary conditions that induce inflammation (other types of bacterial pneumonia, asthma, chronic obstructive lung disease). Thus, despite the non-specific nature of the anti-lipid IgM and IgG responses to CL, the test appears to demonstrate a high degree of specificity if applied to pulmonary diseases.

IgM and IgG levels against all anti-phospholipid antibodies significantly decreased following anti-tuberculosis treatment. Similarly, Goodridge et al. showed that levels of IgM antiphospholipid antibodies decreased among non-cavitary TB patients.[14] However, despite the reduction in Ig levels, our ELISA did not perform as well as the bacteriological test in monitoring response to treatment. This may have been due to the inclusion of TB patients with cavitary disease in our study. The data confirm the observations of Horne et al., who demonstrated that bacteriological tests remain useful for monitoring anti-tuberculosis treatment.[22]

Compared with assays based on cell-mediated responses, antibody detection is considerably simpler and less expensive. However, serological tests still have suboptimal sensitivity and specificity. In the present study, the sensitivity of the IgG test to CL was high (86.5%) compared with the IgM test (77.8% to PI). The sensitivity for most of the lipids reported in other studies was low in TB patients compared with our findings.[23,24] Furthermore, the specificity of the IgG anti-CL ELISA test (87.2%) showed satisfactory results compared with other tests based on lipid antigens.[24]

Finally, the sample size in our study was small; studies with large sample size and longitudinal follow-up, particularly among HHCs infected by *M. tuberculosis*, are required to understand the role of the antiphospholipid antibody in the development of TB disease. It is also important to validate the study in other cohorts with more subjects with clinically indistinguishable disease, such as bacterial pneumonia, from those who have TB and perform IGRAs in non-TB patients. IGRAs are not widely available to test non-TB patients in Brazil.

CONCLUSION

The WHO does not recommend serological tests for the diagnosis of TB based on protein antigens, particularly due to their lack of accuracy. This is likely because the humoral response to *M. tuberculosis* in chronic infection is highly complex and variable. However, based on our results, the clinical utility of serological tests against *M. tuberculosis* phospholipids has potential utility as a rapid screening test for differentiating TB from non-TB pulmonary diseases.

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Conflicts of interest: none declared.

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RÉSUMÉ

CONTEXTE : Salvador, Bahia, Brésil.

OBJECTIF : Evaluer la réponse en anticorps immunoglobuline (Ig) M et en IgG totales a` la cardiolipine (CL), a` la phosphatidylcholine (PTC), a` la phosphatidyléthanolamine (PE), au phosphatidylinositol (PI) et au sulfatide (SL-I) comme une biosignature qui peut être utilisée pour le diagnostic de la tuberculose (TB) pulmonaire et son applicabilite´ au suivi de l'efficacite´ du traitement antituberculeux.

SCHÉMA : Des échantillons de sérum de 37 patients adultes atteints de TB pulmonaire et de 48 témoins dont 16 contacts domiciliaires en bonne santé, 19 contacts domiciliaires atteints d'infection tuberculeuse latente et 13 patients non-TB mais atteints de maladie pulmonaire, ont été dépistés par titrage avec immunoadsorbant lie´ a` une enzyme (ELISA) a` la recherche d'IgM et d'IgG totales vis-à-vis des phospholipides.

RÉSULTATS : Les niveaux d'IgM en réponse à CL, PE et PI et d'IgG en réponse à CL, PE, PI et PTC ont été significativement plus élevés chez les patients TB que dans les groupes témoins. Les IgG anti-CL ont eu les meilleures caractéristiques de performance avec une sensibilite´ et une spécificité de 86,5% et 87,2%, respectivement. Ce test ELISA d'IgG anti-CL a détecté 86,5% (32/37) des patients TB, tandis que le nombre détecté par frottis de crachats a été de seulement 65,9% (24/37). Après traitement antituberculeux, la médiane pour tous les anticorps antiphospholipides a significativement diminué, comparée aux valeurs de départ (P ¸ 0,05).

CONCLUSION : Nos résultats suggèrent que les IgG totales anti-CL pourraient être utiles pour compléter les tests bactériologiques conventionnels pour le diagnostic rapide de la TB pulmonaire des adultes.

RESUMEN

MARCO DE REFERENCIA: Salvador de Bahı´a, en el Brasil.

OBJETIVO: Evaluar la respuesta de anticuerpos inmunoglobulina (Ig) M e IgG total a la cardiolipina (CL), la fosfatidilcolina (PTC), la fosfatidiletanolamina (PE), el fosfatidilinositol (PI) y los sulfátidos (SL-I) como un bioindicador que pueda utilizarse en el diagnóstico de la tuberculosis (TB) pulmonar y analizar su aplicabilidad en el seguimiento de la eficacia del tratamiento antituberculoso.

MÉTODO: Se examinaron muestras séricas de 37 pacientes adultos con TB pulmonar y 48 testigos que incluı´an 16 contactos domiciliarios sanos, 19 contactos domiciliarios con infección tuberculosa latente y 13 pacientes con enfermedades pulmonares diferentes de TB. Mediante un enzimoinmunoanálisis de adsorción (ELISA) se investigó la presencia de IgM y de IgG total dirigidas contra los fosfol´ıpidos.

RESULTADOS: Las concentraciones de IgM contra CL, PE y PI y de IgG contra CL, PE, PI y PTC fueron significativamente más altas en los pacientes con TB que en los grupos testigo. La mejor eficacia diagnóstica se observó con la IgG anti-CL con una sensibilidad de 86,5% y una especificidad de 87,2%. Esta prueba ELISA de IgG anti-CL detectó el 86,5% de los pacientes con TB (32/37), cuando la baciloscopia del esputo solo detectó un 65,9% (24/37). Después de haber iniciado el tratamiento antituberculoso, la mediana de todos los anticuerpos dirigidos contra los fosfolípidos disminuyó de manera significativa en comparación con las concentraciones iniciales (P ¸ 0,05).

CONCLUSIÓN: Estos resultados indican que la IgG total dirigida contra la CL podría ser un complemento útil a las pruebas bacteriológicas corrientes, en el diagnóstico rápido de la TB pulmonar de los adultos.