FUNDAÇÃO OSWALDO CRUZ
CENTRO DE PESQUISAS GONÇALO MONIZ

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

TESE DE DOUTORADO

AVALIAÇÃO DE BIOMARCADORES PARA DIAGNÓSTICO E MONITORAMENTO DO TRATAMENTO DA TUBERCULOSE PULMONAR

IUKARY OLIVEIRA TAKENAMI

Salvador - Bahia
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Orientador: Prof. Dr. Sérgio Arruda

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

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"AVALIAÇÃO DE BIOMARCADORES PARA DIAGNÓSTICO E MONITORAMENTO DO TRATAMENTO DA TUBERCULOSE PULMONAR."

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“As espécies que sobrevivem não são as mais fortes, nem as mais inteligentes, e sim aquelas que se adaptam melhor às mudanças”

Charles Darwin

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RESUMO

INTRODUÇÃO: A tuberculose (TB), doença crônica infecciosa causada por *Mycobacterium tuberculosis*, é considerada um grave problema de saúde pública no país. A caracterização de antígenos protéicos e/ou lipídios que induzem uma resposta imunológica no hospedeiro, torna-se um importante passo para o desenvolvimento de novas ferramentas de diagnóstico e resposta terapêutica. Dentre os diferentes antígenos, em especial a *mammalian cell entry protein 1A* (proteína Mce1A), e os fosfolipídios da parede celular do bacilo como a cardiolipina (CL), os fosfatidilinositol (FI), fosfatidilcolina (FC), fosfatidiletanolamina (FE) e o sulfatide (SL), são, em sua maioria altamente imunogênicos, podendo então ser úteis no sorodiagnóstico. Portanto, o objetivo do estudo é avaliar a produção de anticorpos anti-Mce1A e anti-fosfolipídios como biomarcadores no diagnóstico e no monitoramento do tratamento da TB pulmonar. Além disso, o estudo também objetivou avaliar o perfil de citocinas e quimiocinas produzidas em sobrenadantes de cultura após estímulo *in vitro* com a proteína Mce1A. **PACIENTES E MÉTODOS:** O estudo foi conduzido no 6º Centro de Saúde Rodrigo Argolo e no Instituto Brasileiro para Investigação da Tuberculose (IBIT). A população de estudo foi composta por pacientes recém diagnosticados com TB pulmonar, seus respectivos comunicantes domiciliares (infectados por *M. tuberculosis* e saudáveis) e pacientes diagnosticados com outras doenças pulmonares. **RESULTADOS:** Pacientes com TB produzem uma forte e consistente resposta de anticorpos anti-Mce1A e anti-fosfolipídios (anti-CL, anti-FE, anti-FI e anti-FC) quando comparados com os indivíduos do grupo controle. Além disso, após início do tratamento os níveis de anti-Mce1A e anti-fosfolipídios diminuem significativamente. O sobrenadante de culturas dos pacientes TB, após cultura com Mce1A, induzem uma acentuada produção de TNF, o que não se observa nas demais citocinas e quimiocinas avaliadas. **CONCLUSÃO:** Estes resultados sugerem que os anticorpos anti-Mce1A e anti-fosfolipídios desempenham potencial papel como biomarcadores sorológicos no diagnóstico da TB pulmonar. Além disso, a proteína Mce1A parece desempenhar um papel importante na produção de TNF, que pode contribuir com a indução de necrose pelo bacilo, permitindo sua evasão das respostas imunes e favorecendo a dispersão do bacilo para outras células não infectadas.

Palavras-chave: Tuberculose, Mce1A, Cardiolipina, Fosfolipídios, Diagnóstico, Tratamento.

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ABSTRACT

INTRODUCTION: Tuberculosis (TB), chronic infectious disease caused by *Mycobacterium tuberculosis*, is still a serious public health problem in the country. The characterization of protein and/or lipids antigens that induce an immune response in the host, it is an important step in the development of new diagnostic tools and monitoring TB treatment response. Among the different antigens, particularly mammalian cell entry protein 1A (Mce1A protein), and phospholipids from the cell wall of bacillus such as cardiolipin (CL), phosphatidylinositol (PI), phosphatidylcholine (PTC), phosphatidylethanolamine (PE) and sulfatide (SL), are highly immunogenic and can be used for improvement of the serodiagnosis. Therefore, the aim of the study is to evaluate the production of anti-Mce1A and anti-phospholipids as biomarkers for diagnosis and monitoring of TB treatment response. In addition, the study also aimed to evaluate the profile of cytokines and chemokines produced in vitro after stimulation with Mce1A protein in culture supernatants. **PATIENTS AND METHODS:** The study was conducted on the 6º Centro de Saúde Rodrigo Argolo and the Instituto Brasileiro para Investigação da Tuberculose (IBIT). The study population consisted of newly diagnosed pulmonary TB patients, their household contacts (infected by *M. tuberculosis* and healthy) and patients diagnosed with other lung diseases. **RESULTS:** Patients with TB produce a strong and consistent response to anti-Mce1A and anti-phospholipids (anti-CL, anti-PE, anti-PI and anti-PTC) than those in the control groups. Furthermore, after the beginning of the treatment, anti-Mce1A and anti-phospholipid levels were significantly decreased compared with TB patient at baseline. Culture supernatants of TB patients after stimulation with Mce1A induce a strong TNF production, which is not observed in the other evaluated cytokines and chemokines. **CONCLUSION:** These results suggest that anti-Mce1A and anti-phospholipids play role as potential serum biomarker in the diagnosis of pulmonary TB. Furthermore, Mce1A protein appears to play an important role in TNF production, which may contribute to the induction of necrosis by *M. tuberculosis*, allowing for avoidance of immune responses and facilitating the dispersion of bacillus to other uninfected cells.

Keywords: Tuberculosis, Mce1A, Cardiolipin, Phospholipids, Diagnosis, Treatment

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

6ºCS	6º Centro de Saúde Dr Rodrigo Argolo
BAAR	Bacilo álcool-ácido resistente
BCG	Bacilo Calmette-Guérin
CBA	*Cytometric Bead Array*
CD	*Cluster of differentiation*
CEP	Conselho de Ética em Pesquisa
CFP-10	*Culture filtrate protein-10*
CL	Cardiolipina
CPqGM	Centro de Pesquisa Gonçalo Moniz
DO	Densidade óptica
DP	Desvio padrão
ELISA	*Enzyme linked immuno sorbent assay*
ESAT-6	*Early secretory antigenic target-6*
FC	Fosfatidilcolina
FE	Fosfatidiletanolamina
FI	Fosfatidilinositol
FIOCRUZ	Fundação Oswaldo Cruz
HIV	Vírus da imunodeficiência humana
HTLV	Vírus linfotrópico de células T humanas
IBIT	Instituto Brasileiro para Investigação da Tuberculose
IFN-γ	Interferon-gama
IgA	Imunoglobulina A
IgE	Imunoglobulina E
IgG	Imunoglobulina G
IgM	Imunoglobulina M
IGRA	Ensaio de liberação de interferon-gama
IL	Interleucina
IOC	Instituto Oswaldo Cruz
Mce1A	*mammalian cell entry protein 1A*
MHC	Complexo principal de histocompatibilidade
MS	Ministério da Saúde

NF-κβ	Fator de transcrição nuclear κβ
NLRs	Receptores NOD-like
OMS	Organização Mundial de Saúde
PBMC	Células mononucleares do sangue periférico
PBS	Tampão fosfato-salino
PHA	Fitohemaglutinina
PNCT	Programa Nacional de Controle da Tuberculose
PPD	Derivado protéico purificado
RD1	Região de diferença 1
RNI	Intermediários reativos de nitrogênio
ROC	Característica de Operação do Receptor
ROI	Intermediários reativos de oxigênio
SFB	Soro fetal bovino
SINAN	Sistema de Informação de Agravos e Notificação
SL	Sulfatide
TA	Temperatura ambiente
TB-IRIS	Tuberculose associada a Síndrome Inflamatória de Reconstituição Imune
TB	Tuberculose
TB7.7	antígeno TB7.7
TBL	Tuberculose latente
TCLE	Termo de Consentimento Livre e Esclarecido
TGF-β	Fator de transformação do crescimento beta
Th1	*T helper 1*
Th2	*T helper 2*
TLR	receptores *Toll-like*
TMB	Tetrametilbenzidina
TNF	Fator de necrose tumoral
TT	Teste tuberculínico

SUMÁRIO

1 INTRODUÇÃO

A tuberculose (TB), doença crônica infecciosa causada por *Mycobacterium tuberculosis*, é considerada um grave problema de saúde pública, constituindo uma das principais causas de morbi-mortalidade no país. Embora a incidência e a mortalidade por TB tenham diminuído ao longo dos últimos anos, cerca de 93 mil casos novos e 4,4 mil óbitos são notificados anualmente no país (WHO, 2014). Associados a esse cenário, mudanças no perfil da população brasileira, incluindo o rápido crescimento da população idosa, o aumento da prevalência de diabetes mellitus tipo 2, a infecção pelo vírus da imunodeficiência humana (HIV), pelo vírus linfotrópico de células T humanas (HTLV), bem como TB associada a Síndrome Inflamatória de Reconstituição Imune (TB-IRIS) são fatores que contribuem com a manutenção do elevado índice de endemicidade no país (BISSON; ZETOLA; COLLMAN, 2015; JEON; MURRAY, 2008; KOZLOWSKI et al., 2014, 2014; NARASIMHAN et al., 2013; WILKINSON et al., 2015)

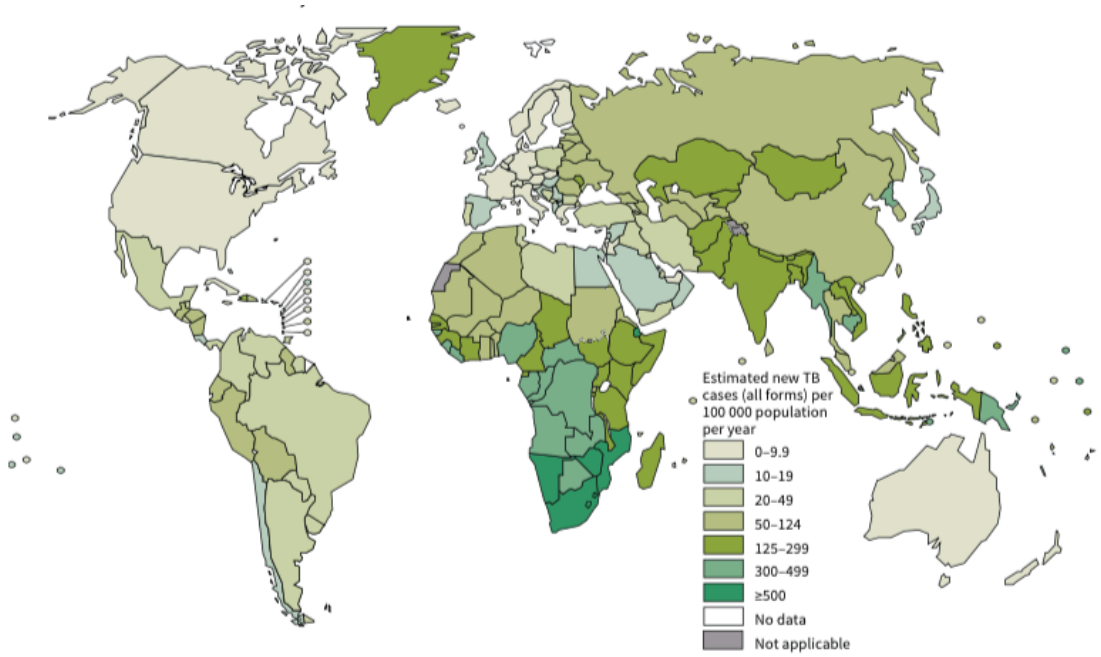
O diagnóstico precoce e o tratamento efetivo são importantes estratégias que visam o controle da TB. Dessa forma, a cadeia de transmissão do bacilo é interrompida e, consequentemente, evita-se a disseminação da doença (JASSAL; BISHAI, 2010). Entretanto, as principais dificuldades encontradas nos serviços de saúde são: 1) a limitação dos atuais testes laboratoriais utilizados na confirmação do diagnóstico da TB; 2) a ineficácia dos métodos diagnósticos para distinguir entre a infecção e a doença ativa; 3) o abandono do tratamento e a falta de um teste que possa predizer de forma eficaz a falha terapêutica. Essas limitações fazem com que novos estudos busquem metodologias capazes de suprir as dificuldades encontradas no combate à esta enfermidade. Por esta razão, a identificação e caracterização de biomarcadores, os quais podem refletir diversos processos em andamento, incluindo processos normais, patogênicos ou respostas farmacológicas às intervenções terapêuticas, constituem o primeiro passo para o desenvolvimento de novas ferramentas de diagnóstico e de avaliação terapêutica.

2 REVISÃO DA LITERATURA

2.1 EPIDEMIOLOGIA

A TB ainda configura-se como um importante e prioritário problema de saúde pública no mundo (Figura 1). A Organização Mundial da Saúde (OMS) estima que cerca de 9 milhões de indivíduos desenvolvem a doença sintomática e, que ocorrem, anualmente, 1,5 milhões de óbitos por ano. Destes, aproximadamente 98% ocorrem em países em desenvolvimento (WHO, 2014).

Figura 1. Número de novos casos de tuberculose registrados no mundo durante o ano de 2013.



Fonte: WHO, 2014.

O Brasil ocupa o 22° lugar entre os 22 países responsáveis por 80% do total de casos de TB no mundo. Além disso, possui o maior número de casos registrados na América do Sul, com uma prevalência estimada em 110 casos por 100.000 habitantes (WHO, 2014). Apesar da redução dos índices de morbidade, a doença ainda permanece como a segunda causa de óbito por doenças infecciosas. Em 2013, segundo o SINAN no estado da Bahia foram registrados

5.743 casos confirmados de TB, o que confere ao estado o primeiro lugar do nordeste em número de casos. Dentre os casos confirmados no estado, Salvador concentra 2.603 (45,3%) casos (BRASIL, 2015).

Atualmente, além dos fatores de risco já conhecidos para o desenvolvimento da TB, como infecção pelo HIV, pobreza, desigualdade social, desnutrição e habitação precária ou com alta aglomeração, outros fatores têm contribuído para a manutenção da doença no país (NARASIMHAN et al., 2013). Dentre eles, destaca-se o envelhecimento progressivo da população, a infecção pelo vírus HTLV, a TB-IRIS, e o aumento da prevalência de diabetes mellitus tipo 2 (BASTOS et al., 2012; JEON; MURRAY, 2008; KOZLOWSKI et al., 2014; NARASIMHAN et al., 2013; WILKINSON et al., 2015). Além de influenciar no padrão de morbidade e mortalidade, estes fatores tornam a TB mais complexa e, consequentemente, mais difícil de ser controlada.

2.2 AGENTE ETIOLÓGICO

O gênero *Mycobacterium* é o único da família Mycobacteriaceae, o qual é constituído pelo complexo *M. tuberculosis* e mais de 150 espécies de micobactérias atípicas, incluindo espécies patogênicas, oportunistas e não patogênicas (SOINI; MUSSER, 2001). O complexo *M. tuberculosis* inclui as espécies: *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. canettii*, *M. caprae*, *M. microti* e *M. pinnipedii*. Entretanto, no homem, a doença é quase exclusivamente causada pelo *M. tuberculosis*, embora as espécies *M. africanum*, *M. bovis,* possam também causar TB (SOINI; MUSSER, 2001).

A espécie *M. tuberculosis* é um bacilo delgado, aeróbio obrigatório, não esporulado, sem flagelos, com dimensões de 0,2 a 0,7 x 1,0 a 4 μm. Não produzem toxinas, são intracelulares facultativos e possuem longos períodos de duplicação (16 a 20 horas) (TORTORA; FUNKE; CASE, 2000). As micobactérias são relativamente resistentes aos procedimentos padrão 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) (RILEY, 2006).

2.3 MODO DE TRANSMISSÃO

A transmissão da TB ocorre pelas vias aéreas, através da inalação de gotículas microscópicas contendo *M. tuberculosis*, expelidas por um indivíduo bacilífero ao falar, espirrar e, principalmente, tossir (FRIEDEN et al, 2003; TOIT; PILLAY; DANCKWERTS, 2006). Quando inalados, parte dos bacilos é retido por mecanismos de defesa inespecíficos do hospedeiro, tais como as barreiras mecânicas do aparelho respiratório como os cílios nasais, reflexo da tosse e depuração mucociliar. Somente o núcleo seco dessas gotículas, chamado de núcleo de Wells, com diâmetro menor que 5 μm e contendo de 1 a 3 bacilos, conseguem ultrapassar os mecanismos de defesa e atingir os bronquíolos (KRITSKI; CONDE; SOUZA, 2000).

Em seguida, o processo da fagocitose realizado pelos macrófagos alveolares residentes no tecido pulmonar, iniciam uma cascata de eventos que pode culminar na contenção bem-sucedida da infeção, caracterizando a infecção tuberculosa latente (TBL) ou pode haver progressão para a doença ativa, evidenciando a TB ativa (BHATT; SALGAME, 2007; FRIEDEN et al., 2003).

O resultado da exposição ao bacilo é consequência de uma série de fatores associados à interação entre a resposta do sistema imunológico do hospedeiro, aspectos ambientais e a virulência micobacteriana (FILHO et al., 2013; NARASIMHAN et al., 2013; VAN CREVEL; OTTENHOFF; VAN DER MEER, 2002). No entanto, a história natural demonstra que apenas 1 a 5% dos indivíduos desenvolvem a TB ativa logo após à exposição (TB primária) e que 10 a 30% tornam-se infectados (TBL). Dentre estes indivíduos, aproximadamente 5 a 10% à infecção progride, transformando-se em TB ativa (TB secundária). É importante ratificar que para estes indivíduos o risco de adoecimento é maior nos dois primeiros anos após a infecção (NORTH; JUNG, 2004).

2.4 RESPOSTA IMUNE

Nos alvéolos, o patógeno interage primeiramente com os macrófagos alveolares que fagocitam os bacilos através dos receptores *Toll-like* (TLR), particularmente o TLR-2 e TLR-4 (BHATT; SALGAME, 2007). Essa interação induz a ativação de uma resposta inata pró-

inflamatória que resulta na ativação do fator de transcrição nuclear NF-κB e na produção de citocinas pró-inflamatórias como fator de necrose tumoral (TNF), interleucina 6 (IL-6) e interleucina 12 (IL-12), quimiocinas e óxido nítrico (DOHERTY; ARDITI, 2004).

Inicialmente, o bacilo *M. tuberculosis* reside em um vacúolo chamado fagossoma. No ciclo normal de maturação, o fagossoma contendo o patógeno, sofre uma série de fusões com vesículas endocíticas que culmina na fusão fagossoma-lisossoma. Os eventos de fusão promovem uma redução do pH interno e ação microbicida através de hidrolases ácidas derivadas do lisossoma. Logo, o ambiente hostil do fagolisossoma [pH ácido, intermediários reativos de oxigênio (ROI) e de nitrogênio (RNI), enzimas lisossomais, e peptídeos tóxicos] contribui com a destruição da maioria dos bacilos (KAUFMANN, 2001; KOUL et al., 2004). Entretanto, micobactérias patogênicas como o *M. tuberculosis* conseguem evitar a destruição pelas enzimas lisossomais interrompendo a maturação normal do fagossoma para fagolisossoma (MALIK et al., 2003). Assim, embora muitos bacilos sejam destruídos, neste momento, um grande número destes conseguem sobreviver, devido, em parte, aos mecanismos de evasão imunológica desenvolvidos por esta micobactéria.

Uma vez no interior do fagossoma, antígenos bacterianos podem ser processados e então apresentados via complexo principal de histocompatibilidade (MHC) classe I e II aos linfócitos T $CD8^+$ e $CD4^+$ respectivamente (HENDERSON; WATKINS; FLYNN, 1997). Aparentemente o controle imunológico deve-se predominantemente aos linfócitos T $CD4^+$ que se diferenciam em células T *helper* 1 (Th1) (Figura 2). Essa diferenciação, dependente da IL-12, resulta na produção de citocinas como interleucina-2 (IL-2) e interferon-gama (IFN-γ). O IFN-γ, além de ser produzido por linfócitos T $CD4^+$ e linfócitos T $CD8^+$, é também secretada por células *natural killers* (NK) (BARNES et al., 1993). O papel fundamental desta citocina na resposta imune ao *M. tuberculosis* foi demonstrado em vários trabalhos (FLYNN et al., 1993; LALVANI; MILLINGTON, 2008). Cooper e colaboradores (1993) demonstraram que camundongos *knouckts* para o gene do IFN-γ são altamente susceptíveis à infecção por *M. tuberculosis* (COOPER et al., 1993). O mesmo foi observado em indivíduos com deficiência nos genes do IFN-γ ou do seu receptor, os quais são mais susceptíveis às infecções micobacterianas (JOUANGUY et al., 1996). A principal função do IFN-γ é ativar macrófagos infectados, e dessa maneira, induzir atividade microbicida, através da produção de reativos de oxigênio e nitrogênio capazes de eliminar o bacilo (CHAN; CHAN; SCHLUGER, 2001)

Além do IFN-γ, outra citocina que desempenha papel importante é o TNF produzido

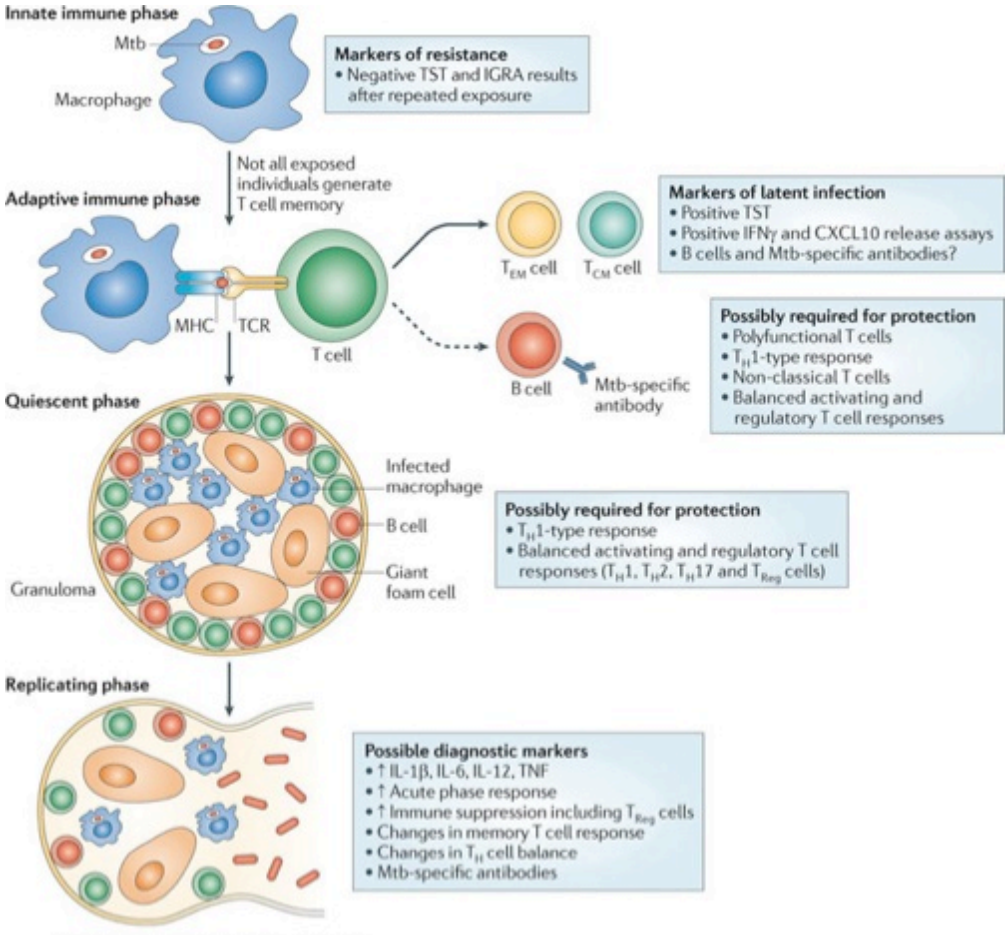
pelos macrófagos. Esta citocina promove à ativação de células endoteliais por meio da expressão de selectinas que facilitam o recrutamento de leucócitos para o sítio de infecção, levando a formação de uma estrutura conhecida como granuloma. O granuloma é essencial para conter a infecção tuberculosa, já que o mesmo funciona como uma barreira, envolta de tecido conjuntivo, delimitando o sítio de infecção (TUFARIELLO; CHAN; FLYNN, 2003). Esta estrutura se caracteriza por um acúmulo focal de macrófagos infectados ou não, células gigantes multinucleadas e células epitelióides, comumente envoltos por um halo de linfócitos T $CD4^+$ e $CD8^+$. Além destas células, o arcabouço do granuloma também é mantido pelo TNF. O papel do TNF na resposta granulomatosa à infecção pelo *M. tuberculosis* foi evidenciado com o aumento da reativação da TB em pacientes com artrite reumatóide submetidos à terapia com anticorpos anti-TNF (YASUI, 2014), sendo, portanto, uma citocina imprescindível para a formação e manutenção da estrutura granulomatosa.

A presença de granuloma no pulmão, associados ao acometimento dos gânglios linfáticos localizados no hilo pulmonar, formam o Complexo de Ghon, caracterizando radiologicamente e patologicamente a primo-infecção tuberculosa. Granulomas resultam da contínua estimulação antigênica dos linfócitos que se aglomeram, mas que não são eficazes na eliminação dos bacilos. A persistência de bacilos vivos, mas dormentes, ou fragmentos de bacilos provavelmente mantém no interior do granuloma à resposta inflamatória tecidual (FILHO et al., 2013). Estes aspectos imunopatológicos caracterizam a TBL. No entanto, em alguns indivíduos, em decorrência de fatores endógenos ou exógenos, o centro do granuloma, torna-se altamente necrótico e posteriormente liquefeito, levando à formação de cavidades. A necrose, resultante da destruição tecidual irreversível, induz futuramente a ruptura desta estrutura e liberação de bacilos viáveis a partir das vias aéreas respiratórias do hospedeiro, resultando na transmissão e surgimento da TB ativa (NORTH; JUNG, 2004; RUSSELL, 2007)

Embora a imunidade mediada por células esteja presente na maioria dos fenômenos de interação bacilo-hospedeiro, a resposta imunológica na TB também envolve a presença de linfócitos B (CHAN et al., 2014) (Figura 2). Os linfócitos B (timo-independentes), ativados por antígenos polissacarídicos e lipídicos, são suficientemente capazes de estimular a proliferação e diferenciação dos linfócitos B, sem necessidade de participação dos linfócitos T auxiliares. Estes linfócitos B, quando estimulados por esses antígenos, se diferenciam em células secretoras de anticorpos da classe IgM e IgG. Por outro lado, linfócitos B (timo-dependentes), ativados por antígenos protéicos, sob a influência de citocinas dos linfócitos T auxiliares, podem se diferenciar em células produtoras de IgG, IgE e IgA (ABBAS;

LICHTMAN; PILLAI, 2008). Nesse contexto, os anticorpos anti-Mtb não são capazes de penetrar no macrófago infectado e destruir o bacilo, contudo, outros mecanismos mediados por esses anticorpos, como opsonização, indução da cascata de ativação do sistema complemento e atuação na citotoxicidade celular podem contribuir com a eliminação do bacilo quando este estiver no meio extracelular (ABEBE; BJUNE, 2009; ACHKAR; CHAN; CASADEVALL, 2015; CHAN et al., 2014).

Figura 2. Caracterização da resposta imunológica desenvolvida na infecção pelo *M. tuberculosis*.



Fonte: WALZL et al., 2011.

2.5 FATORES DE VIRULÊNCIA

A capacidade de um microrganismo persistir no hospedeiro é, dentre outros, também

conferida por fatores ligados à virulência do patógeno, permitindo a evasão do sistema imune e a sua proliferação (KAUFMANN, 2001). Estes fatores são capazes de modular o sistema imunológico permitindo ao *M. tuberculosis* se adaptar ao hospedeiro. O sequenciamento completo do genoma *M. tuberculosis* H37Rv em 1998 por Cole e colaboradores foi imprescindível para auxiliar na compreensão acerca da patogenia induzida pelo complexo *M. tuberculosis* (COLE; BARRELL, 1998). Atualmente, sabe-se que o *M. tuberculosis* não produz fatores de virulência clássicos que possam explicar a manifestação da doença. Estas são predominantemente decorrentes da resposta exacerbada do hospedeiro à infecção e aos antígenos presentes na parede celular do bacilo. Dentre os elementos encontrados na parede celular, destacam-se as proteínas e os diferentes lipídios que compõem a camada mais externa do *M. tuberculosis*. Nesse contexto, o conhecimento destes antígenos e de sua imunogenicidade, é certamente a base para o desenvolvimento de medidas de intervenção mais efetivas.

2.5.1 Proteína Mce1A

A *mammalian cell entry protein 1A* (proteína Mce1A) foi identificada originalmente no *M. tuberculosis* e é expressa pelo gene *mce1a*, constituinte do operon *mce1* (ARRUDA et al., 1993; CASALI; RILEY, 2007). Modelos experimentais de infecção micobacteriana *in vitro* vêm sendo utilizados para determinar o impacto dessa proteína como um fator de virulência na TB. Inicialmente, Arruda e colaboradores demonstraram que a expressão da Mce1A por cepas não invasivas de *Escherichia coli*, promovem a entrada das bactérias em células de mamíferos não fagocíticas (ARRUDA et al., 1993). Posteriormente, foi demonstrado que microesferas de látex revestidas com a proteína Mce1A recombinante eram capazes de penetrar nas células HeLa (CHITALE et al., 2001). Corroborando com esses resultados, a interrupção do gene homólogo mce1A em *Mycobcaterium bovis* BCG reduziu a capacidade das micobactérias invadirem as células HeLa (FLESSELLES et al., 1999), fornecendo evidências, de que, no hospedeiro, a Mce1A despenham um papel na entrada e sobrevivência do bacilo em células de mamífero.

Estudos anteriores demonstram que essa proteína além de estar envolvida na entrada da célula do hospedeiro, também é capaz de promover a manutenção do estágio de latência na TB (MUSTAFA et al., 2014; SHIMONO et al., 2003; UCHIDA et al., 2007). Shimono e

colaboradores demonstraram que camundongos desafiados com cepas mutantes H37Rv *knockout* para o gene responsável pela expressão da proteína, tornou o bacilo mais virulento, induzindo o óbito precoce dos camundongos quando comparado com os que foram desafiados com a cepa virulenta que expressa o gene (SHIMONO et al., 2003). Apesar destes achados, os resultados não podem ser extrapolados para o modelo humano, pois não existe um modelo animal adequado que se aproxime da complexa infecção ocasionada pelo *M. tuberculosis* no homem.

Proteínas de membrana de superfície, a exemplo da Mce1A, exercem um importante papel na interação bacilo-hospedeiro uma vez que estas são as primeiras proteínas a interagir com a célula hospedeira. A Mce1A é, portanto, uma proteína que desempenha um papel relevante durante a resposta imune inata, e que, consequentemente, estimula a resposta imune adaptativa, tendo influência direta na magnitude da resposta desenvolvida. Porém, apesar dos estudos em modelo animal e dos estudos que utilizam linhagens de células específicas, em modelo humano, pouco se conhece sobre o seu papel imunológico, sua capacidade imunomodulatória, ou ainda, como esta modulação da resposta imune pode contribuir ou não para a proteção do hospedeiro.

2.5.2 Lipídios

Muitas proteínas, a exemplo da Mce1A, são conhecidas por serem importantes na compreensão da patogênese e como fatores de virulência. Por outro lado, o *M. tuberculosis* é único entre agentes patogênicos bacterianos que exibe uma grande variedade de lipídios na parede celular. Estes componentes lipídicos compreendem 60% do peso da parede celular e desempenham papel fundamental no ciclo de vida do bacilo, influenciando as respostas metabólicas e imunopatológicas do hospedeiro, além de também representarem fontes de antígenos no desenvolvimento de novos testes diagnósticos (RILEY, 2006). Ancorados à parede destacam-se a glicoproteína lipoarabinoman, ácidos micólicos e glicolipídios (BRENNAN, 2003). Outros possíveis componentes de parede celular são cardiolipina (CL), fosfatidilglicerol, fosfatidilcolina (FC), fosfatidil inositol (FI) e fosfolípidos básicos, tais como fosfatidil etanolamina (FE) e sulfatide (SL). Assim, devido a sua posição na parede celular, estes lipídios são antígenos biologicamente importantes que estimulam a resposta imune do paciente à infecção.

2.6 CONTROLE DA DOENÇA

As principais medidas de políticas públicas para conter o avanço da TB no mundo envolve, dentre outras medidas, o diagnóstico precoce e o tratamento efetivo.

2.6.1 Diagnóstico

Clinicamente, a reação exacerbada do hospedeiro ao processo imunopatológico da doença, pode ser evidenciada através da observação dos sinais e sintomas, os quais podem variar em sua intensidade, frequência e duração. Os sintomas mais frequentemente relatados são: febre, sudorese noturna e tosse. Inicialmente a tosse é seca, posteriormente progride para um aumento no volume de secreções purulentas, apresentando em alguns casos hemoptise. São ainda frequentes: a perda ponderal de peso devido à falta de apetite, astenia, prostração, dor torácica, entre outros (BRASIL, 2011). Estes achados são importantes no diagnóstico clínico, entretanto, são pouco conclusivos, pois podem ser confundidos com outras patologias de manifestação clínica semelhantes.

Além dos sinais e sintomas, o Programa Nacional de Controle da Tuberculose (PNCT) preconiza também a radiografia do tórax e a baciloscopia do escarro como critérios para o diagnóstico da doença (BRASIL, 2011). Embora a radiografia do tórax seja um exame importante, a análise radiológica não é, entretanto, um exame específico para detectar pacientes doentes, visto que lesões pulmonares semelhantes às causadas pelo *M. tuberculosis* podem ocorrer em outras doenças. Além disso, alterações não são demonstradas em até 15% dos casos (BURRILL et al., 2007). Por outro lado, a baciloscopia do escarro é o método mais utilizado rotineiramente, é um método simples e de baixo custo. Contudo, esta técnica apresenta baixa sensibilidade, principalmente em casos paucibacilares, pacientes pediátricos, pacientes com sorologia positiva para HIV e casos em casos de TB extrapulmonar. Além da baixa sensibilidade, que oscila entre 40 e 60%, a baciloscopia não é capaz de discriminar a espécie de micobactéria (GEBRE et al., 1995).

A cultura bacteriológica é o padrão ouro para o diagnóstico, pois é capaz de detectar 80 a 85% dos casos. Além disso, permite a identificação posterior da espécie através de testes bioquímicos, além de testar a sensibilidade às drogas utilizadas no tratamento. A limitação

deste método decorre da morosidade para o crescimento do bacilo, que compreende, aproximadamente, 15 a 60 dias após a coleta do material respiratório (BAYLAN, 2005).

Recentes avanços no campo da biologia molecular têm proporcionado o desenvolvimento de técnicas que reduzem o tempo de detecção e identificação do *M. tuberculosis*. Contudo, o elevado custo e operacionalização para o sistema de saúde pública são fatores que inviabilizam a utilização desta técnica em países em desenvolvimento ou subdesenvolvidos (SOINI; MUSSER, 2001)

Além destes, podem ainda ser utilizados como exames complementares: o teste tuberculínico (TT) e os ensaios de liberação de interferon-gama (IGRA). Ambos os testes são rotineiramente aplicados em países desenvolvidos e subdesenvolvidos, respectivamente, no diagnóstico da TBL. O TT avalia a hipersensibilidade tardia na pele em resposta à inoculação intradérmica do derivado protéico purificado (PPD), que é uma mistura de proteínas de micobactérias de baixo peso molecular. Assim, o TT é usado para classificar os indivíduos de acordo com a probabilidade de ter ou não a infecção, não significando doença ativa (BRASIL, 2011). Portanto, o TT não confirma o diagnóstico da TB, sendo apenas, um exame complementar.

A identificação de regiões do genoma do *M. tuberculosis,* que estão ausentes nas cepas vacinais do *M. bovis* e em outras micobactérias não tuberculosas, permitiu o desenvolvimento de novos marcadores diagnósticos. Estes testes são baseados na quantificação *in vitro* da resposta imune celular pela detecção de IFN-γ, uma citocina liberada pelos linfócitos T sensibilizados após cultivo com os antígenos codificados na região de diferença 1 (RD-1) do *M. tuberculosis*: antígeno alvo precoce de secreção primária com peso molecular de 6kD (do inglês *early-secreted antigenic target, ESAT-6),* proteína do filtrado de cultura com peso molecular de 10kD (do inglês *culture filtrate protein,* CFP-10) – homóloga a proteína L45 e peptídeo da porção do antígeno TB7.7 (MORI et al., 2004; PAI; RILEY; COLFORD, 2004). O IGRA apesar de ser originalmente criado para substituir o TT no diagnóstico da TBL, pode também ser utilizado para o diagnóstico da TB ativa. Alguns estudos demonstram que este teste apresenta uma elevada sensibilidade e especificidade no diagnóstico da TB ativa (LANGE et al., 2009; MENZIES, 2008; PAI; MENZIES, 2007). No entanto, o IGRA não consegue discriminar a doença ativa da infecção causada pelo bacilo e, portanto, não são recomendados para detecção de casos em áreas endêmicas.

2.6.2 Tratamento

O controle eficiente da TB também sustenta-se sobre o pilar do tratamento, o qual deve ser iniciado o mais rápido possível e conduz à cura do doente. O esquema básico utilizado no tratamento consiste na administração de quatro drogas: rifampicina, isoniazida, pirazinamida e etambutol. Os fatores relacionados às reações adversas no tratamento são de natureza multifatorial, mas uma das possíveis causas é precisamente a toxicidade dos fármacos associada ao longo período de terapia. sendo este um fator para a desistência ou abandono do tratamento (CASTELNUOVO, 2010). O objetivo do tratamento é eliminar o bacilo, mas para garantir que esse objetivo seja alcançado, o paciente deverá ser acompanhado mensalmente através da baciloscopia de controle, sendo indispensáveis as do 2º e 6º mês. Se positiva no segundo mês, é indicativa de resistência às drogas usadas no tratamento. Nesses casos é preconizada a realização de cultura e teste de sensibilidade para avaliação da necessidade de mudança de esquema terapêutico (BRASIL, 2011). Nesse cenário, a baciloscopia de controle permite avaliar a eficácia do tratamento adotado. Contudo, há uma real necessidade de maior viabilidade técnica que permita em tempo hábil avaliar a reposta ao tratamento de maneira que permita o melhor direcionamento e eficácia do mesmo. Como mencionado anteriormente, esta técnica carece de sensibilidade, principalmente em casos paucibacilares (DESIKAN, 2013).

3 JUSTIFICATICA

Desde 1993, uma série de estratégias e metas foram adotadas para promover o controle da TB. O diagnóstico precoce e a introdução imediata do tratamento são estratégias importantes para eliminar potenciais fontes de transmissão, contribuir com o rompimento da cadeia epidemiológica e reduzir o número de casos. Contudo, apesar dos grandes investimentos e dos progressos feitos em relação à doença, o desafio de instituir políticas adequadas de tratamento e controle se esbarram nas dificuldades em se realizar um adequado diagnóstico. A baixa detecção dos casos permanece como fator de perpetuação da doença e, consequentemente, como um dos principais obstáculos para o controle global da TB.

Os testes atualmente utilizados deixam a desejar, o que dificulta o rastreamento do doente e o monitoramento da evolução clínica. Além disso, evidencia-se uma grande fragilidade na detecção da doença em crianças e em indivíduos co-infectados, fatores que contribuem para a detecção tardia da TB. Diante desse cenário, a grande preocupação em nível mundial continua a ser a falta de um diagnóstico de fácil execução e de baixo custo (WHO, 2014). Os métodos baseados em biologia molecular, apesar de rápidos, ainda são onerosos e complexos, a utilização comercial em larga escala em países em desenvolvimento e, principalmente, subdesenvolvidos, seria economicamente inviável.

Recentes avanços na área pós-genômica tem permitido a identificação de novos e potenciais antígenos imunogênicos que possam atuar como marcadores da doença. Portanto, a caracterização de antígenos protéicos e /ou lipídios que induzem uma resposta imunológica no hospedeiro, torna-se um importante passo para o desenvolvimento de novas ferramentas de diagnóstico e resposta terapêutica.

4 OBJETIVOS

4.1 OBJETIVO GERAL

Avaliar biomarcadores para o diagnóstico e monitoramento do tratamento da tuberculose pulmonar

4.2 OBJETIVOS ESPECÍFICOS

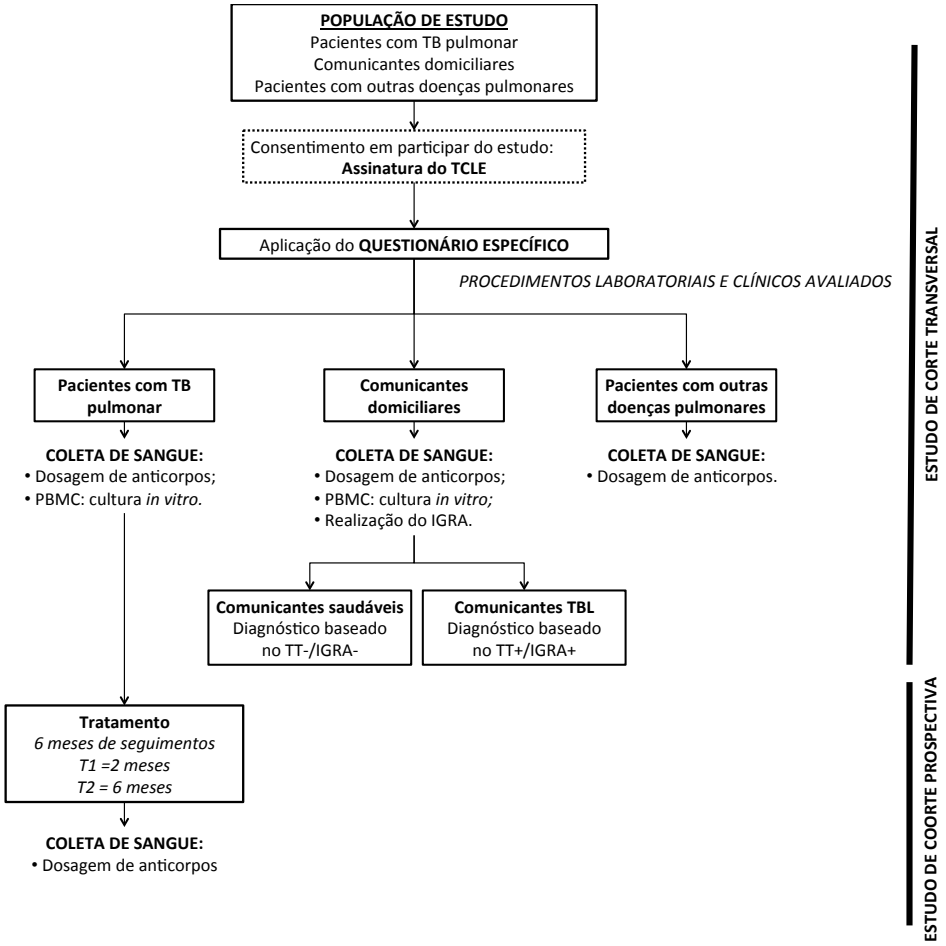
1. Avaliar a imunogenicidade da proteína Mce1A em indivíduos com tuberculose ativa, através da detecção de anticorpos séricos da classe IgM, IgG e IgA;
2. Determinar os níveis séricos de anticorpos anti-Mce1A, antes, durante e após o tratamento dos pacientes com TB pulmonar;
3. Determinar o perfil de citocinas e quimiocinas produzidas após estímulo *in vitro* da proteína Mce1A.
4. Avaliar a imunogenicidade dos fosfolipídios (cardiolipina, fosfatidilcolina, fosfatidilinusitol, fosfatidiletanolamina, sulfatide) em indivíduos com tuberculose ativa, pela detecção de anticorpos séricos da classe IgM e IgG;
5. Determinar os níveis séricos anti-fosfolipídios, antes, durante e após o tratamento dos pacientes com TB pulmonar.

5 PACIENTES E MÉTODOS

5.1 DESENHO DO ESTUDO

O presente trabalho trata-se de um estudo descritivo analítico composto por duas fases. Em sua primeira fase foi realizado um estudo de corte transversal com o objetivo de avaliar e caracterizar o potencial uso de biomarcadores no diagnóstico da TB. Na segunda fase, uma coorte prospectiva formada por pacientes com TB, durante um período de seis meses, a partir da inclusão na primeira fase, foi realizado para avaliar o uso dos biomarcadores na resposta terapêutica (Figura 3).

Figura 3. Desenho experimental do estudo. IGRA: *interferon-gamma release assay*; TT = teste tuberculínico; TCLE = termo de consentimento livre e esclarecido.



5.2 LOCAL DO ESTUDO

Elegeu-se como local de estudo desta investigação dois centos de saúde: o Instituto Brasileiro para Investigação da Tuberculose (IBIT) e o 6º Centro de Saúde Rodrigo Argolo. O IBIT pertence à Fundação José Silveira (FJS) e está localizado no bairro da Federação. O 6º Centro de Saúde Rodrigo Argolo (6ºCS) esta localizado no bairro de Tancredo Neves e pertence ao Distrito Sanitário Cabula-Beiru, da rede pública de saúde do Município de Salvador. Os centros de saúde disponibilizam à população, por meio de uma equipe multidisciplinar, atendimento nas áreas de diagnóstico, tratamento e prevenção da TB. Visando romper a cadeia de transmissão da doença, os centros ainda fornecem avaliação médica, clínica e laboratorial para os indivíduos que convivem com o paciente TB.

5.3 POPULAÇÃO DE ESTUDO

A população de estudo é composta por pacientes recém diagnosticados com TB pulmonar, seus respectivos comunicantes domiciliares e pacientes diagnosticados com outras doenças pulmonares.

Foram definidos como casos de TB pulmonar aqueles indivíduos que apresentavam suspeita clínica de TB associado às seguintes situações: resultado positivo na pesquisa direta do BAAR no escarro; e ou alterações sugestivas de TB na radiografia de tórax; ou ainda, diagnóstico presumido sem confirmação bacteriológica e indicação de tratamento. A radiografia do tórax foi classificada como: 1) lesão cavitária, quando observou-se infiltração pulmonar com cavitação; 2) não cavitária, quando observou-se o infiltrado pulmonar sem cavidade, ou 3) normal, quando não se observou nenhuma lesão pulmonar. O escarro foi realizado através da pesquisa direta do bacilo álcool ácido resistente através da coloração de Ziehl-Neelsen e classificado como negativa, positiva com uma cruz (1+), positiva com duas cruzes (2+) ou positiva com três cruzes (3+).

Como grupo de comparação, comunicantes domiciliares, saudáveis ou com TBL, foram também selecionados. Foram considerados comunicantes domiciliares todos aqueles que convivem no mesmo domicílio, ou que relatassem, pelos menos, 100 horas de contato com o paciente bacilífero. A presença ou não da infecção e, posterior alocação dos comunicantes nos grupos infectados e saudáveis, foi estabelecida pelo TT e pelo IGRA. Para

estes voluntários, procedeu-se a inoculação de 0,1 mL (2 UT) do PPD RT23 (Statens Serum Institut, Copenhagen, Dinamarca), utilizando-se a técnica de Mantoux, por profissionais treinados segundo as normas do Ministério da Saúde. A leitura do resultado do TT foi realizada 48 horas após a aplicação, pelo método de palpação do diâmetro transverso máximo da enduração cutânea. A resposta ao PPD foi categorizada como: 0 a 5 mm, negativo; ≥ 5 mm, positivo e sugestivo de infecção. O IGRA comercial utilizado foi o QuantiFERON-TB Gold in tube (QFT-IT; Cellestis Limited, Carnegie, VIC, Austrália). Todas as etapas para realização do teste seguiram as recomendações do fabricante (MAZUREK et al., 2005). 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 foi considerado positivo. Um valor abaixo ou igual a 0,5 UI/mL de IFN-γ em resposta ao mitógeno foi definido como indeterminado. Para garantir a precisão na alocação dos grupos infectados e saudáveis, voluntários que apresentaram resultado do TT e IGRA discordantes ou indeterminado foram excluídos do estudo.

Pacientes com outras doenças pulmonares que não TB foram também selecionados como grupo de comparação. Pacientes com tosse e/ou expectoração por pelo menos duas semanas, mas, obrigatoriamente, com exclusão do diagnóstico de TB foram convidados a participar do estudo como voluntários.

Os critério de exclusão utilizados no estudo foram: indivíduos em uso de corticosteroides e/ou co-infectados pelo HIV. Todos os voluntários elegíveis para participar do estudo foram atendidos consecutivamente nos centros de saúde. Portanto, para este estudo foi utilizada uma amostra de conveniência.

5.4 CONSIDERAÇÕES ÉTICAS

O estudo foi aprovado pelo Conselho de Ética em Pesquisa (CEP) do Centro de Pesquisa Gonçalo Moniz da Fundação Oswaldo Cruz (CEP-CPqGM/FIOCRUZ) com parecer nº 365/2011. Seguindo a legislação vigente, resolução 466/12, todos os indivíduos elegíveis receberem informações básicas sobre o projeto de pesquisa. Em seguida, atestaram a sua participação como voluntário mediante à assinatura no Termo de Consentimento Livre e Esclarecido (TCLE).

5.5 *ASPECTOS CLÍNICOS E LABORATORIAIS*

Os voluntários que consentiram em participar da pesquisa, foram submetidos a um questionário estruturado e coleta de sangue por punção venosa.

5.5.1 *Questionário*

Após a assinatura do TCLE, todos os voluntários responderam um breve questionário, o qual foi testado e ajustado previamente para esta população. O questionário foi criado com o objetivo de avaliar dados demográficos, sócio-econômico e clínico. Outras informações relevantes foram obtidas mediante a consulta nos prontuários médicos e nos resultados dos exames de procedimento padrão realizados pelos voluntários. Em seguida, todos os participantes foram submetidos à coleta de sangue por punção venosa.

5.5.2 *Coleta de sangue e obtenção de soro*

Por voluntário, foram coletados 15 mL de sangue total, os quais foram distribuídos em duas amostras. A amostra de 5 mL de sangue foi acondicionado em um tubo sem anticoagulante para obtenção do soro. As amostras foram centrifugadas a 1.500 rpm por 10 min. Em seguida, as alíquotas de soro foram devidamente identificadas e armazenadas a – 20ºC até a determinação dos títulos de anticorpos nas amostras. Uma segunda amostra, contendo 10 mL, foi utilizada para os ensaios de cultura celular.

5.5.3 Obtenção da proteína e dos fosfolipídios

A proteína Mce1 foi cedida pelo Prof Dr Lee Riley, através de uma colaboração com a University of California (Berkeley, EUA), e todas as etapas de expressão, clonagem e

purificação são descritas resumidamente a seguir. A proteína recombinante foi expressa em um sistema procarioto, *Escherichia coli*, a partir da clonagem em um vetor de expressão. A confirmação da expressão da proteína foi avaliada através da eletroforese em gel de poliacrilamida e pela técnica de *western blot*. Em seguida, a proteína recombinante foi obtida por cromatografia de afinidade, resultando em um percentual de purificação acima de 90%. Os fosfolipídios utilizados nos ensaios sorológicos foram fosfolipídios sintéticos adquiridos da Avanti Polar Lipids, Inc (Alabaster, AL, EUA).

5.5.4 Ensaio imunoenzimático (ELISA)

Em um segundo momento, as amostras de soro foram submetidas a um ensaio imunoenzimático, ELISA indireto, e são descritas resumidamente a seguir. Microplacas de poliestireno de 96 poços foram previamente sensibilizadas com a proteína Mce1A (10µg/mL) ou com os fosfolipídios CL, FE, FI, FC (10µg/mL) ou SL (1µg/mL) diluídas em álcool anidro P.A.

Após 12h de incubação a 4ºC as placas foram lavadas com tampão fosfato-salino (PBS 1x) e os sítios livres foram bloqueados com PBS-BSA-1% por 1h à temperatura ambiente (TA). Após três lavagens com PBS 1x, as amostras de soro, no volume de 100µL/poço, foram diluídas a 1:100 em PBS 1x e incubadas à 2h por TA. Após esse período as placas foram lavadas e incubadas com segundo anticorpo (IgA, IgG ou IgM) humano conjugada a peroxidase (Sigma, USA), diluída a 1:10.00, 1:50.000 ou 1:10.000, respectivamente em PBS 1x. Após incubação por 1h à TA, foram feitas três lavagens. A reação enzimática desenvolveu-se após adição do cromógeno 3,3', 5,5'-tetrametilbenzidina – TMB (Sigma, USA). Posteriormente, a reação foi interrompida com adição de 50µL/poço de H_2SO_4 (2M). Todos os ensaios foram feitos em triplicata e os valores de densidade óptica (DO) foram obtidos em espectrofotômetro (405nm).

5.5.5 Ensaio de cultura celular e dosagem das citocinas

Em condições estéreis, após a coleta de 10mL de sangue heparinizado, o mesmo foi submetido à separação de células mononucleares do sangue periférico (PBMC) por gradiente de densidade (ficoll-hypaque). Em seguida, as células foram contadas em câmara de Neubauer através do corante Azul de Trypan. As células foram cultivadas em uma placa de cultura de 96 poços em meio RPMI 1640 completo com 2nM glutamina, 1nM piruvato de sódio, 1nM HEPES, 1% antibiótico e antimicótico e 10% soro bovino fetal (SFB) por 48 horas à 37ºC na ausência (controle negativo) ou presença dos seguintes antígenos: proteína Mce1A (10µg/mL), antígeno bruto do *M. tuberculosis* (H37Ra; 10µg/mL) e fitohemaglutinina (PHA) (5µg/mL; controle positivo). Cada condição foi realizada em triplicata. Após período de incubação, o sobrenadante das culturas foi transferido para eppendorfs previamente identificados e estocados no freezer a -80ºC para posterior dosagem de citocinas.

Por fim, as citocinas foram dosadas pelo método colorimétrico multi-ensaio utilizando esferas de látex (ensaio multiplex *Cytometric Bead Array* – Becton-Dickinson). O sobrenadante das culturas celulares foram incubados com esferas de látex contendo anticorpos específicos para as citocinas avaliadas, também chamadas de *beads* (anticorpos primários) e anticorpos secundários correspondentes fluoresceinados. Para este experimento foi utilizado o Kit Human Th1/Th2 Cytokine CBA e o Kit Human Chemokine (BD Biosciences) para dosagem de ensaio das citocinas: IL-2, IL-4, IL-6, IL-10, IFN-γ e TNF, e das quimiocinas: CXCL8/IL-8, CXCL-9, CXCL10/IP-10, CCL2/MCP-1 e CCL5/RANTES. Todos os procedimentos citados acima foram realizados conforme preconizado pelo manual do fabricante.

5.6 COORTE PROSPECTIVA

Seis meses de tratamento diário com isoniazida, rifampicina, pirazinamida e etambutol, na dosagem de 5 mg/Kg/dia, foi oferecido aos pacientes recém-diagnosticados com TB pulmonar, esquema de tratamento padrão preconizado pelo MS (BRASIL, 2011). A droga foi oferecida sem custos adicionais. Todos foram convidados a retornar a cada 30 dias para reposição dos comprimidos e no 2º e 6º mês uma nova coleta de sangue foi realizada para as dosagem sorológicas.

5.7 ANÁLISE ESTATÍSTICA

Todos os dados coletados foram inseridos em planilha eletrônica do programa Excel e posteriormente analisados, utilizando o programa GraphPad Prism v.5.0 (GraphPad Inc., San Diego, CA). As variáveis qualitativas nominais foram descritas como frequência relativa (%) e absoluta (n). As demais variáveis contínuas foram expressas em média ± desvio padrão (DP). Para verificação da distribuição de normalidade das variáveis quantitativas foi utilizado o teste de *Kolmogorov-Smirnov.* Em seguida, os dados foram submetidos ao test *t* Student ou análise de variância simples (*one way* ANOVA) para comparar a média de dois grupos ou, entre três, ou mais grupos, respectivamente. Além disso, outros testes como correlação de Pearson, análise de variância composta, chi–quadrado, foram realizadas para verificar possíveis correlações entre as variáveis estudadas. Uma curva de Característica de Operação do Receptor (ROC) foi construída para avaliar a capacidade das imunoglobulinas predizer a TB pulmonar. Os valores de sensibilidade e especificidade para cada escore foram distribuídos em um gráfico bidimensional (xy), no qual o eixo y equivale à sensibilidade e o eixo x, a 100-especificidade. As diferenças foram consideradas estatisticamente significantes para valores de $p \leq 0,05$.

6 RESULTADOS

Os resultados obtidos durante o meu doutoramento serão apresentados na forma de manuscritos a serem submetidos à publicação por revistas indexadas. O primeiro artigo é uma revisão de literatura de caráter exploratório, a qual tem como objetivo sintetizar os principais estudos sobre a proteína Mce1A desde a sua descoberta (1993) até os dias atuais. A análise da literatura publicada auxiliou na estruturação conceitual que fornece sustentação ao desenvolvimento dos objetivos específicos 1, 2 e 3. O protocolo de revisão deste estudo tem como base as ferramentas metodológicas utilizadas em revisões sistemáticas e metanálises, descritas seguindo as recomendações *Preferred Reporting Items for Systematic Reviews and Meta-Analyses* (PRISMA).

O segundo e o terceiro manuscrito são trabalhos experimentais em humanos envolvendo a proteína Mce1A. Atendendo aos objetivos específicos 1 e 2, o segundo manuscrito aborda os resultados obtidos sobre a imunogenicidade da proteína através da detecção de anticorpos séricos como potenciais biomarcadores para o diagnóstico e monitoramento da TB pulmonar. O terceiro manuscrito contempla o objetivo específico 3 e avalia a produção de citocinas e quimiocinas por PBMC após estímulo *in vitro* com a proteína. Por último, o quarto manuscrito que corresponde ao objetivos específicos 4 e 5, aborda a utilização de anticorpos anti-fosfolipídios como potenciais biomarcadores para o diagnóstico e tratamento da TB pulmonar.

6.1 MAMMALIAN CELL ENTRY PROTEIN 1A (MCE1A): UMA REVISÃO DA LITERATURA

Manuscrito em processo de submissão: **TAKENAMI, I**.; PETRILLI, J.; RILEY, L. W.; ARRUDA, S. Mycobacterial mammalian cell entry protein 1A (Mce1A): a literature review 23 years after its discovery

Mycobacterial mammalian cell entry protein 1A (Mce1A): a literature review 23 years after its discovery

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Summary

The mammalian cell entry gene (mce-1) and its product, mammalian cell entry protein 1A (Mce1A), are associated to entry and survival of *Mycobacterium tuberculosis* within human macrophages, a process central to the pathogenesis of tuberculosis (TB). However, the precise mechanisms involving mce1 gene and its protein remain unclear. Thus, this literature review aims at summarizing existing research findings about Mce1A protein since its discovery in 1993 until nowadays. Using tools that are designed for use in systematic reviews we identified 48 relevant articles indexed in databases which includes MEDLINE/PubMed, Scopus, Embase and LILACS. The wide distribution of the mce1 gene in *M. tuberculosis,* including Mycobacterium genus, and other bacterial species (pathogenic and non-pathogenic), implicates that the presence of this virulence gene is not an indicator for the pathogenicity. Instead, the pathogenicity might be determined by its expression during infectious process. Although Mce2A protein exhibits 67% of homology with Mce1A, the immune response is triggered by Mce1A by immunogenic epitope based on sequence KRRITPKD, located just on the surface of this protein, indicating that the sequence carries a unique functionality of *M. tuberculosis* Mce1A. Further, the Mce1A protein is also possibly associated with granuloma formation and metabolism and transport of lipids. Taken together, these findings provide evidences that the Mce1A protein plays an important role in the pathogenesis of TB. In order to promote novel strategies for diagnosis, prevention and treatment, additional studies involving *M. tuberculosis* Mce1A-host interaction are required to assess the clinical relevance of Mce1A in humans.

Keywords: Mammalian cell entry gene; Mce1A; tuberculosis.

1. Introduction

Tuberculosis (TB) is an infectious disease caused by mycobacterial species belonging to the *Mycobacterium tuberculosis* complex, including *M. tuberculosis* that is the most common etiological agent of human TB. Infection by *M. tuberculosis* can result in active disease or, more commonly, latent infection [1]. One third of the world's population is infected with *M. tuberculosis*, and this population represents an enormous reservoir for disease reactivation [2]. The investigation of mechanisms of immune response that leads to control of initial infection and prevent reactivation of latent infection is crucial to combating this disease. In that context, a specific protein namely mammalian cell entry protein 1A (Mce1A) has an important role during infectious process [3] and could represent a source of antigen for breakthrough in the diagnosis and control of disease.

Arruda et al. discovered the first mce gene, now known as mce1, which encodes the putative Mce1A protein from *M. tuberculosis* in 1993 [3], even before the genome sequence of *M. tuberculosis* H37Rv has been revealed [4] Nowadays the mce genes have become important to the research community due their crucial role in the pathogenesis of the disease. However, despite numerous studies, the precise mechanisms involving mce1 gene and its Mce1A protein remain unclear. Thus, this literature review aims at summarizing existing research findings about Mce1A protein since its discovery in 1993 until nowadays, in order to help understand its role in bacillus-host interactions and pathogenicity.

2. Methods

2.1 Search strategy and inclusion criteria

The literature review was carried out following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. Using rigorous methods, we seek to identify, evaluate and synthesize the scientific evidence of mce1 gene and its Mce1A protein. The searches were carried out in the following databases MEDLINE, SCOPUS, EMBASE and LILACS. Search topic included “tuberculosis”, “*Mycobacterium*”, “Mce1A protein” and “mce1 gene” using the search terms described in Table 1. We restricted our selection criteria to include only articles published since the discovery of the protein in 1993 to the present day (May 2015). Then, the relevance of the studies were assessed in details by hierarchical approach based on title, abstract, and full-text article. The study flow chart depicting the literature search and selection is presented in Fig. 1. We did not use any restrictions for the language of the published studies. One review author (IT) extracted the following data from included studies and the second author (JP) checked the extracted data.

2.2 Study selection

The initial search identified 226 articles, which were reduced to 106 after eliminating duplicates. After reviewing, 56 studies were excluded as irrelevant based on title and abstract. Of the 50 remaining, by reading full papers, a total of 48 articles were finally included for review (Fig. 1).

2.3 Categories of analysis

Five categories were developed for the analysis of findings: 1) Discovery of the mce1 gene and its protein product; 2) Role of Mce1A protein and its molecular structure; 3) Presence of gene and protein in Mycobacterium genus and other bacterial species; 4) Immunological aspects and granuloma formation and 5) Influence on the *Mycobacterium* growth and its lipid metabolism.

3 Results

3.1 Discovery of the mce1 gene and its protein product

The putative *M. tuberculosis* virulence gene was first identified in 1993 when the recombinant expression of Mce1A encoded by the mce1 gene in *Escherichia coli* allowed this non-pathogenic bacterium the ability to invade non-phagocytic HeLa cells After complete sequencing of the genome, the mammalian cell entry (mce) gene was subsequently localized in the strain *M. tuberculosis* H37Rv and termed mce1. However, the analysis of the whole genome also revealed the presence of three copies of others mce (mce2, mce3 and mce4) located in distinct operons, each consisting of eight genes, which were organized in a highly similar manner with extensive homology. The mce1 gene, located a 13-gene mce1 operon, encodes a number of proteins (MceA-F), including Mce1A protein, which is localized to the cell wall surface of the *M. tuberculosis* [4–6] (Table 2).

3.2 Role of Mce1 protein and its molecular structure

Several studies have demonstrated the important role of the mce1 gene and their product (Table 2). The expression of mce1 gene is important for the virulence of mycobacteria, because the protein facilitates invasion into nonphagocytic cells of non-pathogenic *Escherichia coli* and latex microspheres [3,7–10]. Flesselles et al. showed that a BCG strain mutated in mce1 exhibited reduced ability to invade the non-phagocytic HeLa cells [11]. Moreover, *M. tuberculosis* knockout mutants deficient in the expression of the

mce1 operon reported alteration on their ability to multiply when inoculated in mice [12,13]. Chandolia et al. recently demonstrated that an antisense strategy, method for gene silencing, reduced by approximately 60% of Mce1A expression by *M. tuberculosis*, resulting in attenuation of the bacilli in cell culture model of infection [14]. These findings have been endorsed by some authors to demonstrate that Mce1A protein is immunogenic and elicit specific antibody response, suggesting that it is expressed during natural infection with *M. tuberculosis* [15–17]. On the other hand, polyclonal antibody against conserved sequences of Mce1A protein can be used to block mycobacterial infection in human macrophages [18]. Taken together, these results suggest that Mce1A protein plays an important role during the pathogenesis of the bacillus.

The crystal structure of protein revealed that the Mce1A is an α/β protein consisting of two major (α and β) domains connected by a long α-helix. Furthermore, *in silico* modeling of Mce1A suggests its structure resembles that of Colicin N, a pore-forming bacterial toxin. The model predicts that binding of Mce1A to its cognate receptor is followed by a conformational change of the protein and perforation of target cell membrane [19].

The protein's uptake activity contains to a 58-amino acid domain in its central region located between positions 106 and 163 of the protein. Das et al. showed that the epitope involved in binding and uptake of the *M. tuberculosis* could be narrowed down to a core of four amino acids (TPKD) and that upstream flanking residues (KRR) also contributed to binding [16,19]. In this respect, some authors suggest that cell uptake by latex beads coated with Mce1A seems to involve a receptor-mediated process [7,9]. Additionally, other authors also suggest that there may be a receptor for Mce1A on host cells [12,19]. However, despite extensive investigation, the receptor for Mce1A remains elusive.

Previous studies have shown that Mce1A is highly homologous to Mce2A, Mce3A, and Mce4A [7,20]. However, the epitope associated to promote uptake of *M. tuberculosis* in host cells is not conserved in any these proteins, implying that the proteins bind to different receptors and possibly target different cells [19]. Sequential alignments showed that Mce1A and Mce2A are highly homologous (67% similarity), while the other combinations provide only about 30% similarities [7,20]. Nevertheless, the Mce1A protein, which includes the putative receptor-binding domain, promotes the uptake of latex beads by HeLa cells, whereas recombinant Mce2A protein does not display this property [7,10]. These findings indicate that the sequence carries a unique functionality of Mce1A protein.

3.3 Presence of gene and protein in Mycobacterium genus and other bacterial species

Parker et al. were the first to report that the mce operon genes, including mce1 gene, are distributed throughout the genus *Mycobacterium* [21]. *Polymerase Chain Reaction* (PCR) and sequence analysis showed the presence of a mce1 homology in members of the *M. tuberculosis* complex [6,22–24]*, M. avium* complex including *M. avium*, *M. scrofulaceum* and *M. intracellulare* [21] and of the *M. smegmatis* [24–26]. Subsequently, the presence of mce1 was also detected in *Mycobacterium leprae* [22,26,27]. This finding suggests that possibly in addition to cell entry, *mce* operons perhaps have other functions in Mycobacterium genus. Nevertheless, some studies also reported other bacterial species containing mce genes including the genus Nocardia, Janibacter, Nocardiodes, Amycolatopsis and Streptomyces [6] and the species *L. interrogans* [28] (Table 2).

Although these data show that there is homology between *M. tuberculosis* and other bacterial species, the wide distribution of the mce operons in pathogenic and nonpathogenic bacteria implicates that the presence of these putative virulence genes is not an indicator for the pathogenicity bacterial. Since the presence or absence of mce operons does not correlate with pathogenicity, it is possible that the expression profile of the mce operons is associated with virulence of pathogenic mycobacteria [29–33]. Corroborating these findings, Kumar et al. showed that differential expression of the operons was dependent on the growth conditions and nutrient availability [30]. Haile et al. also noted that mce1 gene are differentially expressed under aerobic conditions when compared to oxygen-limited conditions [29]. Furthermore, *in silico* analysis reveals that non-synonymous single nucleotide polymorphisms (nsSNPs) at mce1 operon could play a pivotal role in causing functional changes in *M. tuberculosis* that may reflect upon the biology of the bacteria [34]. On the other hand Joon et al. demonstrated the presence of two functional promoters for mce1 operon in *M. tuberculosis,* which could potentially segregate different functions of a single operon [35]. Thus, it can be concluded that the pathogenicity of mce1 operon genes might be determined by their expression and that can be related with nsSNPs.

3.4 Immunological aspects and granuloma formation

In addition to the uptake of bacteria in non-phagocytic cells, Mce1A protein is also possibly associated with granuloma formation [12,13,36,37] (Table 2). In the murine model of infection, a *M. tuberculosis* mce1 operon mutant elicits an aberrant granulomatous response, resulting in uncontrolled replication and failure to enter a persistent state [13,38]. Then, all mice infected with mce1 mutant died more quickly than mice infected with the wild type or complemented strains [13,38]. The hypervirulence of the mutant strain may be resulted from

its inability to induce a proinflammatory response that plays an important role in granuloma formation, leading to containment of viable bacilli into state of dormancy [13,36]. These results suggest that the expression of the mce1 products, including Mce1A protein, is required for organized granuloma formation, which is protective to the host and required for the persistence of bacilli.

To determine whether differential cytokine induction might explain the abnormal granuloma formation, Shimono et al. infected mice macrophages with mce1 mutant. The mutant strain showed a decreased ability to induce tumor necrosis factor (TNF), interleukin 6 (IL-6), monocyte chemoattractant protein 1 (MCP-1), and nitric oxide (NO), but not interleukin 4 (IL-4) [13]. This finding, coupled with other studies [39–41], provide evidence that Mce1A protein up-regulating expression of proinflamatory cytokine and chemokine maybe link to the invasion and survival capacity of *M. tuberculosis* in macrophages. Indeed, Kohwiwattanagun et al. reported that the Mce1A alone are not able to modulate the chemokine response but that recombinant protein may promote chemokine induction by augmenting the interaction between bacteria and mammalian cells [40].

In order to investigate the role of regulating the mce1 operon, Casali et al. created a gene that encodes a negative transcriptional regulator of the mce1 operon, which was designated mce1R. The outcome of infection in mice demonstrated that the overexpression of mce1R result in repression of the mce1 genes *in vitro*, showing that mce1R acts as a negative regulator for the mce1 operon [42]. According to the results of Uchida et al., a *M. tuberculosis* mutant disrupted in mce1R showed hypervirulent phenotype, causing a premature death compared with mice infected wild type [36]. It is likely that the hypervirulence mechanism is due to diminished Th1-type cytokine production [36,42,43]. In another study published by Miyata et al, when administered intraperitoneally the recombinant Mce1A protein, as a post-exposure vaccine, provide a immune response that prevents reactivation of latent infection [44].

In summary, these observations indicate that products of the mce1 operon, including Mce1A protein, may be involved in modulating the host inflammatory response. Indirectly the inflammatory response may induce granulomas formation that restricts the spread of the tubercle bacilli. In this way, the bacterium can enter a persistent state (dormancy) without being eliminated. In contrast, the absence of these products can cause diffuse granuloma formation and aberrant proinflammatory cell migration, this resulting in a faster progression to death. Thus, this proinflammatory response appears to play an important role in granuloma induction and support the theory of an involvement of this protein in the formation of an

organized and protective granuloma.

3.5 Influence on the Mycobacterium growth and its lipid metabolism

It has been proposed that mce1 operon can also encode ABC-like lipid transporters and substrate-binding proteins [6,45]. This has already been demonstrated by Stavrum et al., the Mce1 protein complex has a significant role in the transcriptional activation of genes involved in substrate trafficking during the initial phase of macrophage infection by *M. tuberculosis* [46].

Interestingly, the cell wall of *M. tuberculosis* disrupted in the mce1 operon contains more than 10-fold greater amounts of mycolic acids than that of wild-type *M. tuberculosis* [47], suggesting that mce1 mutant accumulates more mycolic acids than the wild type and complemented strains. Subsequently, this accumulation of mycolic acid is exacerbated when bacteria are cultured in the presence of palmitic acid or arachidonic acid, demonstrated that the mce1 operon may serve as a mycolic acid re-importer [48].

In order to investigate the effect of free mycolic acids on toll-like receptor-2 (TLR-2), Sequeira et al. compared the interaction of *M. tuberculosis* and its mce1 operon mutant with macrophages and epithelial cells line [49]. They demonstrated that wild type induce significantly higher levels of IL-8, MCP-1, chemokine ligand 5 (CCL5), and interferon gamma-induced protein 10 (IP-10) in both cell types than did by mce mutant. In contrast, free mycolic acids reduced the ability of the mammalian cells to respond to a TLR-2 agonist, suggesting that differences in mycolic acid abundance in the *M. tuberculosis* cell wall can affect TLR-2-mediated pro-inflammatory response in both cells. It is possible that the constitutive expression of the mce1 genes is necessary to maintain lipid metabolism, enabling it to survive long term and proliferate inside granuloma.

Furthermore, some studies have demonstrated that Mce1A expression can be regulating by aerobic conditions. The RT-PCR results showed that mce1 was expressed throughout the cultivation period and this study together with the results of mce2, mce3 and mce4 contributes to understanding of the bacilli involved in latent tuberculosis [29,50]. Beste et al recently reported that in *M. bovis* BCG, the *mce1* operon appears to be a component of the switch to slow growth rate, which is consistent with the proposed role in virulence of *M. tuberculosis*. These results suggest novel perspectives for unraveling the mechanisms involved in the switch between acute and persistent TB infections and provide a means to study aspects of this important phenomenon *in vitro* [51].

4. Conclusion

There are a number of challenges that need to be elaborated for progress in the investigation of Mce1A protein. In summary, the results of this literature review provide evidence that the Mce1A protein plays an important role in the immunopathogenesis of TB. However, the immune response against Mce1A protein, particularly in humans, is poorly understood. Although determinants of virulence of *M. tuberculosis* contribute to the development of infection, the disease is also critically dependent on the immune status of the host. Thus, much of what is known is based on studies done in animals, particularly in animal model [7,12,13,36,38,39,42–44] or in human cell lines [3,8–11,15,18,19,27,49]. Until this moment, there have been no studies evaluating the ability of the Mce1A protein to stimulate T-cells response in peripheral blood mononuclear cell (PBMC) culture or in infection of human macrophages. Research in human is equally needed to understand the host-protein interaction by providing news perspectives as a potential target for diagnostic and therapy of TB.

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Table 1. Search strategy.

Search terms
#1 (Tuberculosis OR tb). #2 (Mycobacterium). #3 ("Mammalian cell entry protein 1A" OR "Mce1A"). #4 ("mammalian cell entry 1 gene" OR "mce1 gene").
#1 OR #2 AND #3.
#1 OR #2 AND #4.
#1 OR #2 AND #3 OR #4.

Figure 1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flowchart.

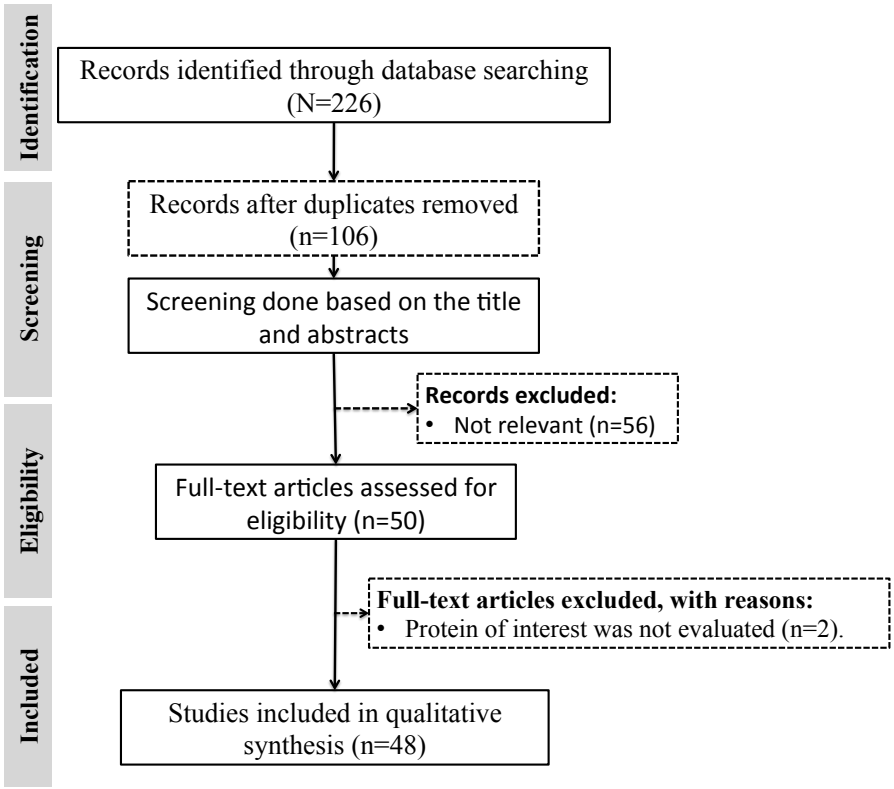

Figure 2. Main findings described in the literature on mce1 gene and their Mce1A protein

Author / year / reference	Mains results
Arruda et al. 1993 [3]	The Mce1A protein was able to promote uptake of *Escherichia coli* non-pathogenic into HeLa cells and survive inside human macrophages.
Cole et al. 1998, Tekaia et al. 1999, Casali et al. 2007 [4–6]	Sequencing of the *M. tuberculosis* genome revealed that mce1A was part of an operon that encoded eight putative membrane-associated proteins: YrbEA-B, MceA-F.
Chitale et al 2001, Casali et al 2002, Lu et al 2006, El-shazly et al. 2007 [7–10]	Latex beads coated with Mce1A protein or peptides containing the Mce domain are internalized by HeLa cells.
Flesselles et al. 1999, Gioffré et al. 1999, Shimono et al. 2003 [11–13]	Inactivation of *mce* genes can attenuate *M. tuberculosis* virulence in a murine model, which could be explained as a reduced ability to invade and/or persist in host cells.
Ahmad et al. 1999, Harboe et al. 1999 , Harboe et al. 2002 [15–17]	Mce1A protein and synthetic peptides are recognized by antibodies from TB patients.
Sivagnanam et al. 2012 [18]	Antibody against conserved region of Mce1A protein blocks *M. tuberculosis* infection in human macrophages.
Das et al. 2003 [19]	The predicted molecular structure of Mce1A is similar to the structure of bacterial toxin colicin N. Moreover, the immunogenic linear epitope of Mce1A protein is based in the KRRITPKD region located on the surface of Mce1A.
Chitale et al 2001, Mitra et al 2005 [7,20]	Mce1A and Mce2A are highly homologous, which has 67% similarity, while the other combinations (Mce3A, Mce4A) gave only about 30% similarities.
Chitale et al 2001, Casali et al 2002, Lu et al 2006, El-shazly et al. 2007 [7–10]	Latex beads coated with Mce1A protein or peptides containing the Mce domain are internalized by HeLa cells.
Parker et al 1995, Flesseles et al 1999, Wiker et al 1999, Harboe et al 1999, Zumárraga et al 1999, Haile et al 2002B, Casali et al 2007, Zhang et al 2012, Sato et al 2007, Santhosh et al 2005 [6,11,17,21–24,26–28]	Analysis of the complete genome sequence showed the presence of a mce1 homology in members of the *Mycobacterium genus* including *M. avium, M. scrofulaceum and M. intracellulare, M. smegmatis, M. leprae, M. bovis*; other bacterial genus including Nocardia, Janibacter, Nocardiodes, Amycolatopsis and Streptomyces and *L. interrogans*.
Kumar et al 2005, Haile et al 2002, Lam et al 2008, Beste et al 2009 [29,30,32,51]	The presence of multiple copies of the operon may suggest that there is a differential expression of the operon depending on the nutrient availability and oxygen conditions.
Shimono et al 2003, Lima et al 2007, Uchida et al 2007, Cheigh et al 2010, Gioffré et al 2005 [12,13,36,38,43]	The *mce1* products, including Mce1A, are required to stimulate T helper 1-type immunity and for organized granuloma formation, which is both protective to the host and necessary for the persistence of *M. tuberculosis*.
Shimono et al 2003, Kohwiwattanagun 2007 [13,40]	The operon mce1 mutant strain showed a decreased ability to produce TNF, IL-6, MCP-1, NO. On the other hand, induce high levels of IL-4.

6.2 DETECÇÃO DE ANTICORPOS SÉRICOS ANTI-MCE1A COMO POTENCIAIS BIOMARCADORES PARA O DIAGNÓSTICO E MONITORAMENTO DA TB PULMONAR

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Immunoglobulin G response to mammalian cell entry 1A (Mce1A) protein as biomarker of active tuberculosis

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Summary

Cell wall components are major determinants of virulence of *Mycobacterium tuberculosis* and they contribute to the induction of both humoral and cell-mediated immune response. *The mammalian cell entry protein 1A (Mce1A), in the cell wall of M. tuberculosis, mediates entry of the pathogen into mammalian cells*. Here, we examined serum immunoglobulin levels against Mce1A as a potential biomarker for diagnosis and monitoring tuberculosis (TB) treatment response. A total of 39 pulmonary TB patients and 66 controls (including 15 healthy *household contacts*, 19 latently infected household contacts, 14 non-TB and 18 leprosy patients) were examined. After new diagnosis, TB patients were followed until the end of treatment. Serum samples were screened by ELISA for IgA, IgM and total IgG to the Mce1A protein. The mean levels of IgA, IgM and total IgG were significantly higher in TB patients when compared with control groups ($p<0.0001$). Sensitivities of IgA, IgM and IgG test were 59, 51.3 and 79.5%, respectively, while the specificities observed were 77.3, 83.3 and 84.4%, respectively. Combination of IgG with IgA and/or IgM did not improve the sensitivity. Routine sputum smear examination diagnosed 26 (66.7%) of 39 TB cases, while total IgG ELISA identified 32 (82%) of 39 patients. After anti-TB treatment, the mean for IgA and total IgG levels decreased significantly compared to baseline ($p<0.0001$). The positive test results for IgA, IgM and total IgG against Mce1A were 61.5, 53.8 and 82%, respectively, before treatment, and 46.2, 30.8 and 43.6%, respectively, at the end of treatment. For comparison, the sputum smear test was negative in 100% of the TB cases after the treatment. These results suggest that circulating total IgG antibody to Mce1A could be a complementary tool to diagnosis pulmonary TB. However, the sputum smear was more effective to monitor the TB treatment.

Keywords: Mce1A protein, pulmonary tuberculosis, immunoglobulin A, immunoglobulin M, immunoglobulin G, diagnosis, treatment.

1. Introduction

Tuberculosis (TB) is a chronic bacterial infection, caused primarily by the obligate human pathogen *Mycobacterium tuberculosis*. Although nearly one-third of the human population is infected with *M. tuberculosis*, only 10% of these individuals develop active disease during their lifetime [1]. Early diagnosis and effective treatment of TB cases is the most effective tool available to control the disease.

The identification of the bacillus by microscopic examination of sputum smear or by culture has several limitations. Approximately 40% of TB patients test negative by microscopy, and culture requires a long time for the growth of *M. tuberculosis*, which delays diagnosis [2, 3]. Additionally, other pulmonary non-TB diseases such as cancer, pneumonia, pulmonary abscess, bronchitis and bronchiectasis may present with similar clinical symptoms and radiographic patterns [4, 5]. Therefore, a rapid diagnostic test with both high sensitivity and specificity is still needed. The most significant advance in last few years was the development of real time PCR assay (Xpert® MTB/RIF) for detection of *M. tuberculosis* DNA and mutations associated with resistance to rifampicin. However, the higher cost and sophisticated infrastructure requirements have remained major barriers for their large-scale implementation for routine use. Further, the test does not eliminate the need for conventional tests, which are required to monitor treatment success and detect resistance to drugs other than rifampicin. Currently the standard method to monitor treatment response is still sputum smear microscopy conversion after 2 months of treatment. However, this test is not very sensitive, and such a test is often not reliably performed in most TB-endemic settings.

M. tuberculosis survives and multiplies inside the host's macrophages by modulating the cells' antimicrobial effector response. In 1993, Arruda et al. reported that recombinant mammalian cell entry protein (Mce1A) expressed in *Escherichia coli* allows this non-pathogenic bacterium to invade HeLa cells and survive inside macrophages [6]. Mce1A is encoded by *mce1A*, which is one of 13 genes that comprise an operon. Shimono et al. showed that *M. tuberculosis* disrupted in the *mce1* operon failed to elicit a strong Th1-type immune response and caused a formation of poorly organized mouse lung granulomas comprised mostly of foamy macrophages [7]. Casali et al. and Uchida et al. have showed that *mce1*A expression is regulated when *M. tuberculosis* is intracellular or *in vivo*. Taken together, these results suggest an essential role of Mce1A protein for the immunopathogenesis of TB.

In the present study, we evaluated humoral response (IgA, IgM and total IgG) of TB patient against Mce1A as a potential biomarker for diagnosing TB and monitoring TB treatment response in Salvador, Brazil, a setting with a high prevalence of TB.

2. Study population and methods

2.1 Setting

This prospective study was conducted at Centro de Saúde Rodrigo Argolo (CSRA) in Salvador, Brazil. A total of 105 eligible participants were recruited using convenience sampling from January 2012 to October 2013.

2.2 Study population

The participants were categorized into five groups, as follows.

Pulmonary TB patients (n=39): All cases of pulmonary TB attending in the CSRA were invited to participate in this study. The diagnosis of pulmonary TB was established by symptoms consistent with TB and one or more of the following characteristics: 1) chest radiography (CXR) suggestive of TB opacities, 2) sputum samples that contained acid-fast bacilli on microscopy, 3) individuals who responded to anti-tuberculosis drugs. Sputum smear microscopy was performed by Ziehl-Neelsen staining and the results were grouped as negative, 1+, 2+, or 3+.

Household contacts (n=35): At the time of the TB case identification, their household contacts (HHC) were enrolled into the study. HHC included all those who lived in the same household as the TB patient, or who have reported at least 100 hours of contact with the patient. The tuberculin skin test (TST) was performed on all HHC. TST was done by the Mantoux procedure with 2TU of RT23 purified protein derivate (RT23 PPD) (Staten Serum Institute, Copenhagen, Denmark). Reading was performed after 72 hours by a trained nurse. The TST response was categorized as: 0 to 5 mm, negative; ≥ 5 mm, positive and indicative of infection by *M. tuberculosis*. To confirm *M. tuberculosis* latent infection (LTBI), interferon-gamma release assay (IGRA) was also performed. We used QuantiFERON-TB Gold In Tube (QFT-IT; Cellestis Limited, Carnegie, Victoria, Australia). The test was performed according to the manufacturer's instructions [8]. The cut-off value for a positive response was 0.35 IU/mL. Samples that gave indeterminate or discordant TST and IGRA results were excluded. Blood was drawn for the baseline IGRA before the TST was administered; both were conducted on the same day. Then, household contacts were stratified into two groups: 1) TST-negative and IGRA-negative [healthy household contact; HHC(-)] or 2) TST-positive and IGRA-positive [latently infected household contacts; HHC(+)]. Those with TB-like symptoms were further evaluated, including by sputum examination and/or chest radiography to exclude disease.

Non-TB patients (n=13): This category included subjects with symptoms of TB (cough, fever, loss of appetite) but with other lung diseases. TB was ruled out in this group by sputum smear and culture. Among patients who presented with other lung diseases, five had bacterial pneumonia, two had lung cancer, one had bronchial asthma and the remaining five subjects had other pulmonary infections.

Leprosy patients (n=18): An additional control group included cases of leprosy. These cases were confirmed bacteriologically using smear microscopy and histological examination. The sera from these leprosy patients were obtained from Hospital Couto Maia.

Exclusion criteria were volunteers who tested positive for human immunodeficiency virus (HIV) and patients taking immunosuppressive drugs. The study was previously approved by the Human Subject Ethics Committee of Oswaldo Cruz Foundation in Salvador, Brazil. All subjects provided informed consent to participate in this study according to our institutional guidelines.

2.3 Serum specimens and ELISA

Serum specimens were obtained upon recruitment and stored at −80 °C until tested. Patients who had a confirmed diagnosis of TB received anti-tuberculosis treatment with isoniazid, rifampin, pyrazinamide and ethambutol for 2 months followed by isoniazid and rifampin for 4 months. Serum samples were prospectively collected at baseline, 2 months and 6 months after starting the treatment.

Measurement of total IgG, IgM, and IgA against Mce1A protein was performed with an indirect enzyme-linked immunosorbent assay (ELISA). The recombinant protein used in this study was kindly provided by Dr L. W. Riley (University of California, Berkeley, CA, USA). Mce1A protein (10µg/ml) was diluted to 1:1000 in ethanol and 50µl of the solutions were dried overnight in polystyrene ELISA well plates (Greiner bio-one). ELISA plates were blocked with 100µl of 3% low fatty-acid bovine serum albumin (BSA) (US Biologicals) and washed with phosphate buffered saline (PBS, pH 7.4) (GIBCO, Invitrogen). Frozen serum samples were thawed twice and diluted 1:100 in 3% BSA. The diluted sample was added to the plate and incubated for 1 hour at room temperature (RT), followed by three washes with 1x PBS. Then, 100 µl of 1:10.000, 1:50.000, 1:10.000 goat-derived anti-human IgM, total IgG and IgA, respectively, labeled with horseradish peroxidase (HRP) (Sigma–Aldrich) diluted in 3% BSA/PBS was added, followed by incubation at RT for 1 hour and washed again with 1x PBS. Finally, 100 µl of tetramethylbenzidine substrate (TMB) (Invitrogen *Life Technologies*)

was added and the reaction was stopped immediately with 100 µl of 2N sulfuric acid. Reactions were read within 10 min at 450 nm in a spectrophotometer (Thermo Scientific). The results were read out as the average of optical density (OD) of triplicate assays and samples were re-run if >10% coefficient of variance was observed.

2.4 Statistical analysis

Data were analyzed by GraphPad Prism v.5.0 (GraphPad Inc., San Diego, CA). The immunoglobulin levels were expressed as mean and standard deviation (mean ± SD). Statistical variations were analyzed by Student's *t* test or one-way analysis of variance (ANOVA) followed by the Tukey test. For longitudinal analysis of immunoglobulin levels on anti-TB treatment, differences between time points were first assessed by two-way ANOVA and Tukey's *post hoc* test. Pearson's correlation test was used to assess correlation between immunoglobulin levels and clinical data. The ability of immunoglobulin levels to discriminate active TB from non-TB disease was performed with receiver operating characteristic (ROC) curve and the area under curve (AUC) was also analyzed. The significance of association for categorical variables was estimated by Chi-squared test or Chi-square test for linear trend. The level of statistical significance was set at $P < 0.05$.

3. Results

3.1 Characteristics of study population

The active TB group included 39 pulmonary TB patients. Of these, 26 (66.7%) had a positive sputum smear result. From these TB patients' households, 57 HHC were enrolled into the study. Of these, 23 (40%) were excluded due to the following reasons: six (10.5%) did not return for TST reading; three (5.3%) had indeterminate IGRA and fourteen (25%) had discordant results between TST and IGRA. Of the 34 eligible HHC, 19 (56%) were latently infected (TST and IGRA positive) and 15 (44%) were healthy controls (TST and IGRA negative). Besides the HHC group, we included non-TB (n=13) and leprosy (n=18) patients. The demographic and clinical characteristics of these study subjects are listed in Table 1. Chi-squared tests demonstrated no significant differences (p>0.05) in these demographic characteristics among the study groups.

3.2 IgA, IgM and total IgG response against Mce1A protein

The anti-Mce1A IgA, IgM and total IgG ELISA results are shown in Fig.1. All immunoglobulin patterns were significantly higher in untreated TB patients than in the control

groups ($p<0.0001$). The mean values of IgA against Mce1A protein were significantly higher in pulmonary TB patients (0.874 ± 0.734) than in HHC (0.310 ± 0.202), HHC(-) (0.462 ± 0.179), HHC(+) (0.190 ± 0.126), non-TB (0.354 ± 0.153) and leprosy patients (0.347 ± 0.198; Fig.1A). IgM levels were also able to discriminate TB patients (0.643 ± 0.353) from leprosy patients (0.068 ± 0.061; Fig.1B).

Further, TB patients were found to have extremely high levels of total IgG against Mce1A (0.814 ± 0.331) than those in the control groups [HHC, HHC(-), HHC(+), non-TB and leprosy patients (Fig.1C)]. The mean values and SD for the control groups were 0.501 ± 0.239, 0.427 ± 0.112, 0.559 ± 0.290, 0.446 ± 0.149 and 0.353 ± 0.062, respectively. Interestingly, seven (36.8%) of 19 HHC with LTBI had mean IgG levels similar to those of the TB patients.

3.3 Correlation between IgA, IgM and total IgG response against Mce1A protein and clinical data

When TB patients were categorized by chest X-ray status, anti-Mce1A total IgG levels were higher in pulmonary TB patients with cavitary lesions than in those without cavitary lesions ($p=0.046$). On the other hand, no significant differences were observed for other immunoglobulins (IgA and IgM, $p=0.745$ and $p=0.645$, respectively). Furthermore, no difference was found in immunoglobulins levels when compared with sputum smear status, sex, race, age, BCG, use of alcohol, tobacco and drugs in the TB patients group ($p>0.05$).

Additionally, immunoglobulin's levels were also evaluated in HHC according to their TST and IGRA results. There was no correlation between IgM, IgG or IgA levels and TST induration diameter (r=-0.002, $p=0.988$; $r=0.080$, $p=0.657$; $r=-0.196$, $p=0.266$, respectively). Similarly, there was no relationship between IFN-γ levels measured by IGRA and the IgM levels ($r=0.099$, $p=0.578$). However, negative and positive correlation was observed between TST diameter and IgA levels ($r=-0.625$, $p<0.0001$) or IgG levels ($r=0.358$, $p=0.041$), respectively.

3.4 Qualitative ELISA results and diagnostic values for pulmonary TB

Based on the significant difference found in the quantitative analysis of serological tests, we performed an ROC analysis. For this purpose, we defined pulmonary TB group as the ''diseased group'' and their HHC, non-TB and leprosy patients as the ''control group''. AUC analysis results were obtained for all, independent of the immunoglobulin used. With the cut off value of 0.482 OD, established according to the ROC method, the IgA test was

positive in 24 (61.5%) of 39 TB patients. Overall sensitivity and specificity of the test was 59% and 77.3%, respectively (Fig 2A, 2B). With the cut off value of 0.585 OD, the IgM test was positive in 21 (54%) of 39 TB patients. The sensitivity and specificity was 51.3 and 83.3%, respectively (Fig. 2C, 2D). The ROC curve determined that the optimal index cutoff value for the anti-Mce1A total IgG ELISA was 0.546 OD, with a corresponding sensitivity of 79.5% and a specificity of 84.4% (Figure 2E, 2F). The total IgG anti-Mce1A was positive in 32 (82%) of 39 TB patients. Further, when we considered the combination of immunoglobulins (IgG+IgA, IgG+IgM or IgG+IgA+IgM) results, the sensitivity was not improved (data not shown). Interestingly, seven latently infected HHC with positive IgG test results showed a negative correlation with the IFN-g production ($r=-0.857$, $p=0.024$). All of them had a TST reaction of ≥ 15 mm of induration.

3.5 Time-course changes in IgG, IgM and IgA antibody titers after initiation of anti-TB treatment

The mean levels of IgG, IgM and IgA antibodies against Mce1A protein in TB patients decreased during treatment. The mean of IgA and IgM levels decreased significantly between the first and third (6 months) serum samples ($p=0.036$ and $p=0.019$, respectively; Fig. 3A and 3B). Similarly, the mean IgG levels decreased significantly between the first, second (2 months) and third (6 months) serum samples ($p<0.0001$, Fig. 3C). Furthermore, after the completion of treatment, no statistical difference was observed between mean IgM and total IgG levels in TB patients and HHC(-) or HHC(+) ($p=0.290$ and $p=0.185$, respectively; Fig. 3B and 3C)

The frequency of positive test results for IgA, IgM and total IgG is shown in the Table 3. There was also a significant negative linear trend in the positive test results among the TB group before the treatment, at the end of 2 months and 6 months of treatment (IgA, $p=0.023$; IgM, $p=0.001$ and IgG, $p<0.0001$.

4. Discussion

Here we assessed the humoral response to Mce1A as a potential biomarker for the diagnosis of TB and response to treatment. We found that immunoglobulin levels were significantly higher in new pulmonary TB patients than those of the control groups. In this study, a cut-off value of 0.546 for the total IgG ELISA provided the best result, detecting 82% of the TB patients compared with IgA (61.5%) and IgM (53.8%) results (Table 2, Fig. 2). However, the combination of immunoglobulin tests did not increase the positive test results.

In the control groups, very few individuals had a positive serology; 22.7% were positive for IgA, 16.7% for IgM and total IgG. Other studies that examined humoral response against other *M. tuberculosis* proteins in the control population found responses ranging from 4.1 to 23.4% for IgA, 7 to 19.5% for IgM and 12 to 24.7% for IgG [9-11].

In the present study, routine sputum smear examination diagnosed 26 (66.7%) of 39 TB cases. The IgG ELISA identified 10 (76.9%) additional cases among those who tested sputum-smear negative. ROC-curve analysis showed high sensitivity and specificity of the IgG ELISA. These results are better than those previously reported in other studies using different *M. tuberculosis* protein antigens for TB serodiagnosis [9-11]

The response to Mce1A is associated with advanced cavitary TB (IgG: cavitary vs non-cavitary, p=0.046). Mizusawa et al reported that titers of IgG to glycolipid were higher in pulmonary TB patients with cavitary lesions than in those without cavitary lesions [12]. This increase of total IgG in cavitary TB patients may reflect liquefied caseum containing high numbers of the tubercle bacilli. Here, we hypothesize that the *mce1* gene is upregulated during growth and multiplication of the bacilli in the host, leading to higher expression of Mce1A protein and thereby promoting antibody response to the protein. Hence, IgG antibody could play a role as an inflammatory marker in pulmonary TB.

Interestingly, a group of seven HHC with LTBI with TST induration diameter >15 were found to be positive by the IgG ELISA. It is known that latently infected individuals produce high levels of IFN-γ, and that this cytokine is a key determinant in protection against TB [13]. We wonder if low-level IFN-γ production associated with high-level anti-Mce1A IgG response may indicate a predictive biomarker for progression from LTBI to active disease. We will follow these seven HHC and expand our study to identify more such HHC to see if any of them develop TB over time.

Mycobacterium leprae has a homologue of the mce1A gene, as do nontuberculous mycobacteria (NTM) [14-16]. Das et al demonstrated that an immunodominant linear epitope KRRITPKD (residues 131-138 in Mce1A) is highly conserved in *M. tuberculosis* [16]. This may explain the difference in response we observed between TB and leprosy patients. The Mce1A protein is also present in cell wall of *Mycobacterium bovis* BCG [17-19]. However, we did not observe any statistical difference in the anti-Mce1A ELISA response between BCG-vaccinated and non-vaccinated patients. Since BCG is given at birth in Brazil, such a difference is unlikely to be observed in adults. The high anti-Mce1A IgG response indicates active disease but perhaps also recent infection in those with LTBI (as observed among HHC with LTBI).

Alternative ways to monitor TB treatment response are needed. We found that changes in serum IgG anti-Mce1A antibodies appear to be a useful marker to monitor treatment response. The decline in total IgG may reflect decrease in bacterial burden and healing of lung lesions in these patients. However, despite the decrease in the immunoglobulin levels, 69.2% of TB patients had IgG test that remained positive after 2 months of anti-tuberculosis treatment.

Serologic tests based on *M. tuberculosis* are not recommended by World Health Organization (WHO) for the diagnosis of TB. However, our test based on response to Mce1A may be usable as an initial screening test, especially in those who are sputum-smear test negative. It may also have utility superior to smear tests for monitoring treatment response. The limitation of this study includes the sample size and exclusion of TB patients co-infected with HIV. The sensitivity of serologic tests in acquired immune deficiency syndrome (AIDS) patients or immunosuppressed patients is unacceptably low in tests based on most *M. tuberculosis* proteins. A larger study including HIV-infected patients is necessary to further evaluate the validity of this new test.

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Table 1. Demographic and clinical characteristics of pulmonary TB patients, their household contacts, non-TB and leprosy patients.

	TB patients (n = 37)	HHC (n = 35)	HHC infection status		Non-TB patients (n = 13)	Leprosy Patients (n=18)
			Uninfected (n = 16)	Infected (n = 19)		
Age, years, mean ± SD	40.8 ± 15.5	30.4 ± 20.8	29.9 ± 18.3	30.7 ± 22.8	44.6 ± 13.6	40.3 ± 14.9
Male, n (%)	23 (62.2)	15 (42.8)	7 (43.8)	8 (42.1)	5 (38.5)	8 (44.4)
BCG scar, n (%)	24 (64.9)	26 (74.3)	11 (68.8)	15 (78.9)	11 (84.6)	16 (88.9)
History of tuberculosis, n (%)	2 (5.4)	1 (2.8)	0	1 (6.2)	1 (7.7)	0
Sputum density of index, n (%)						
Negative	13 (35.1)	-	-	-	13 (100)	-
1+	11 (29.7)	-	-	-	0	-
2+	7 (19.0)	-	-	-	0	-
3+	6 (16.2)	-	-	-	0	-
CXR*, n (%)						
Cavities	16 (57.1)	-	-	-	-	-
No cavities	12 (42.9)	nd	nd	nd	-	-

*CXR not available for nine TB patients.

BCG = bacille Calmette-Guérin; CXR = chest radiograph; HHC = household contacts; SD = Standard deviation; TB = Tuberculosis; nd = not done.

Figure 1. IgA (A), IgM (B) and IgG (C) antibody levels against Mce1A protein in different groups of sera tested. Statistical significance was determined by Kruskal-Wallis followed by Dunn´s test; significance was considered at $p<0.01$, $p<0.001$ or $p<0.0001$ as represented by *, ** and ***, respectively. TB = tuberculosis patients (n=39), HHC = household contacts of pulmonary tuberculosis patients (n=34); HHC(-) = Healthy household contacts (n=15); HHC(+) = latently infected household contacts (n=19); Non-TB = patients without tuberculosis (n=14); LP = leprosy patients (n=18); OD = optical density. The height of the line within each bar represents the median OD value.

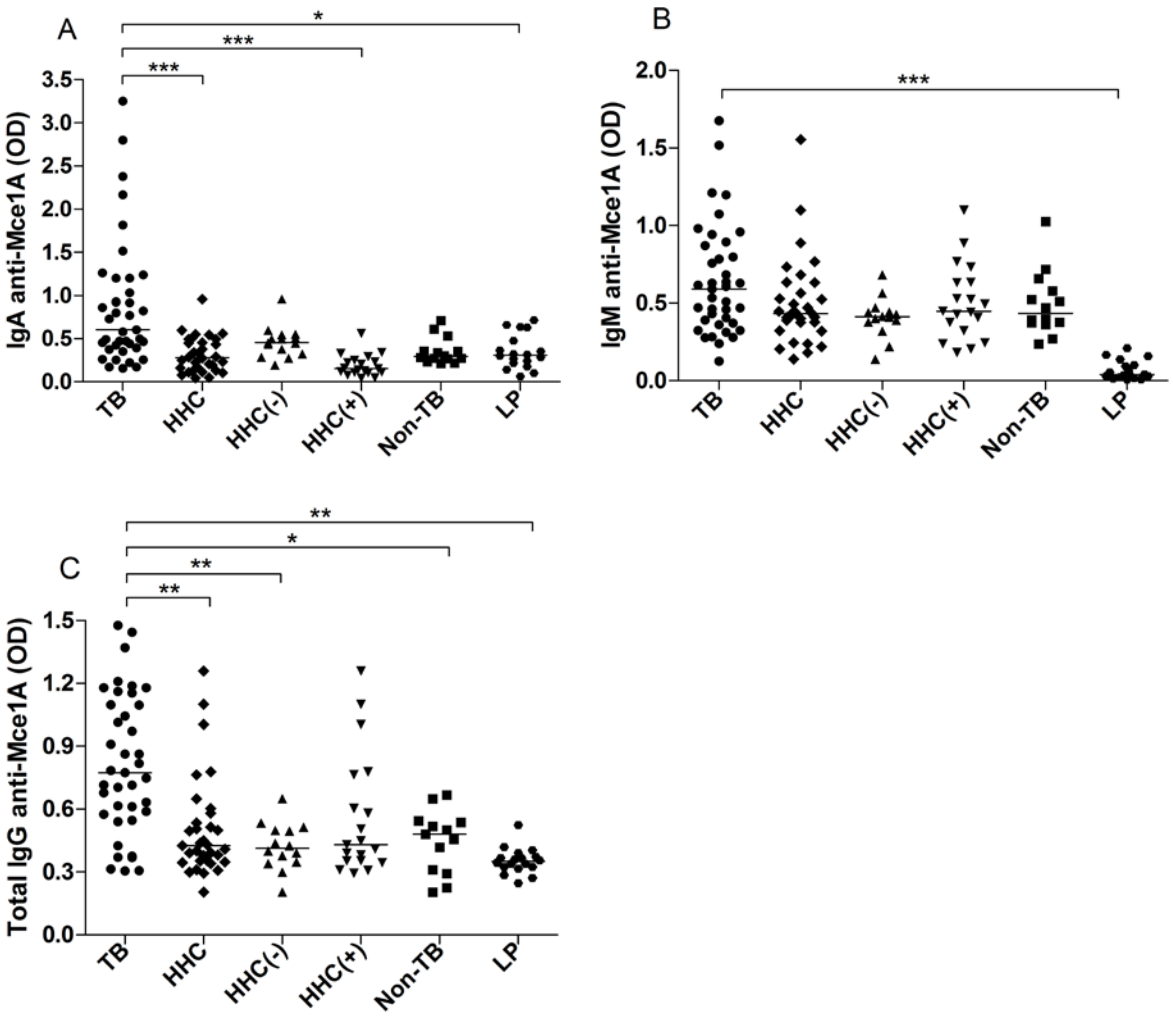

Figure 2. Receiver operating characteristic analysis for comparison of IgA anti-Mce1A (A, B), IgM anti-Mce1A (C, D) and total IgG anti-Mce1A (E, F) between active pulmonary TB patients (n=39) and control group (n=66). AUC = area under the curve; CI = confidence interval; Se = sensitivity; Sp = specificity; LR+ = positive likehood ratio; TB = tuberculosis

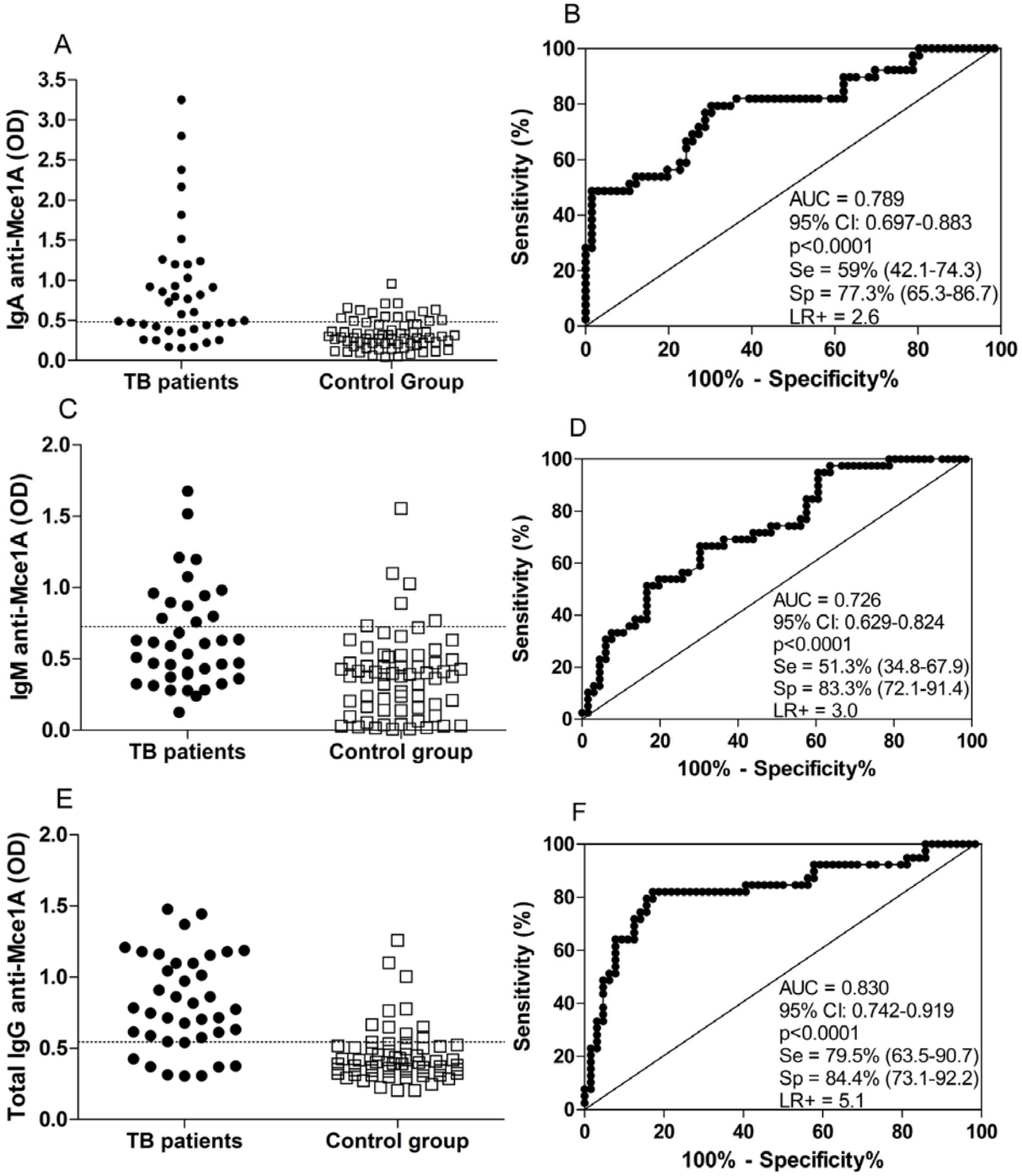

Table 2. Positivity to Mce1A protein in different groups of study. TB = tuberculosis; HHC = household contacts.

Study groups	No. of cases	No. of seropositive patients (%)		
		IgA	IgM	IgG
TB patients	39	24 (61.5)	21 (53.8)	32 (82.1)
Healthy HHC	15	7 (46.7)	2 (13.3)	1 (6.7)
Latently infected HHC	19	1 (7.14)	6 (42.8)	7 (36.8)
Non-TB patients	14	3 (21.4)	3 (21.3)	3 (21.4)
Leprosy patients	18	4 (22.2)	0 (0)	0 (2)

Chi-squared test, $p < 0.0001$ for *all* immunoglobulins.

Figure 3. Levels of serum IgA (A), IgM (B) and total IgG (C) against the Mce1A protein in active TB patients during the anti-tuberculous treatment. Sampling time (0) just before treatment, (2) after 2 months of treatment, (6) after 6 months of treatment. Statistical significance was determined by Friedman test (between TB patients) or Kruskall-Wallis test (between groups), followed by Dunn's posttest; significance was considered at p<0.01, p<0.001 or p<0.0001 as represented by *, ** and ***, respectively. The height of the line within each bar represents the median OD value. TB = tuberculosis; HHC = household contacts; Mo = months; OD = optical density.

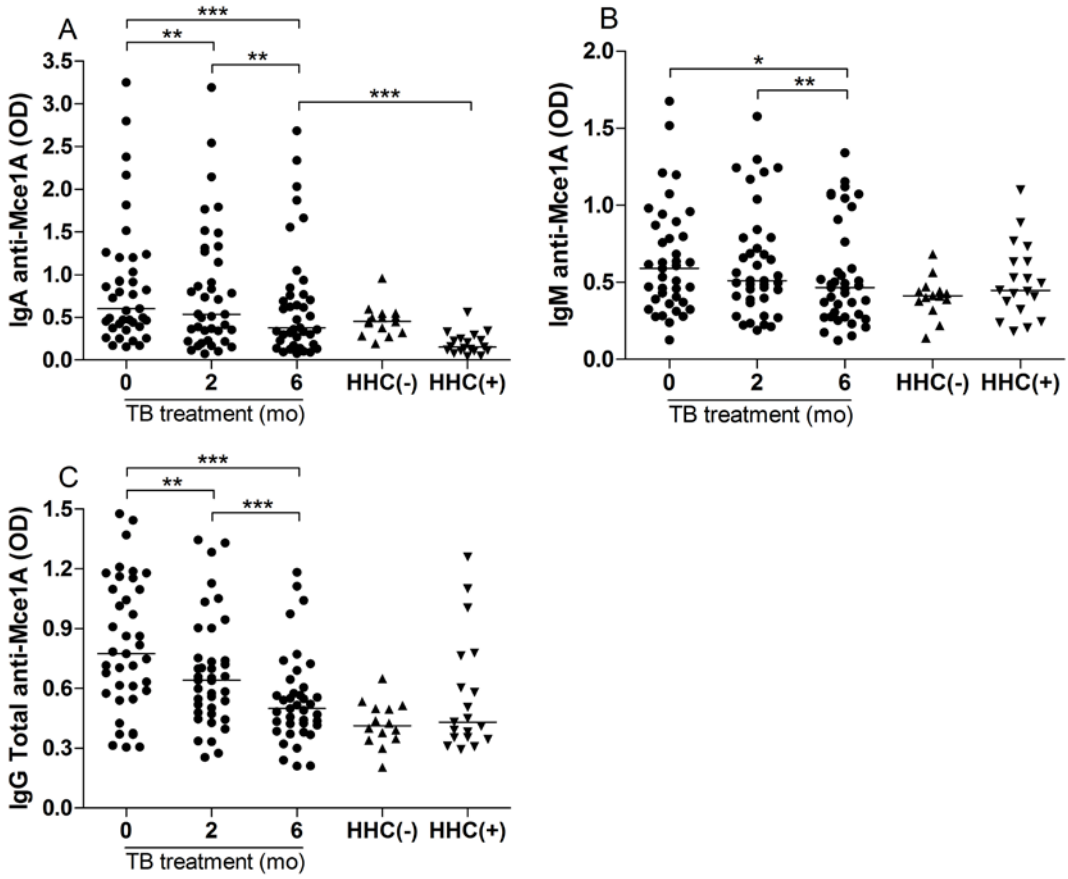

Table 3. Positivity to Mce1A protein during TB treatment (N=39).

TB patients	No. of seropositive patients (%)		
	IgA	**IgM**	**IgG**
At baseline	24 (61.5)	21 (53.8)	32 (82.1)
After 2 months of treatment	23 (59)	17 (43.6)	27 (69.2)
At the end of treatment	18 (46.2)	12 (30.8)	17 (43.6)

Chi-squared test for trend: IgA: $p>0.05$; IgM: $p=0.039$; IgG: $p=0.0004$.

6.3 CITOCINAS E QUIMIOCINAS INDUZIDAS PELA MCE1A APÓS ESTÍMULO IN VITRO

Manuscrito em processo de submissão: **TAKENAMI, I.**; SOARES, J.; RILEY, L. W.; ARRUDA, S. Mammalian cell entry protein 1A-induced TNF production in the peripheral blood mononuclear cells from tuberculosis patients

Mammalian cell entry protein 1A-induced TNF production in the peripheral blood mononuclear cells from tuberculosis patients

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Summary

The *mammalian cell entry protein 1A (Mce1A),* localized on cell wall of *Mycobacterium tuberculosis,* is involved in the uptake of bacteria in non-phagocytic cells. However, it is not exactly clear what cytokines and chemokines are induced in the interaction between *Mycobacterium* Mce1A and mammalian cells. To pursue this objective, we evaluated cytokines and chemokines production in cell culture by peripheral blood mononuclear cells (PBMC) after Mce1A protein stimulation. PBMC were isolated from TB patients and healthy community controls and cultured with Mce1A protein and phytohemagglutinin for 48 hours. The cell culture supernatants were collected and levels of cytokines and chemokines produced were measured. TNF levels obtained in the supernatants of PBMC culture in response to Mce1A protein were higher in TB pulmonary patients than control groups ($p=0.01$). In contrast, all of the other cytokines and chemokines analyzed were not increased in pulmonary TB patients as compared to controls. There was a clear association between disease pathogenesis and the TNF production. In order to obtain reliable results, further investigations using large population size is necessary.

Keywords: Tuberculosis, Mce1A protein, PBMC, cytokine, chemokine.

1. Introduction

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis*. TB most commonly affects the lungs and is responsible for high morbidity and mortality worldwide. Central to an understanding the pathogenesis of *M. tuberculosis* infection in human is the ability of the bacillus to evade macrophages responses by inhibiting phagosome-lysosome fusion [1]. The initial alveolar macrophage infection results in a pro-inflammatory response and the recruitment of other cells of the immune response, including more macrophages to the infection site [2]. These events lead to the formation of a granuloma, where the pathogen can survive for decades, persisting in a special dormant state [2, 3]. The immune response contains *M. tuberculosis* infection in 90-95% of infected subjects; just 5-10% of infected people will develop active disease during their lifetimes [4]. Despite that, little is known about the mechanisms of pathogen and host that lead to development of active disease.

The *mammalian cell entry gene 1A* (*mce1A*) was initially described its ability to promote uptake of *Escherichia coli* noninvasive to enter nonphagocytic mammalian cells [5]. Nowadays, its coding gene is located in the mce1 operon. Subsequent investigation, whole genome analysis revealed that *M. tuberculosis* H37Rv possesses three other related operons designated mce2, mce3, mce4. All four mce operons carry six mce genes (mceA-F) and two yrbE genes (yrbEA-B) [6-8]. Among these, previous studies have shown that recombinant expression of Mce1A protein by *E. coli* and Mce1A-coated polystyrene latex beads are efficiently internalizeds by HeLa cells.

Shimono et al. have shown that mice infected with *M. tuberculosis* H37Rv or Erdman strain disrupted in the mce1 operon contributed to aberrant granuloma formation in the lungs due to inability to stimulate a T helper type 1 immune. Furthermore, the mice die significantly earlier than mice infected with the wild type *M. tuberculosis* [9]. Gioffre et al. have shown that disorganized granuloma was also observed in the lungs of mice infected with Δmce mutant whereas the mutation attenuated the virulence [10]. These studies suggest that mce1 gene products stimulate the host immune response resulting in the granuloma formation at the site of infection. Additionally, some authors have demonstrated that the mce operon are expressed at different stages of infection and that the mce1 is expressed during initial phase of infection by *M. tuberculosis*, suggesting an important role in the early events of host-bacillus interaction [11, 12]. On the other hand, Mce1A-coated latex beads did not induce the chemokine production. Instead, the chemokine and cytokine production is dependent to on

interaction between Mce1A protein and epithelial cells and this relation may be important event that influences the host inflammatory response [13].

On the basis of these observations, we hypothesized that fluctuations in expression of the *mce1* operon during the course of infection can modulate the host's cytokine and chemokine production to favor bacillus survival, contributing to long-term infection. To this end, we evaluated cytokines and chemokines production by mononuclear cells from latent and active tuberculosis patients after stimulation with Mce1A protein.

2. Material and methods

2.1 Subjects

From april 2014 to march 2015, smear positive pulmonary TB patients were recruited from Instituto Brasileiro para Investigação da Tuberculose (IBIT). According to the national guideline for TB, patients with cough lasting more than 2 weeks are screened for TB. The diagnosis of smear positive pulmonary TB was made when at least two out of three consecutive sputum smear examinations were positive for acid fast bacilli. Screening for HIV infection was done according to the national guideline and only those without HIV infection were included. At the same time, we recruited healthy volunteers with latent tuberculosis infection (LTBI) and healthy controls (HC). The TST was injected intradermally and indurations were read after 72h according to the standard technique (Mantoux technique). Volunteers who had a positive Mantoux reaction (≥ 5mm) were classified as latent tuberculosis infection (LTBI) and volunteers who had a negative Mantoux reaction (< 5mm) were classified as healthy controls (HC). All volunteers were HIV-1 negative. Informed consent was obtained from all subjects, and the study was approved by the local research ethics committee.

2.2 Isolation of peripheral blood mononuclear cells (PBMCs) and cell culture experiments

Heparinized venous blood (10ml) was drawn from subjects into sterile blood collection tubes. PBMCs were isolated by ficoll-hypaque density gradient centrifugation at 1,500 rpm for 30 min at room temperature. PBMCs were cultured in RPMI 1640 medium (Gibco) (Sigma Chemical Co., with L. glutamine and without bicarbonate sodium) supplemented with 10% heat inactivated fetal bovine serum (Sigma Chemical Co.), penicillin (100 U/ml)/ streptomycin (100μg/ml) (LIFE Technologies. Gibco BRL). The cell suspension

was adjusted to 1×10^6 cells/ml and were cultured in 96-well plates at 37°C in 5% CO_2 in the presence of either Mce1A antigen (10 μg/ml), M. tuberculosis H37Ra (10 μg/ml; Freund's complete adjuvant, Sigma Chemical, St. Louis, M O) or PHA (10 μg/ml) or PBMC were left unstimulated (baseline). Cell cultures were terminated after 48 hours, and cell-free supernatants were collected and stored below −20°C until further use.

2.3 Cytokine/chemokine production

The cytokines and chemokine were measured in culture supernatants using the Human Th1/Th2/Th17 Cytometric Bead Array (CBA) kit (BD Biosciences, San Diego, CA), which allowed for the simultaneous detection of IL-2, IL-4, IL-10, TNF, IFN-γ and IL-17A or using Human Chemokine CBA kit (BD Biosciences, San Diego, CA), wich allowed for the simultaneous detection of CXCL8, CXCL9, CXCL10, CCL2/MCP-1 and CCL5/RANTES. Cytokine and chemokine were quantitated by flow cytometry (Becton-Dickinson FACSAria™) using fluorescent antibodies according to the manufacturer's instructions.

2.4 Statistical Analysis

Statistical analysis was performed using GraphPad Prism v.5 software (GraphPad Inc., San Diego, CA). The results are presented as the mean ± S.D The cytokines/chemokines levels were compared by one-way analysis of variance (ANOVA) and accomplished by Tukey post-test. All statistical tests were two tailed, and the differences were considered significant at $P \leq 0.05$.

3. Results

Nine pulmonary TB suspects were recruited during the study period. All patients had sputum smear grading as 3+ (67%), 2+ (22%) or 1+ (11%). In addition, five LTBI subjects and six HC were included (Table 1).

Cytokine levels measured in untreated TB patients, LTBI subjects, and HC are shown in Table 2. Mce1A-stimulated TNF levels were significantly higher in untreated TB patients compared to those of LTBI subjects and HC (p=0.01). There was no significant change among any of the other cytokines and chemokine levels between untreated TB patients and control groups (Table 3). PHA induced similar cytokine titers in both control groups and TB patients (Table 2, Table 3).

4. Discussion

Mce1A protein of M. tuberculosis is involved in bacterial entry and survival in macrophages, which has been shown to induce production of TNF [9, 13]. We observed that TNF levels obtained in the supernatants of PBMC culture in response to Mce1A protein were significantly higher in the TB patients than those in the control group.

Previous studies have demonstrated that Mce1A protein and many other M. tuberculosis components, such as liparabinomannan (LAM), mycolylarabinogalactan peptidoglycan complex (mAGP), and even total lipids, cause macrophages to secrete TNF [9, 14]. This protein has a dual role in cell survival; it can promote granuloma formation necessary for the containment of intracellular infections and on the other hand can induce necrosis instead of apoptosis in infected macrophages, and this has been suggested as a mechanism for evading the host immune response [15]. Early clinical deterioration before starting treatment is associated with a selective increase of TNF in plasma. Moura et al evaluating the immune response of patients prior to and after treatment demonstrated that TB patients produced increased levels of TNF [16]; however they did not observe significant difference in these cytokine levels after treatment, concluding that these results reinforce this cytokine's role at both the physiopathology and in the protective immunity of the disease.

In addition, we could not detect any other cytokine production. Some authors suggest that TB patients show increased TNF-α and IL-10 and decreased IL-12 levels compared with controls [17, 18]. IL-12 has a role in host defense against M tuberculosis and is essential for inducing IFN-γ production [3], which plays a critical role in resistance to *M. tuberculosis* infection. Is it possible that this protein is capable of inducing TNF-production only, which leads to necrosis at the site of infection and favors the spread of bacillus to other uninfected cells. On the other hand, Mce1A protein is unable to stimulate IFN-γ production from T cells, which participates in killing of *M. tuberculosis* [1, 3].

Furthermore, no differences in chemokine levels were observed between study groups. The chemokines are involved in cell migration and are logical candidates for a role in granuloma formation. However, the chemokines induced during *M. tuberculosis* infection have been studied to a limited degree, the precise role is still unclear. Although there was no statistical difference in Mce1A-stimulated chemokine levels, CCL2 and CCL5 were lowered in TB patients as compared to the controls. CCL2 and CCL5 are produced primarily by macrophages and are involved in cellular trafficking in TB [19]. CCL2-deficient mice had higher mycobacterial burdens in the lung and spleen after three weeks of M. tuberculosis infection [20]. According to Shadidi et al (2003), blocking of either of the chemokines, CCL5 or CCL2, strongly inhibited migration of the Th1 cells [21]. We believe that the low levels of

chemokines is a survival strategy to evade the host immune response. However, in vitro studies indicate that the degree of production of chemokines may vary with the bacterial strain and is not frequently associated with resistance [22]. The relative contribution of chemokines can vary significantly, depending on the virulence of the pathogen and its involvement in escape mechanisms [23, 24].

In summary, we evaluated the production of 11 different cytokines and chemokines in PBMC from TB patients and controls. There was a clear association between disease pathogenesis and the TNF production. However, the data available are very limited; is important to increase population size and correlate the differences between cytokine and chemokine production. To further investigation, larger studies in high-transmission settings must be conducted. Data based on the T-cell proliferation and cytokine/chemokine response before; during, and after TB treatment should be collected.

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Table 1. Demographic information.

Group	No.(n)	Sex (male/female)	Average Age	Clinical diagnosis
HC	6	3/3	28	Healthy
LTBI	5	1/4	37.2	Tuberculin skin test positive
TB	9	6/3	41.8	Pulmonary TB

Table 2. Cytokine production by PBMC from patients and control subjects.

Cytokine (pg/mL)	Study population			p value
	HC	LTBI	TB	
TNF				
Mce1A	432.2 ± 303.0	436.9 ± 440.9	**2394 ± 1779**	**.01**
PHA	237.3 ± 184.5	54.1 ± 39.86	410.8 ± 369.7	.21
IFN-g				
Mce1A	13.41 ± 8.90	12.08 ± 6.08	33.40 ± 70.08	.65
PHA	83.73 ± 106.6	12.45 ± 5.49	58.81 ± 59.73	.27
IL-2				
Mce1A	3.04 ± 0.60	2.93 ± 0.27	4.03 ± 3.10	.57
PHA	3.92 ± 0.57	2.76 ± 0.22	9.43 ± 8.58	.10
IL-4				
Mce1A	6.33 ± 0.68	6.60 ± 0.82	6.88 ± 0.71	.38
PHA	7.43 ± 0.74	6.80 ± 0.72	10.94 ± 7.60	.29
IL-10				
Mce1A	772.7 ± 579.0	573.9 ± 555.6	653.8 ± 1020	.92
PHA	26.71 ± 41.80	13.99 ± 28.82	43.65 ± 66.63	.59
IL-17				
Mce1A	30.34 ± 1.73	31.77 ± 2.85	31.66 ± 1.59	.40
PHA	32.4 ± 2.85	30.93 ± 1.26	42.71 ± 31.07	.53

Table 3. Chemokine production by PBMC from patients and control subjects.

Cytokine (pg/mL)	Study population			p value
	HC	LTBI	TB	
CXCL-8				
Mce1A	6905 ± 2983	4865 ± 2160	6582 ± 2270	.36
PHA	7790 ± 2000	6815 ± 3836	8196 ± 2638	.68
CXCL9				
Mce1A	16.72 ± 3.15	17.68 ± 3.07	30.96 ± 33.13	.42
PHA	6189 ± 2849	4500 ± 5102	2030 ± 1788	.06
CXCL10				
Mce1A	0	0	0	---
PHA	673.4 ± 363.3	399.8 ± 414.4	662.0 ± 834.2	.72
CCL2				
Mce1A	3809 ± 1875	3293 ± 3002	2751 ± 1890	.66
PHA	4340 ± 1447	3640 ± 3302	5441 ± 520	.21
CCL5				
Mce1A	1005 ± 712.2	939.8 ± 998.9	687 ± 708.8	.72
PHA	997.7 ± 565.1	459.4 ± 386.9	694.2 ± 389.9	.16

6.4 DETECÇÃO DE ANTICORPOS SÉRICOS ANTI-FOSFOLIPÍDIOS COMO POTENCIAIS BIOMARCADORES PARA O DIAGNÓSTICO E MONITORAMENTO DA TB PULMONAR

Manuscrito em processo de submissão: **TAKENAMI, I.**; OLIVEIRA, C. C.; PETRILLI, J.; SOARES, J.; MACHADO JR; A. S.; RILEY, L. W.; ARRUDA, S. Serum antiphospholipid antibody levels as biomarkers for diagnosis and monitoring treatment of pulmonary tuberculosis patients

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

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Summary

Mycobacterium tuberculosis is characterized by a complex cellular envelope composed of various lipids that have important functions in survival of mycobacterial bacilli. Several different types of these lipids behave as antigens capable of stimulating specific B-cells to produce immunoglobulins. The aim of this study was to evaluate the IgM and total IgG antibodies response against cardiolipin (CL), phosphatidylcholine (PTC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and sulfatide (SL) to diagnosis pulmonary tuberculosis (TB) and its applicability for monitoring the efficacy of antituberculous treatment in these patients. A total of 37 pulmonary TB patients and 48 controls, including, 16 healthy *household contacts*, 19 latently infected household contacts and 13 non-TB patients, were selected. After baseline diagnosis, TB patients were followed until the end of treatment. Serum samples were screened by enzyme linked immunosorbent assay (ELISA) for IgM and total IgG against the phospholipids. Levels of IgM against CL, PE and PI and IgG against CL, PE, PI, PTC and SL were significantly higher in TB patients than control groups. Anti-CL IgG had the best performance characteristics, with a sensitivity and specificity of 83.3% (CI 95%: 68-93.8) and 95.6% (CI 95%: 85.2-99.5), respectively. This IgG anti-CL ELISA test detected 84% (31/37) of the TB patients, whereas the number detected by sputum smear was only 65.9% (24/37). After anti-tuberculous treatment, the mean for all antibodies anti-phospholipids decreased significantly compared to baseline. However, among 37.8% and 59.4% of TB patients remains positive after 2 months of treatment. Our results suggest that the total IgG anti-CL can be potential biomarker for the diagnosis of pulmonary TB. On the other hand, despite reduction in the frequency of positive results, the IgG test not useful for monitoring the clearance of the bacterial load.

Keywords: pulmonary tuberculosis, cardiolipin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, sulfatide, antibody, diagnosis, treatment.

1. Introduction

Mycobacterium tuberculosis (Mtb) kills approximately two million people each year and is thought to latently infect one-third of the world's population [1]. Despite advances in therapies, the tuberculosis (TB) remains an urgent public health problem in many other parts of the world, especially in 22 countries designated by World Health Organization (WHO) as high TB burden countries. The co-infection between TB and human immunodeficiency virus (HIV) or Human T-cell lymphotropic virus (HTLV), associated with increased incidence of multidrug-resistant TB and diabetes have rendered the TB disease more complex and consequently more difficult to be control [2–5].

The early diagnosis and adequate treatment of pulmonary TB patients are considered essential to reduce transmission of Mtb and to achieve the disease elimination currently, the diagnosis of TB is based on clinical features, sputum smear and isolation of Mtb from clinical specimens. Therefore, these techniques have limitations of speed, sensitivity and specificity [6].

The most significant advance in last few years was the development of real time PCR assay (Xpert® MTB/RIF) for detection of *M. tuberculosis* DNA and mutations associated with resistance to rifampicin (adicionar ref). However, the higher cost and sophisticated infrastructure requirements have remained major barriers to the test's introduction in low-and middle-income countries. Furthermore, the test does not eliminate the need for conventional tests, which are required to monitor treatment response of patients with TB detected by Xpert® MTB/RIF. Thus, new rapid diagnostic techniques are urgently needed for early detection and correct treatment. There is also the need of new methods to monitor TB treatment, aiming to better treatment results and preventing cases of multidrug-resistant.

Alternatives to traditional methods are the serological tests. Serological testing based on the humoral immune response is simple, economical and non-invasive and it is applicable to smear-negative patients diagnosed clinically. The mycobacterial cell wall has unique features, it is basically consists of proteins and a high lipids content that constituting up to 40% of their dry weight. These lipids behave as antigens capable of stimulating specific B-cells to produce immunoglobulins and, therefore, can be a source of antigens used in serological tests. A unique feature of mycobacterial cell wall lipids includes the presence of lipoarabinomannan (LAM), mycolic acids, phenolic glycolipids, poly acyl trehalose and lipooligosaccharides [7]. Other possible components of the cell wall are cardiolipin (CL), phosphatidylglycerol, phosphatidylcholine (PTC), phosphatidyl inositol (PI) and basic

phospholipids such as phosphatidyl ethanolamine (PE) and sulfatide (SL) [8]. Thus, the aim of the present study was to evaluate IgM and total IgG antibody response against CL, PE, PI, PTC and SL to diagnosis of pulmonary TB and its applicability in monitoring treatment of these patients.

2. Material and methods

2.1 Subjects and serum specimens

A total of 85 subjects from 6º Centro de Saúde Rodrigo Argolo entered into this study. The participants were categorized as pulmonary TB patients or control groups. The control group consists of healthy subjects, infected by Mtb and non-TB patients.

Pulmonary TB patients: Thirty-seven patients with clinical symptoms suggestive of TB and one or more of the following characteristics: 1) chest radiography (CXR) suggestive of TB opacities, 2) sputum samples that contained acid-fast bacilli on microscopy, 3) individuals who responded to anti-tuberculosis drugs. Sputum smear microscopy was performed by Ziehl-Neelsen staining and the results were grouped as negative, 1+, 2+, or 3+. All the patients had negative HIV serology.

Household contacts (HHC): Thirty-five individuals who had been exposed to TB patients were invited to participate in the study. We considered HHC all those who lived in the same household as the TB patient or who have reported at least 100 hours of contact with the patient during the latter's symptomatic period. Active pulmonary TB was excluded from HHC by chest X-ray and sputum smear. This group included only HHC who underwent both tuberculin skin test (TST) and interferon-gamma release assay (IGRA) tests. The TST was carried out by the Mantoux procedure with 2TU of RT23 purified protein derivate (RT23 PPD) (Staten Serum Institute, Copenhagen, Denmark). Reading was performed after 72 hours by a trained nurse. The TST response was categorized as: 0 to 5 mm, negative; ≥ 5 mm, positive and indicative of infection by *M. tuberculosis*. The commercial IGRA performed was QuantiFERON-TB Gold In Tube (QFT-IT; Cellestis Limited, Carnegie, VIC, Australia). The test was performed according to the manufacturer's instructions [9]. The cut-off value for a positive response was 0.35 IU/mL. Samples that gave indeterminate or discordant TST and IGRA results were excluded. Blood was drawn for the baseline IGRA before the TST was administered; both were conducted on the same day. Then, HHC were stratified in two groups: 16 were TST and IGRA negative [healthy household contact; HHC(-)] and 19 TST and IGRA positive [latently infected household contacts; HHC(+)]).

Non-TB patients: Thirteen patients with various chronic diseases other than TB, who declared that they not had any contact with patients with pulmonary TB, were considered as a control group. All patients were excluded the possibility of TB disease by sputum smear, X-ray and culture. Among patients who presented with other lung diseases, five had bacterial pneumonia, two had lung cancer, one had bronchial asthma and the remaining 5 subjects had other pulmonary infections.

Exclusion criteria included patients and controls that tested positive for HIV and patients taking immunosuppressive drugs. All patients and control subjects filled informed consent. The study protocol was approved by Human Subject Ethics Committee of Centro de Pesquisa Gonçalo Moniz (CPqGM/FIOCRUZ).

2.2 ELISA (Enzyme -Linked Immunosorbent Assay)

Serum specimens were obtained from both TB patients and controls upon recruitment and stored at −80 °C until tested. Patients who had a confirmed diagnosis of TB received anti-tuberculosis treatment with isoniazid, rifampin, pyrazinamide and ethambutol for 2 months followed by isoniazid and rifampin for 4 months. Serum samples were prospectively collected at baseline, 2 months and 6 months after starting the treatment.

Measurement of total IgG and IgM was performed with an indirect enzyme-linked immunosorbent assay (ELISA). Lipids were diluted to 10 mg/ mL (CL, PE, PI and PTC) and 1mg/mL (SL) in ethanol anhydro and 50 µl of the solutions were dried overnight in polystyrene ELISA well plates (Greiner bio-one). The coated wells were washed one time with 1x phosphate-buffered saline, pH 7.4 (1x PBS, GIBCO, Invitrogen), blocked with 100 µl of 3% low fatty acid bovine serum albumin (BSA), and incubated for 1h at room temperature. Plates were washed two times with 300 µl 1x PBS and 100 µl of the sera diluted 1:100 in 3% BSA were added. After incubation for 1h at room temperature, the sera were removed and the plates washed 3 times with 1x PBS. The anti-IgM (Sigma-Aldrich) and anti-IgG total (Sigma-Aldrich) enzymatic conjugate diluted 1/10,000 and 1/50,000 respectively, in the same diluent as the serum was then added, 100 µl /well. After 1h of incubation a new cycle of washes and 100 µl /well of the chromogenic substrate tetramethylbenzidine (TMB) (Invitrogen *Life Technologies*) was added and the reaction was stopped immediately with 100 µl of 2N sulfuric acid. Reactions were read within 10 min at 450 nm in a spectrophotometer (Thermo

Scientific). The results were read out as the average of optical density (OD) of triplicate assays and samples were re-run if >10% coefficient of variance was observed.

2.3 Statistical Analysis

Statistical analyses were performed using the GraphPad Prism v.5.0 (GraphPad Inc., San Diego, CA, USA). The immunoglobulin levels as mean optical density ± standard deviation (SD). Group comparisons were performed by one-way analysis of variance (ANOVA) followed by the Tukey test and Student's *t* test. For longitudinal analysis of immunoglobulin levels on anti-TB treatment, differences between time points were first assessed by two-way ANOVA and Tukey's *post hoc* test. The diagnostic power of the IgM and IgG in serum of TB patients was calculated using receiver-operating characteristic (ROC) curve analysis. The significance of association for categorical variables was estimated by Chi-squared test or Chi-square test for linear trend. All tests for statistical significance referred to a significance level with a $p<0.05$.

3. Results

3.1 Characteristics of study subjects

During the period from January 2012 to October 2013 were recruited a total of 85 volunteers to participate in the study. Out of 37 (43.5%) pulmonary TB patients, 24 (64.9%) had sputum samples smear positive for acid-fast bacilli (AFB). From these TB patients' households, 16 were healthy controls (uninfected) and 19 latently infected individuals. The mean induration size of the TST positive was 17.2 ± 6.4 mm. In addition to these groups, we included 13 non-TB patients. No statistically significant differences in demographic characteristics were observed among the study groups ($p>0.05$, Table 1).

3.2 IgM and total IgG response against CL, PE, PI, PTC and SL phospholipids

The IgM response to CL, PE and PI were consistently elevated in TB patients than in the control groups (Table 2). No difference was observed in the anti-PTC or anti-SL IgM ($p=0.443$ and 0.06, respectively). On the other hand, the mean levels of total IgG against all phospholipids were significantly elevated in TB patients than in the control groups (Table 3).

3.3 IgM and total IgG test

Based on the significant difference found in the quantitative analysis of serological tests, we performed an ROC analysis to evaluate its potentials for TB diagnostics. For this purpose, at the beginning we used pulmonary TB patients as the disease group and their HHC and non-TB patients as the control group. Significant AUC analysis results were obtained, independent of the immunoglobulin used. The overall sensitivity, specificity and likelihood ratios (LR) are show in Fig. 1.The optimum cut off value to IgM against CL, PE and PI was 0.505, 0.474, 0.382 OD, respectively, whereas to total IgG against CL, PE, PI and PTC was 0.620, 0.497, 0.459 and 0.353 OD, respectively. The frequencies of positive results are summarized in Table 4. Total IgG demonstrated the best performance overall, IgG anti-CL ELISA test detected 83.8% (31/37) of the TB patients, whereas the number detected by sputum smear was only 65.9% (24/37). Additionally, when the results of IgM and IgG tests were considered, the number of positive results did not increase in TB patients, i.e. this combination did not improve the sensitivity of any test.

3.5 IgM and IgG test to monitoring TB treatment

The results before, during and after TB treatment are presented in Table 5. The mean levels of IgM to CL, IgG to CL, PE and PTC decreased significantly during TB treatment ($p=0.04$, $p=0.0008$, $p=0.004$ and $p<0.0001$ respectively). However, despite the decrease in antibody levels, even after completion of treatment, TB patients still have higher levels of IgM or IgG to CL, PE and PI when compared with HHC or non-TB patients. There was also a significant negative linear trend in the rates positive test among the TB group before the treatment, at the end of 2 months and 6 months of treatment (Table 6).

4. Discussion

In the present study, we observed that anti-phospholipids levels, including anti-CL, anti-PE, anti-PI and anti-PTC, were significantly higher in new pulmonary TB patients than those of the control groups. The IgG test demonstrated the best performance. In fact, the frequency of positive TB patients by total IgG test (CL: 83.8%, PE: 70.3% and PI: 81.1%) was higher than sputum smear results (AFB: 64.9%) or than IgM test (CL: 54.1%, PE: 64.9.3% and PI: 75.7%). In post-primary (chronic) pulmonary TB, low titers of IgM and high titers of IgG could explain high rates of positivity of IgG [10]. Additionally, in the control groups, very few individuals had a positive serology (ranging from 0 to 15.4%). Thus, anti-phospholipid antibodies could be useful to complement conventional bacteriological tests for

the rapid diagnosis and to discriminate pulmonary TB from other pulmonary diseases having similar symptomatology.

Although the role of these Mtb cell wall phospholipids during infection is uncertain, the cardiolipin is essential for *Mycobacterium tuberculosis* growth in vitro [11]. Antibodies against Mtb cardiolipin were also found in sera from TB patients [12,13]. Similarly to our results, there were increased levels of anti-CL antibodies in TB patients than other control groups. On the other hand, anti-CL antibodies are also considered autoantibodies produced by the immune system that mistakenly targets the body's own cardiolipins which are found almost exclusively in the inner mitochondrial membrane [14]. The high prevalence of anti-CL antibodies in TB patients has rarely been investigated and thus, it is not clear if the antibodies are autoantibodies or are result from polyclonal B cell activation after stimulation by mycobacterial cardiolipins. Therefore, if the anti-CL antibodies are autoantibodies, there should be tested based on the corresponding clinical symptoms and signs. Taken together our findings suggest that increased levels of anti-CL antibodies during pulmonary TB may not be associated to the autoimmune diseases, because the patients were investigated to other diseases and they did not reported any symptoms of autoimmune disorders. On the other hand, it is known that, *in vitro*, virulent Mtb strain can favor necrosis over apoptosis in infected macrophages [15]. It seems plausibly that this mechanism of cell death is a strategy used by Mtb to induce tissue destruction, causing release of free fatty acids and phospholipids of the human cells, which will be used as sources of cell wall and membrane synthesis during growth of bacilli. The massive necrotic cell death can be recognized by the immune system as antigens, contributing to the increased production of autoantibodies. Thus, further studies are required to identify the origin of these antibodies, i.e. if the anti-CL antibodies are autoantibodies or Mtb cell wall cardiolipin.

Levels of IgM and IgG against all anti-phospholipids antibodies significantly decreased following anti-TB treatment. Similarly, Amador et al showed that IgM anti-phospholipids antibodies decreased among non-cavitary TB patients [16]. Despite frequency reduction of positive results of IgM and total IgG levels, the ELISA test did not show a good performance to monitor the response to treatment. After two months, 37.8-59.4% of TB patients remain positive for the test whereas only 16.2% (6/37) of the TB patients had positive sputum smear results. The bacteriological results indicated that could be more sensitive than serological test to monitor the response to treatment. The data confirm what was previously observed by Horne et al who demonstrated that, bacteriological test still remain useful for monitoring TB treatment [17].

The immune response induced in TB is cell mediated, but an antibody response is common and may be correlated with the lack of an effective cell-mediated response. Compared to assays of cell-mediated response, detection of antibodies is considerably simpler and less expensive. However, the serological tests still have suboptimal sensitivity and specificity. In this study, sensitivities of the IgG test 83.3% to CL were very high compared to those of the IgM test (77.1% to PI). The sensitivity for most of the lipids reported in other studies was low in TB patients when compared with ours findings [18,19]. Additionally, the specificity of the IgG anti-CL ELISA test showed satisfactory results, compared with others tests based on lipid antigens [19]. Finally, the *sample size* may have been too small, and further larger studies are required to confirm these results.

World Health Organization (WHO) does not recommend serologic tests for the diagnosis of TB, especially because of the lack of accuracy of the tests. This is likely because the humoral response to Mtb is highly complex. However, based on these results, the clinical utility of serological testing with these phospholipids could be useful for differentiating TB from non-TB diseases.

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Table 1. Demographic and clinical characteristics of pulmonary TB patients, their household contacts and non-TB patients.

	TB patients (n = 37)	HHC (n = 35)	HHC infection status		Non-TB patients (n = 13)
			Uninfected (n = 16)	Infected (n = 19)	
Age, years, mean ± SD	40.8 ± 15.5	30.4 ± 20.8	29.9 ± 18.3	30.7 ± 22.8	44.6 ± 13.6
Male, n (%)	23 (62.2)	15 (42.8)	7 (43.8)	8 (42.1)	5 (38.5)
BCG scar, n (%)	24 (64.9)	26 (74.3)	11 (68.8)	15 (78.9)	11 (84.6)
History of tuberculosis, n (%)	2 (5.4)	1 (2.8)	0	1 (6.2)	1 (7.7)
Sputum density of index, n (%)					
Negative	13 (35.1)	-	-	-	13 (100)
1+	11 (29.7)	-	-	-	0
2+	7 (19.0)	-	-	-	0
3+	6 (16.2)	-	-	-	0
CXR*, n (%)					
Cavities	16 (57.1)	-	-	-	-
No cavities	12 (42.9)	35 (100)	16 (100)	19 (100)	-

*CXR not available for nine TB patients.
BCG = bacille Calmette-Guérin; CXR = chest radiograph; HHC = household contacts; SD = Standard deviation; TB = Tuberculosis.

Table 2. IgM antibody against CL, PE, PI, PTC and SL in different groups of sera tested.

Mean ± SD	**TB patients (n = 37)**	**HHC (n = 35)**	**HHC infection status**		**Non-TB patients (n = 13)**
			Uninfected (n = 16)	**Infected (n = 19)**	
Anti-CL	0.559 ± 0.283[a]	0.401 ± 0.257	0.431 ± 0.301	0.376 ± 0.219	0.312 ± 0.175
Anti-PE	0.586 ± 0.304[b]	0.417 ± 0.263	0.445 ± 0.302	0.393 ± 0.231	0.275 ± 0.173
Anti-PI	0.558 ± 0.284[b]	0.327 ± 0.225	0.370 ± 0.288	0.291 ± 0.153	0.215 ± 0.147
Anti-PTC	0.280 ± 0.197	0.335 ± 0.194	0.379 ± 0.222	0.297 ± 0.162	0.321 ± 0.249
Anti-SL	0.334 ± 0.196	0.461 ± 0.304	0.561 ± 0.374	0.376 ± 0.204	0.302 ± 0.196

The one-way ANOVA and Tukey multiple comparison tests were used to compare data among the groups.
[a] p=0.0012; [b] p<0.0001
CL = cardiolipin; HHC = household contacts; IQR = interquartile range; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PTC = phosphatidylcholine; SL = sulfatide; TB = tuberculosis.

Table 3. Total IgG antibody against CL, PE, PI, PTC and SL in different groups of sera tested.

Median (IQR)	**TB patients (n = 37)**	**HHC (n = 35)**	**HHC infection status**		**Non-TB patients (n = 13)**
			Uninfected (n = 16)	**Infected (n = 19)**	
Anti-CL	0.917 ± 0.325[a]	0.393 ± 0.241	0.486 ± 0.124	0.315 ± 0.288	0.200 ± 0.083
Anti-PE	0.733 ± 0.423[a]	0.337 ± 0.180	0.316 ± 0.124	0.355 ± 0.218	0.339 ± 0.160
Anti-PI	0.599 ± 0.171[a]	0.386 ± 0.193	0.403 ± 0.094	0.371 ± 0.250	0.279 ± 0.150
Anti-PTC	0.504 ± 0.240[a]	0.227 ± 0.103	0.188 ± 0.062	0.261 ± 0.120	0.291 ± 0.174
Anti-SL	0.534 ± 0.470[b]	0.319 ± 0.164	0.306 ± 0.102	0.330 ± 0.205	0.370 ± 0.194

The one-way ANOVA and Tukey multiple comparison tests were used to compare data among the groups.
[a] p<0.0001; [b] p=0.017
CL = cardiolipin; HHC = household contacts; IQR = interquartile range; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PTC = phosphatidylcholine; SL = sulfatide; TB = tuberculosis.

Figure 1. Receiver operating characteristic analysis for comparison of anti-CL (A), anti-PE (B) and anti-PI (C) IgM, and anti-CL (D), anti-PE (E), anti-PI (F) and anti-PTC total IgG between pulmonary TB patients (n=37) and control group (n=48). AUC = area under the curve; CI = confidence interval; Se = sensitivity; Sp = specificity; LR+ = positive likehood ratio.

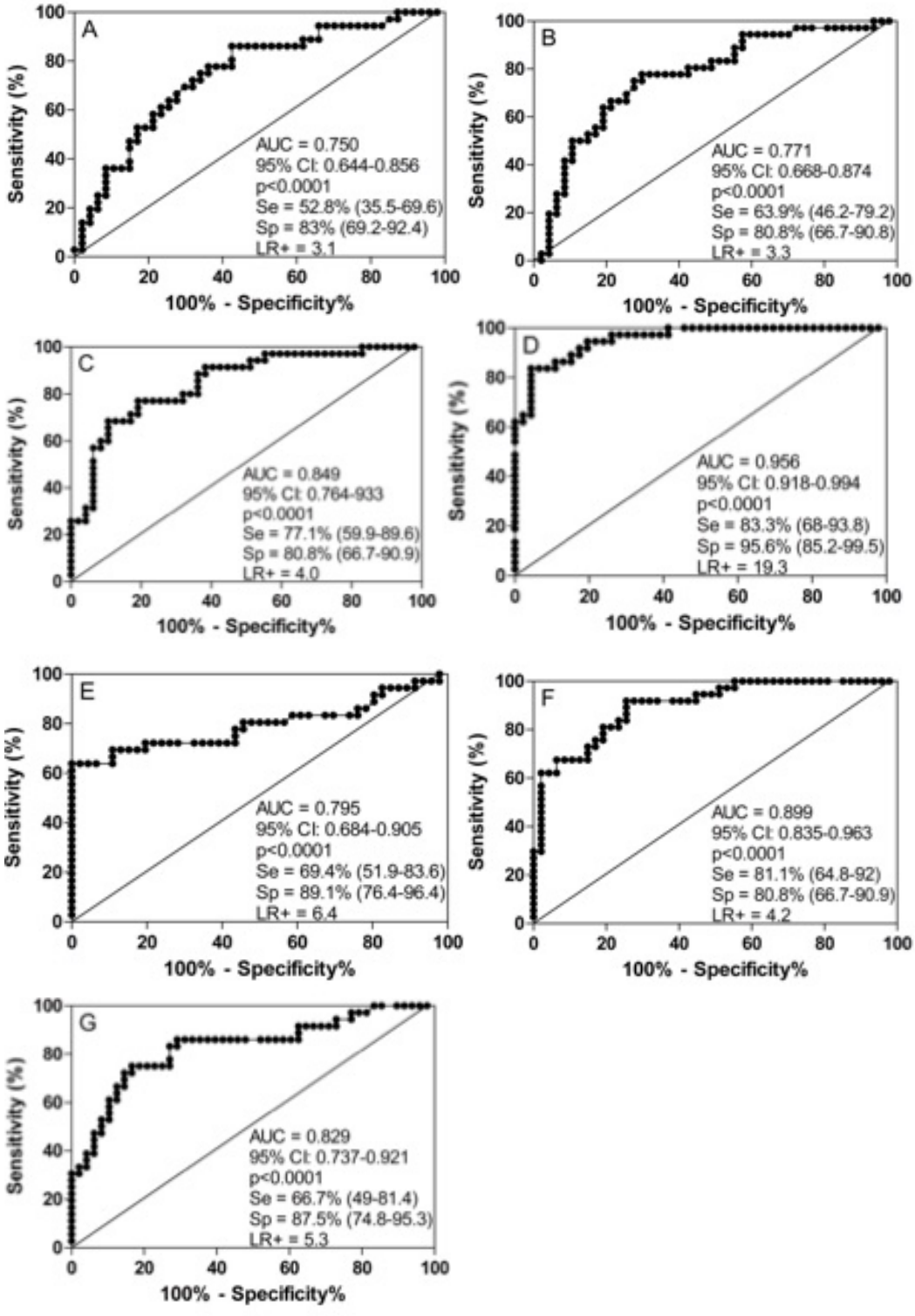

Table 4. Frequency of positive samples to phospholipids between groups.

IgM	TB patients n (%)	HHC n (%)	HHC infection status		Non-TB patients n (%)	TB vs controls p-value*
			Uninfected n (%)	Infected n (%)		
CL	20 (54.1)	8 (22.9)	4 (25)	4 (21.1)	1 (7.7)	**.001**
PE	24 (64.9)	9 (25.7)	5 (31.2)	4 (21.1)	1 (7.7)	**<.0001**
PI	28 (75.7)	9 (25.7)	5 (31.2)	4 (21.1)	1 (7.7)	**<.0001**
IgG						
CL	31 (83.8)	3 (8.6)	1 (6.2)	2 (10.5)	0	**<.0001**
PE	26 (70.3)	4 (11.4)	1 (6.2)	3 (15.8)	2 (15.4)	**<.0001**
PI	30 (81.1)	9 (25.7)	4 (21.1)	5 (31.2)	1 (7.7)	**<.0001**
PTC	24 (64.9)	2 (5.7)	0	2 (10.5)	4 (30.8)	**<.0001**

*p value by Chi-square test.
CL = cardiolipin; HHC = household contacts; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PTC = phosphatidylcholine; TB = tuberculosis.

Table 5. Mean levels during TB treatment and compared to HHC or non-TB-patients.

IgM	TB patients			0 vs 2 vs 6 p-value*	TB patient (6 mo) vs HHC or Non-TB patients p-value**
	Baseline	2 months	6 months		
	Mean ± SD				
CL	0.559 ± 0.283	0.514 ± 0.229	0.466 ± 0.243	**.040**[a]	**.044**[d,e]
PE	0.586 ± 0.304	0.538 ± 0.224	0.526 ± 0.246	.288	**.0008**[d,e]
PI	0.558 ± 0.284	0.522 ± 0.234	0.472 ± 0.260	.070	**<.0001**[d,e]
IgG					
CL	0.917 ± 0.325	0.779 ± 0.302	0.711 ± 0.342	**.0008**[a,b]	**<0.0001**[d,e]
PE	0.733 ± 0.423	0.604 ± 0.323	0.499 ± 0.254	**.004**[a]	**0.011**[d]
PI	0.599 ± 0.171	0.538 ± 0.232	0.526 ± 0.161	.117	**.0003**[d,e]
PTC	0.504 ± 0.240	0.410 ± 0.200	0.307 ± 0.140	**<.0001**[a,b,c]	0.060

* The two-way ANOVA and Tukey multiple comparison tests were used to compare data among TB patients
** The one-way ANOVA and Tukey multiple comparison tests were used to compare data among the groups.
[a] baseline > 6 months.
[b] baseline > 2 months.
[c] 2 months > 6 months.
[d] 6 months > HHC.
[e] 6 months > non-TB patients.
CL = cardiolipin; HHC = household contacts; SD = standard deviation; Mo = months; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PTC = phosphatidylcholine; TB = tuberculosis.

Table 6. Frequency of positive samples to phospholipids during TB treatment.

IgM	TB patients, n (%)			p-value*
	0M	2M	6M	
CL	20 (54.1)	19 (51.4)	10 (27)	**.019**
PE	24 (64.9)	22 (59.4)	17 (45.9)	.101
PI	28 (75.7)	27 (73)	22 (59.4)	.130
IgG				
CL	31 (83.8)	22 (59.4)	19 (51.4)	**.004**
PE	26 (70.3)	21 (56.8)	17 (45.9)	**.034**
PI	30 (81.1)	21 (56.8)	20 (54.1)	**.016**
PTC	24 (64.9)	14 (37.8)	10 (27)	**.001**

* Chi-square test for linear trend.
CL = cardiolipin; HHC = household contacts; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PTC = phosphatidylcholine; TB = tuberculosis.

7 DISCUSSÃO

A busca por anticorpos como biomarcadores sorológicos na TB data desde 1951 (ROTHBARD, 1951). Desde então, muitos estudos foram realizados, impulsionados principalmente pelos avanços na área pós-genômica, proteômica e lipidômica na tentativa de identificar potenciais marcadores para uso como antígenos em testes sorológicos (COLE; BARRELL, 1998; JENA et al., 2013; SARTAIN et al., 2011). Contudo, o MS e a OMS não preconiza o uso dos testes sorológicos na rotina e na clínica da TB. Os testes, amplamente difundidos em países em desenvolvimento como a Índia e a China, apresentam dados inconsistentes e imprecisos de sensibilidade e especificidade, fatores que dificultam e inviabilizam a sua utilização no diagnóstico e na resposta terapêutica à doença (STEINGART et al., 2011, STEINGART et al., 2012). A complexidade da resposta imune do hospedeiro ao *M. tuberculosis*, o diferente espectro clínico, a influência dos determinantes sociais e ambientais são alguns dos fatores responsáveis por esta grande variação na reatividade aos diferentes antígenos em diversas regiões do mundo.

Apesar destas limitações, o baixo custo e a simplicidade, passíveis de serem implantados nos países subdesenvolvido e em desenvolvimento, são fatores atrativos que incentivam os estudos à otimizar o desempenho dos testes sorológicos através da identificação de antígenos imunogênicos capazes de estimular uma forte e consistente resposta imune humoral. Em nosso estudo, os níveis de anticorpos anti-Mce1A e anti-fosfolipídios, incluindo anti-CL, anti-FE, anti-FI e anti-FC foram significativamente maiores em pacientes com TB pulmonar do que nos indivíduos do grupo controle, demonstrando o potencial papel destes anticorpos como biomarcadores sorológico no diagnóstico da TB pulmonar.

O IgG total anti-Mce1A proporcionou o melhor resultado na detecção dos casos de TB (82,1%), em comparação com as outras classes de imunoglobulinas avaliadas (IgA: 61,5%, IgM: 53,8%), ou até mesmo em comparação com os anticorpos anti-fosfolipídios (CL: 83.8%, FE: 70.3%, FI: 81,1% e FC: 64,9%). No grupo controle, poucos indivíduos apresentaram sorologia positiva para IgG anti-Mce1A, variando de 0 a 16,7%. Estudos que avaliaram a resposta imune humoral contra outras proteínas do *M. tuberculosis*, demonstraram que a prevalência da soropositividade pode variar de 4,1 a 24,7% entre os indivíduos do grupo controle (BEN-SELMA et al., 2010; KEYGHOBAD GHADIRI, 2008; SINGH et al., 2012).

A baciloscopia, o atual exame utilizado no diagnóstico bacteriológico, foi capaz de detectar apenas 26 (66,7%) dos 39 casos de TB. O IgG total anti-Mce1A identificou 10

(76,9%) casos adicionais entre aqueles que apresentaram resultado de baciloscopia negativo. Além disso, o desempenho deste teste, avaliado através da curva ROC, em termos de sensibilidade e especificidade foi também superior aos estudos reportados na literatura (BEN-SELMA et al., 2010; KEYGHOBAD GHADIRI, 2008; SINGH et al., 2012). Estes resultados sugerem que a adoção do teste IgG total anti-Mce1A, como método complementar, seria uma ferramenta útil na detecção dos casos de TB, especialmente nos casos com baciloscopia negativa.

Embora tenha-se observado que pacientes com TB pulmonar produzam altos níveis de anticorpos anti-Mce1A, um grupo de sete comunicantes domiciliares com TBL e com TT > 15 mm também produziam altos níveis de IgG anti-Mce1A e, consequentemente, foram considerados positivos pelo teste. Estes mesmos indivíduos foram identificados como baixos produtores IFN-γ. Reforçando a importância da resposta imune celular frente à infecção, sabe-se que os indivíduos com TBL produzem altos níveis de IFN-γ, e que esta citocina é um fator determinante na proteção contra a TB (FLYNN et al., 1993). Assim, a correlação negativa entre a produção de anticorpos e os níveis de IFN-γ sugerem que o desenvolvimento da doença pode estar associada à diminuição da resposta Th1, em detrimento do aumento da resposta humoral, mediado por anticorpos.

Alguns autores têm demonstrado, através da análise por *immunoblotting*, que a proteína Mce1A recombinante reage com soro de pacientes TB (AHMAD et al., 1999; HARBOE et al., 1999). Além disso, anticorpos policlonais direcionados contra a proteína Mce1A podem bloquear à infecção por micobactérias em macrófagos humanos (SIVAGNANAM; NAMASIVAYAM; CHELLAM, 2012). Kumar e colaboradores também demonstraram que o gene responsável pela codificação da proteína esteá amplamente distribuído no gênero *Mycobacterium*. Ou seja, a presença ou ausência do gene não se correlaciona com a patogenicidade do bacilo. Por outro lado, é possível que o perfil de expressão gênica do *M. tuberculosis* durante a infecção seja crucial para determinar a manutenção da virulência micobacteriana (KUMAR et al., 2005). Em conjunto, os resultados sugerem que o aumento da expressão desta proteína pelo *M. tuberculosis* na infecção natural, especialmente durante a fase ativa da doença, contribui para o desenvolvimento de uma resposta imune humoral persistente nos pacientes TB. No entanto, apesar da produção dos anticorpos anti-Mce1A estar diretamente associada aos pacientes com TB pulmonar, pouco se conhece sobre o papel destes anticorpos na imunopatogênese da doença. Estudos futuros deverão ser realizados para esclarecer o papel protetor ou imunopatológico dos anticorpos anti-Mce1A na evolução clínica e no prognóstico dos pacientes.

A caracterização do perfil de citocinas frente à Mce1A, demonstrou que os pacientes TB produzem altos níveis de TNF. Estudos prévios demonstraram que a proteína Mce1A e diversos outros componentes de *M. tuberculosis*, tais como LAM, ácido micólicos e glicolipídios estimulam a produção de TNF por macrófagos (SHIMONO et al., 2003; RAJNI et al., 2010). Abebe e Bjune sugerem que esta citocina apresenta um duplo papel na sobrevivência celular; o TNF pode promover a formação de granulomas necessários para a contenção de infecções intracelulares mas, por outro lado, pode também induzir necrose em vez apoptose em macrófagos infectados (ABEBE; BJUNE, 2009). Este último tem sido sugerido como um mecanismo de evasão da resposta imune do hospedeiro.

A deterioração clínica precoce antes de iniciar o tratamento também está associada a um aumento seletivo de TNF no plasma. Moura e colaboradores, ao avaliar a resposta imunológica de pacientes antes e após o tratamento, demonstraram que os pacientes TB produzem elevados níveis de TNF (MOURA et al., 2004); no entanto, não foi observada nenhuma diferença significativa após o tratamento, concluindo que estes resultados reforçam o papel deste citocina, tanto na fisiopatologia como na imunidade protetora da doença.

Não foi possível detectar qualquer outra produção de citocinas. Alguns autores sugerem que os pacientes TB apresentam altos níveis de TNF e IL-10 e baixos níveis de IL-12 em comparação com os indivíduos do grupo controle (WALZL et al., 2011; YASUI, 2014). A IL-12 tem um papel na defesa do hospedeiro contra o *M. tuberculosis* e é essencial para a indução da produção de IFN-γ, que desempenha um papel crítico na resistência à infecção por *M. tuberculosis.* É possível que esta proteína seja capaz de induzir somente a produção de TNF, o que leva à necrose no local da infecção e favorece a propagação do bacilo para outras células não infectadas. Por outro lado, a proteína Mce1A é incapaz de estimular a produção de IFN-γ, que participa diretamente na destruição bacilar.

Nenhuma outra diferença foi igualmente observada na produção das quimiocinas entre os grupos de estudo. As quimiocinas estão envolvidas na migração de células e, possivelmente desempenham um papel na formação de granulomas (ALGOOD; CHAN; FLYNN, 2003). No entanto, ainda existem lacunas de conhecimento sobre as quimiocinas induzidas durante a infecção por *M. tuberculosis,* o papel exato não está claro e representa grande interesse para o entendimento da regulação da imunidade inata.

Pacientes com TB apresentaram baixos níveis de CCL2 e CCL5 quando estimulados com a proteína Mce1A. Embora não haja diferença estatística, estudos demonstram que estas quimiocinas são produzidas principalmente por macrófagos e estão envolvidos no tráfico celular na TB (ALGOOD; CHAN; FLYNN, 2003). Camundongos deficientes em CCL2

apresentaram um aumento na carga bacilar no pulmão e baço após três semanas de infeção (SCOTT; FLYNN, 2002). De acordo com Shadidi e colaboradores (2003), o bloqueio de qualquer uma destas quimiocinas, inibe fortemente a migração das células Th1. Assim, acreditamos que os baixos níveis de quimiocinas é uma estratégia de sobrevivência para evadir a resposta imune do hospedeiro, levando a uma cronicidade persistente da infecção. No entanto, estudos *in vitro* indicam que o grau de produção de quimiocinas pode variar com a cepa bacteriana e não é frequentemente associada com a resistência (RHOADES et al., 1995). A contribuição relativa das quimiocinas pode variar significativamente, dependendo da virulência do agente patogênico e o seu envolvimento nos mecanismos de escape (CHENSUE et al., 2001; HAGGE et al., 2009).

Além da proteína Mce1A, o *M. tuberculosis* também exibe uma variedade de lipídios na parede celular. Estes lipídios são, em sua maioria, altamente imunogênicos e podem servir como ferramentas para o sorodiagnóstico. Nesse sentido buscamos caracterizar o perfil de anticorpos anti-CL, anti-FI, anti-FE, anti-FC e anti-SL nos pacientes com TB pulmonar. A prevalência de pacientes com TB detectados pelo teste de IgG total anti-fosfolipídios (CL: 83,8%, FE: 70,3% e FI: 81,1%) demonstrou um desempenho superior ao da baciloscopia (64,9%) ou da classe de imunoglobulina IgM (CL: 54,1%, FE: 64.9.3% e FI: 75,7%). Independente do estudo, observa-se que a combinação das imunoglobulinas IgM com IgG não melhora o desempenho dos testes sorológicos. O IgG demonstrou ser a melhor imunoglobulina na detecção da doença ativa. A pesquisa de IgM, assim como em outros estudos (GUPTA, 2002), mostrou-se insatisfatória para uso clínico. Isto pode estar associado ao fato de que a TB é uma doença com características clínicas crônicas, portanto um paciente doente, somente apresentaria esta classe de anticorpo, de forma a ser pesquisado, na fase inicial da doença. Tendo em vista que muitos dos pacientes, somente procuram o serviço quando apresentam um quadro clínico mais avançado, é razoável afirmar que esta imunoglobulina não oferece segurança na discriminação da doença, já que na fase crônica os níveis séricos de IgM seriam praticamente vestigiais.

Dentre os fosfolipídios avaliados, a CL demostrou ser o melhor antígeno na avaliação sorológica. Embora haja lacunas sobre o papel dos fosfolipídios na parede celular do *M. tuberculosis* durante a infecção, alguns estudos relatam que a CL é essencial para o crescimento de *M. tuberculosis in vitro* (FISCHER et al., 2001). Os anticorpos anti-CL já foram observados no soro de pacientes com TB pulmonar em outros estudos (ELKAYAM et al., 2013; SANTIAGO et al., 1989). De modo semelhante aos nossos resultados, houve um aumento dos níveis de anticorpos anti-CL em pacientes TB quando comparado com os

indivíduos do grupo controle. Por outro lado, os anticorpos anti-CL são também considerados auto-anticorpos, produzidas pelo sistema imunitário que por engano tem como alvo as CLs do próprio corpo, que são encontrados quase exclusivamente na membrana mitocondrial interna. A alta prevalência de anticorpos anti-CL em pacientes com TB raramente tem sido investigada e, assim, não esta claro se esses anticorpos são auto-anticorpos ou são provenientes da ativação de linfócitos B policlonais após estimulação pelas CLs micobacterianas. Por conseguinte, se os anticorpos anti-CL são auto-anticorpos, deve ser avaliado com base nos sinais e sintomas clínicos correspondentes. Em conjunto, nossos resultados sugerem que os níveis aumentados de anticorpos anti-CL durante a TB pulmonar não pode ser associado às doenças auto-imunes, uma vez que os pacientes não relataram quaisquer sintomas de doenças auto-imunes. Por outro lado, sabe-se que, *in vitro*, a cepa virulenta de *M. tuberculosis* pode favorecer necrose ao invés da apoptose em macrófagos infectados (ABEBE et al., 2011). Parece plausível pensar, que este mecanismo de morte celular é uma estratégia utilizada pelo *M. tuberculosis* para induzir a destruição do tecido, causando a liberação de ácidos graxos livres e fosfolipídios de células humanas, que serão utilizados como fontes de substrato para a síntese de parede celular durante o crescimento do bacilo. A morte celular por necrose massiva pode ser reconhecida pelo sistema imune como antígeno, contribuindo para o aumento da produção de auto-anticorpos. Assim, mais estudos são necessários para identificar se os anticorpos anti-CL são auto-anticorpos ou contra a CL da parede celular do *M. tuberculosis*.

Os anticorpos anti- Mce1A e anti-fosfolipídios foram também avaliados quanto a sua capacidade de monitorar o tratamento da TB. Durante o tratamento, observa-se que o níveis de anticorpos e a frequência de sorologia positiva para Mce1A e fosfolipídios diminuem significativamente. No entanto, os testes sorológicos baseados na Mce1A e nos fosfolipídios não apresentaram um bom desempenho para monitorar à resposta terapêutica quando comparado com a baciloscopia de escarro. Depois de dois meses, entre 37,8 e 59,4% dos pacientes com TB apresentaram sorologia positiva para os fosfolipídios, enquanto que apenas 16,2% (6/37) dos pacientes apresentavam resultados de baciloscopia positiva. Estes resultados sugerem que os testes bacteriológicos ainda são mais sensíveis do que os testes sorológicos para monitorar a resposta ao tratamento. Os dados confirmam o que anteriormente foi observado por Horne e colaboradores, ao demonstrar que, os testes bacteriológicos são capazes de levar à negativação logo após o início do tratamento, pois detectam diretamente a presença do microrganismo na amostra biológica.

A resposta imune induzida em TB é mediada por células, mas uma resposta de anticorpos é comum e pode ser correlacionado com a falta de uma resposta celular eficaz. Em comparação com os ensaios de resposta mediada por células ou baseados na detecção do DNA do *M. tuberculosis*, a detecção de anticorpos é consideravelmente mais simples e menos dispendiosa. No entanto, os testes sorológicos disponíveis comercialmente ainda têm sensibilidade e especificidade abaixo do ideal, o que contribuiu para oposição da OMS. No nosso estudo, demonstramos que os anticorpos sorológicos encontram-se fortemente elevados nos pacientes com TB pulmonar, demonstrando um nível de detecção superior à baciloscopia. A limitação destes estudos inclui o tamanho da amostra e a exclusão dos pacientes com TB co-infectados com HIV. Um estudo mais amplo, incluindo pacientes infectados pelo HIV é necessário para avaliar ainda mais a validade destes novos biomarcadores.

8 CONSIDERAÇÕES FINAIS

Pacientes com TB produzem uma forte e consistente resposta humoral mediada por anticorpos anti-Mce1A e anti-fosfolipídios quando comparados com os indivíduos do grupo controle, sugerindo o potencial papel desses anticorpos como biomarcadores sorológicos no diagnóstico da TB pulmonar. Nesse contexto, o ensaio imunoenzimático poderia ser utilizado como um teste de triagem inicial, especialmente em pacientes com baciloscopia negativa. No entanto, apesar da diminuição dos níveis de anticorpos e da frequência de resultados positivos ao longo do tratamento, os testes sorológicos não apresentam valor no monitoramento da resposta terapêutica.

Por fim, a proteína Mce1A, induz uma acentuada produção de TNF que pode contribuir com a indução de necrose pelo bacilo, permitindo sua evasão das respostas imunes e favorecendo a dispersão do bacilo para outras células não infectadas. Além disso, a revisão da literatura demonstra também que a proteína, apesar dos poucos estudos em modelo humano e em células de linhagem, desempenha uma papel importante na imunopatogênese da TB pulmonar.

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APÊNDICE

Durante o desenvolvimento do doutorado, foi possível publicar um artigo científico na revista indexada *The Brazilian Journal of Infectious Diseases*. O artigo científico intitulado "*Revisiting the Rich's formula: an update about granulomas in human tuberculosis*" faz uma breve atualização da fórmula de Rich, proposta em 1951, com base nos novos e velhos conceitos sobre os mecanismos patogênicos envolvidos no desenvolvimento da TB, incluindo a importância do gene mce1A no controle da latência/persistência e da virulência do bacilo.

Revisiting the Rich's formula: an update about granulomas in human tuberculosis

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Special article

Revisiting the Rich's formula: an update about granulomas in human tuberculosis

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ABSTRACT

The formula proposed by Rich in 1951 explained the formation in a tuberculous lesion in a period that was unknown cellular functions, cytokines and other immunological aspects involved in granuloma formation by tuberculosis; its components are assembled conceptually to explain the pathogenic mechanisms involved in the granulomatous lesion in tuberculosis. In this manuscript, we report an update of Rich's formula based on the new and old concepts about pathogenic mechanisms involved in the granulomatous lesion in tuberculosis. Current knowledge allows us to conclude that the balance between the characteristics of the bacillus and host protective response is necessary to indicate the outcome of pathogenesis, infection or active disease and the necrosis degree of the tuberculosis lesion.

Introduction

Tuberculosis (TB) is the most frequent cause of granulomas and is the most prevalent infectious disease in Brazil, remaining one the most important problems of public health in the world. In 2010, 71,000 cases were reported, with a mortality of 4800/year.[1] Brazil is the 19th in rank among countries with the majority of TB cases in the world. Bahia, the northeast State of Brazil, is ranked 3rd in number of cases, after São Paulo and Rio de Janeiro. TB incidence in Bahia is 39.5/100,000 habitants.[1–3]

TB is a granulomatous disease characterized by a cell-mediated hypersensitivity reaction in response to components of the wall structure of *Mycobacterium tuberculosis* especially cord factor and lipoarabinomannan.[4] Granuloma was first described in 1679 by Sylvius, and etymologically comes from the original word tubercle.[5] Nowadays, granulomas are much better understood based on the knowledge of cellular immunology (cell mediated hypersensitivity reaction) and its interaction with cytokines and chemokines.

In 1951, Rich proposed that the pathogenesis of tuberculous lesion was directly dependent of inoculum, bacillus number (N), bacilli virulence (V) and host hypersensitivity (H), by counterbalancing and decreasing the lesion size, the natural and acquired resistance of the host (R_{n+a}). This formula can be represented by[6]:

$$L = \frac{N + V + H}{R_{(n+a)}}$$

Rich's formula shows components that are assembled conceptually to explain the pathogenic mechanisms involved in

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the granulomatous lesion in TB. Considering the TB pathogenesis in two different clinical forms, infection and disease, where in infection, the balance depends on the host natural resistance. In contrast, in disease, the balance depends on the bacilli properties such as virulence and hypersensitivity phenomenon. In both clinical forms, either infection or disease, granulomas are detected in the tissue.[7–9] Both stages are taking place inside of the granuloma, where the components of Rich's formula occur. The ancient concept was that a granuloma was a cellular agglomerate walling of the bacilli; thus in Rich's formula this should be considered in the denominator. This can be observed in some host, in which the granuloma formation is impaired and therefore the bacillus growth is faster. In fact, in these almost 60 years since the proposition of Rich's formula, the science has advanced significantly in the knowledge of the properties of *M. tuberculosis*, as well as in understanding of host defense mechanisms. These new concepts have been incorporated into the above formula.

Initiation of infection and granuloma formation

After mycobacterial arrival in the tissues of the respiratory tract, an unspecified reaction occurs in which neutrophils try to phagocytize the bacillus. This initial phase is not well studied in humans.[10] Three weeks later, there is a development within these tissues of a more specific reaction, when mononuclear cells reach the focus of the infectious lesion, modulating the granuloma formation.[9,11,12] Mononuclear cells including monocytes/macrophages and lymphocytes, cells with their predefined morphologies and functions, participate in the recognition and presentation of components of *M. tuberculosis* to the immune system.[11–13] During and after mononuclear cells naïve starts cells differentiation into specific cell types such as macrophages, dendritic cells, CD4 and CD8 T helper.[14]

In the process of granuloma formation, many macrophages with large cytoplasm and organelles with high phagocytic and microbicidal functions were observed.[15–17] These cells can fuse into multinucleated giant cells; however, these changes are not completely understood.[12,17] Mature macrophages can undergo a transformation into epithelioid cells (epithelioid histiocytes), which interdigitated their membranes aggregating into other cells as the epithelial barrier.[16] CD4 and CD8 T helper (Th) lymphocytes as well as epithelioid monocytes and giant multinucleated cells participate in this granuloma formation that is orchestrated by INF-γ produced by CD4 and TNF-α secreted by macrophages.[8,11,12,15,18] Other cells, such as neutrophils, join this granuloma via a dynamic process.[9,19] All these facts have dynamic morphology that is influenced by the balance between host resistance and the *M. tuberculosis* virulence gene expression.[9,11]

Cellular apoptosis of caseous necrosis can also be detected within the centers of granulomas. Progress has been made in determining these mechanisms; however, much remains not completely understood.[9] One interesting point that has been found regards the function of the granuloma. Although the main consensus is that the granuloma that surrounds the *M. tuberculosis* is doing so in an attempt to restrict it from spreading.[20] Other experiments in the zebrafish have shown that granuloma also contribute to bacilli spreading. During the granuloma's formation the tissues are injured and necrosis develops. This mechanism of lesion development depends on the acquired resistance versus the hypersensitivity developed by the patient to the bacillus during the infection.[9]

Granulomas seen in tuberculous pleurisy allow to distinguish the cellular components comparing the cells present in pleura fluid.[21] Fayyazi et al. showed a possible apoptosis of epithelioid cells and the presence of CD4 T lymphocytes in the proximity of the focus of the necrosis. Such findings could suggest a direct relation between the cells, their secretions and the central necrosis of granulomas, in addition to the epithelioid cell's apoptosis.[22] The interaction of all these aspects and also the elements of the extracellular matrix[9] are part of the final product, the granuloma with central caseous necrosis.[23,24]

Bacilli virulence

The genome sequence of *M. tuberculosis* allowed to conduct studies about the role of unknown genes through functional genomics. The identification of genes present in hypervirulent strains of *M. tuberculosis*,[25] but absent in pathogenic strains such as *Mycobacterium bovis* BCG, allowed a better understanding of the pathogenesis of TB.

Currently, the virulence of *M. tuberculosis* is defined by region of difference (RD1) genes, which are absent in BCG strains and are present in all *M. tuberculosis* virulent strains. Several studies have demonstrated that deletion of RD1 from *M. tuberculosis* results in attenuation of virulence, suggesting a dominant role in immune responses.[26,27] Thus, deletion mutants of virulent *M. tuberculosis* strains for RD1 resemble BCG in their infectivity and attenuation.[27]

Furthermore, another group of genes have been associated with virulence of *M. tuberculosis* in animal model. It is suggested that the mammalian cell entry genes (mce), which encodes Mce protein, were originally identified in *M. tuberculosis*[28] and have been associated with survival within macrophages and an increase in virulence.[29,30] Disruption of the mce gene of BCG results in a mutant that exhibits reduced invasiveness epithelial cells, impairing their ability to survive and multiply inside phagocytic cells.[31]

Inhibition of macrophage apoptosis by *M. tuberculosis* strain H37Rv has been proposed as another virulence mechanism.[32] This hypothesis is supported by studies that show that bacilli can cause necrosis instead of apoptosis in infected macrophages to avoid innate host defense. The macrophage cell death pathway by necrosis could be an exit mechanism for *M. tuberculosis* to facilitate the spread of infection and contribute to the formation of necrotic lesions in TB.[33,34] Indeed, infection of macrophages with the attenuated *M. tuberculosis* causes apoptosis, which limits bacterial replications and promotes T-cell cross priming by antigen-presenting cells.[35]

Host hypersensitivity

There is a strong relationship among immunity and hypersensitivity in TB. T lymphocytes can produce distinct types of

immune responses associated with different cytokine profiles and have opposing effects on resistance and susceptibility.[14] The result of this balance will determine the degree of lesion in infected individuals and TB disease. Latent infection is associated with protective cellular immune responses whereas advanced disease is associated with immune suppression and exacerbated hypersensitivity reaction, causing tissue damage. Thus, T lymphocytes may also mediate harmful tissue-destroying hypersensitivity reactions that cause progression of disease.[36]

Natural and acquired resistance

Macrophages, dendritic cells and neutrophils principally mediate the natural host resistance to *M. tuberculosis*. The available data on the role of neutrophils are controversial. Neutrophils are the first cells to be mobilized and arrive within hours at the site of infection. They are not professional phagocytes such as macrophages, but contribute substantially to innate resistance to infection. Neutrophils can also form an extracellular fibril matrix known as NETs (neutrophil extracellular traps). These NETs were described recently.[37] They are formed by activated neutrophils and consist of a DNA backbone with embedded antimicrobial peptides and enzymes. Thus, NETs represent a distinct innate defense mechanism to control and eliminate microbial infections.[38] On the other hand, the poor ability of neutrophils to restrict mycobacterial growth compared to alveolar macrophages indicates that the prevalence of neutrophils in TB inflammation contributes to the development of a nonspecific cellular reaction, rather than protection of the host, and that neutrophils may play the role of a "Trojan horse" for *M. tuberculosis*.[10,39] Indeed, interferon-inducible neutrophil-driven blood transcriptional signature has been described in patients with active TB, which correlates with lung radiographic disease severity and implicates neutrophils directly in the pathogenesis of active TB.[40]

Molecular studies show that the human genetic are responsible for natural resistance to *M. tuberculosis*. There is consensus in the literature that humans exposed to *Mycobacterium* millennially were evolutionarily selected, providing relatively more resistant cohorts. One example of this is the infection of Native Americans when Europeans first came to North America where the Apaches tribe was rapidly infected by acute and deadly TB.[41] Also, indigenous Brazilians show a high TB rate, which can be attributable to an intrinsic susceptibility of these individuals.[42]

The resistance mechanism in TB involves a great number of cellular interactions (macrophage, T cell). Several different T-cell populations are required for the successful control of the pathogen. This dynamic interplay underlying protection is the reason for the long-term persistence of *M. tuberculosis*. From the cellular point of view the T lymphocytes are differentiated in the thymus. CD4 and CD8 T-cells activate macrophages against *M. tuberculosis*. These cells recognize specific parts of the bacillus and secrete cytokines, which activate macrophages.[9,11–15,18] T cells also act, induced by components of the bacillus, producing defense mechanisms. By reacting with proteins, a state of hypersensitivity is created that can be measured by the tuberculin test. In this context, cytokines play an important role in the pathogenesis of TB. The cytokines are weighted molecules of about 8–14 kDa whose production is a function of the components of *M. tuberculosis*. These cytokines attract macrophages that transform into epithelioid cells.[8,9,14] IFN-γ, TNF-α and interleukin-(IL)-12 are the most fundamental cytokines, but there are currently about 40 classified cytokines and other chemokines that act as receptors involved in the cellular dynamic of granulomas.[43]

The events underlying the structural reorganization of immune cells to form a stable granuloma or progress to a pathologic lesion are dependent on other components. It is related with some components of the structure of *M. tuberculosis*, including some parts of its genetic material. These elements can mobilize, recruit, and amplify T cells reaction through the influence of cytokines.[44] Glycolipids, components of the *M. tuberculosis* wall (sero-lipidic membrane), also involve the bacillus and DNA. They contain a 6 kDa protein, designed as an ESAT-6, that mobilizes the T cell and activates the production of TNF-α and IFN-γ. Both of these activations lead the macrophages to destroy the *Mycobacterium*.[19,45] Following the above events, as well as the arrival of bacilli in the tissues (for example, pulmonary alveoli), the formation of a granuloma with central caseous necrosis is completed.

Directed by T lymphocytes, modified monocytes aggregate and transform into epithelioid cells. With this fusion, giant multinucleated cells (Langerhans cells) are created. The dynamic previously described is also influenced by TNF-α.[44] In addition to Langerhans cells, TNF-α also plays a role in the formation of caseous necrosis within the center of the granuloma.[9,44] The anaerobic environment and the action of phagolysosomes within the granulomas inhibit the spread of bacilli. The hypersensitivity promotes the progression to necrosis, making the cells more sensitive to the components of the bacillus (for example, tuberculostearic acid). The final differentiation of the granuloma will depend on the interplay of all the factors cited in this paper. When there is high immunity and low hypersensitivity there will be a low level of necrosis and the granuloma will most likely scar under the action of miofibroblasts. If the hypersensitivity is dominant, the necrosis will spread and the lesion will grow with an increase in the proliferation of bacilli.[46]

In conclusion, although Rich's formula was proposed in the 1950s, it still holds some relevance to the distinct components within the granulomatous lesions. Even after incorporation of current knowledge about pathogenesis of *M. tuberculosis*, the balance between the characteristics of the bacillus and the host protective response is necessary to indicate the outcome of infection.

Conflict of interest

The authors have no conflict of interest to declare.

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PARECER DO COMITÊ DE ÉTICA EM PESQUISA

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FIOCRUZ
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Comitê de Ética em Pesquisas
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CEP FIOCRUZ-BA

Comitê de Ética em Pesquisa – CPqGM/FIOCRUZ

PARECER FINAL Nº 256/2012

Protocolo: 365/ 2011

Projeto de Pesquisa: "DISTINGUINDO TUBERCULOSE ATIVA E LATENTE: UM ESTUDO DE COORTE".

Pesquisador(a) Responsável: Dr. Sérgio Marcos Arruda
Instituição ou Departamento: Centro de Pesquisas Gonçalo Moniz

Considerações:

Após análise ética do projeto e realização dos esclarecimentos solicitados ao pesquisador responsável, o CEP considera que o projeto atende aos princípios éticos de autonomia, beneficência, não maleficência, equidade e justiça.

Diante do exposto, o Comitê de Ética em Pesquisas do Centro de Pesquisas Gonçalo Moniz da Fundação Oswaldo Cruz (CEP-CPqGM/FIOCRUZ), conforme atribuições conferidas pela CONEP/CNS/MS (Carta Doc.32-04/97), com base na Resolução 196/96 e suas complementares, julga **aprovado** o projeto supracitado, com algumas recomendações a seguir:

O CEP/CPqGM-FIOCRUZ especifica abaixo, o período de vigência, bem como, determina as datas para o envio dos relatório parcial e final, referentes ao desenvolvimento do protocolo de pesquisa aprovado.

Vigência: 03/08/2012 a 02/08/2017.

Relatórios parciais: 02/08/2013 - 02/08/2014 - 02/08/2015 - 02/08/2016.

Relatório final: 02/09/2017.

Salvador, 21 de Setembro de 2012.

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IORG-0002090 / IRB-00002612