UNIVERSIDADE FEDERAL DA BAHIA
FACULDADE DE MEDICINA
FUNDAÇÃO OSWALDO CRUZ
INSTITUTO GONÇALO MONIZ

UFBA FIOCRUZ

Programa de Pós-Graduação em Patologia

TESE DE DOUTORADO

AVALIAÇÃO DA RESPOSTA IMUNE CELULAR INDUZIDA POR EXTRATOS LIPÍDICOS DE BCG MOREAU E *MYCOBACTERIUM TUBERCULOSIS*

ALICE SARNO MARTINS DOS SANTOS

Salvador - Bahia
2023

UNIVERSIDADE FEDERAL DA BAHIA
FACULDADE DE MEDICINA
FUNDAÇÃO OSWALDO CRUZ
INSTITUTO GONÇALO MONIZ

Programa de Pós-Graduação em Patologia

AVALIAÇÃO DA RESPOSTA IMUNE CELULAR INDUZIDA POR EXTRATOS LIPÍDICOS DE BCG MOREAU E *MYCOBACTERIUM TUBERCULOSIS*

ALICE SARNO MARTINS DOS SANTOS

Tese apresentada ao programa de Pós-graduação em Patologia Humana para obtenção do grau de Doutora.

Orientador: Prof. Dr. Sérgio Marcos Arruda
Coorientador: Prof. Dr Adriano Queiroz Silva

Salvador - Bahia
2023

Ficha Catalográfica elaborada pela Biblioteca do
Instituto Gonçalo Moniz/ FIOCRUZ – Bahia - Salvador

S237a Santos, Alice Sarno Martins dos.

Avaliação da resposta imune celular induzida por extratos lipídicos de BCG moreau e *Mycobacterium Tuberculosis*./Alice Sarno Martins dos Santos. _ Salvador, 2023.

112 f.: il.: 30 cm

Orientador: Prof. Dr. Sérgio Marcos Arruda
Coorientador: Prof. Dr. Adriano Queiroz Silva

Tese (Doutorado em Patologia Humana) – Universidade Federal da Bahia, Faculdade de Medicina, Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, 2023.

1. BCG. 2. Microbactéria. 3. Extrato lipídico apolar. 4. Expressão gênica. 5. Atenuação genômica. I. Título.

CDU 616-002.5

"AVALIAÇÃO DA RESPOSTA IMUNE CELULAR INDUZIDA POR EXTRATOS LIPÍDICOS DE BCGMOREAU E MYCOBACTERIUM TUBERCULOSIS".

ALICE SARNO MARTINS DOS SANTOS

FOLHA DE APROVAÇÃO

Salvador, 16 de maio de 2023

COMISSÃO EXAMINADORA

Dra. Valéria Cavalcanti Rolla
Pesquisadora
IOC/FIOCRUZ

Dr. Paulo Renato Zuquim Antas
Pesquisador
IOC/FIOCRUZ

Dra. Bruna Aparecida Souza Machado
Professora
SENAI/CIMATEC

Dra. Natália Machado Tavares
Pesquisadora
IGM/FIOCRUZ

Dr. Sérgio Marcos Arruda
Pesquisador
IGM/FIOCRUZ

FONTES DE FINANCIAMENTO

"O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Código de Financiamento 001".

Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Fundação de Amparo à Pesquisa doEstado da Bahia (FAPESB).

Ministério da Saúde.

AGRADECIMENTOS

À minha família e amigos, com os quais escolheria dividir a vida de novo e de novo, por meensinarem, todos os dias, sobre a sorte do amor tranquilo em sua força.

Ao meu orientador, Dr. Sérgio Arruda, e ao meu coorientador, Dr. Adriano Queiroz, pelaconfiança no meu trabalho, suporte e incentivo, nos últimos quatro anos.

Às Dra. Lilian Pimentel e MSc. Luanna de Ângelis pela colaboração imprescindível na extraçãodos extratos lipídicos de *Mycobacterium tuberculosis* utilizados neste trabalho.

Aos colegas de Fiocruz, em especial ao Grupo TB LASP, pelo apoio, companheirismo eamizade constantes e fundamentais nesta caminhada.

Aos professores e colegas do PgPAT, pelo entusiasmo na troca de conhecimento e experiências.A todos que, de alguma forma, participaram deste momento.

À CAPES pelo fomento, apoio financeiro e consolidação do programa de pós-graduação em Patologia Humana.

À FAPESB pelo financiamento do projeto e concessão de bolsas de estudo.

A Universidade Federal da Bahia.

Ao Departamento de Patologia e Medicina Legal, Faculdade de Medicina - UFBA.

SANTOS, Alice Sarno Martins dos. **Avaliação da resposta imune celular induzida por extratos lipídicos de BCG Moreau e *Mycobacterium tuberculosis***. 2023. 112 f. Tese (Doutorado em Patologia) – Universidade Federal da Bahia. Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, 2023.

RESUMO

INTRODUÇÃO: Bacillus Calmette-Guérin (BCG) é a única vacina licenciada disponível para o uso contra tuberculose, apesar de sua eficácia variável. A heterogeneidade genômica entre cepas atenuadas e cepas virulentas de *Mycobacterium tuberculosis* (Mtb), e a perda de lipídios importantes para sua virulência, podem ajudar a explicar a proteção vacinal reduzida diante da possibilidade de ampliar a resposta já conferida pela BCG. **OBJETIVO**: Comparar a resposta imune celular induzida por extratos de lipídios de BCG e Mtb. **MATERIAL E MÉTODOS**: As regiões não-homólogas entre Mtb e seis cepas de BCG foram categorizadas funcionalmente após alinhamento das sequências genômicas *in silico*. A resposta imune celular induzida pelos lipídios de BCG e Mtb, em macrófagos RAW 264.7 e células mononucleares do sangue periférico, foi avaliada por RT-qPCR, Citometria de Fluxo e Ensaio Imunoenzimático (ELISA). **RESULTADOS**: Foram identificados 14 genes relacionados a lipídios, não-homólogos em BCG, que indicam metabolismo similar à dormência, o que pode estar relacionado à proteção vacinal reduzida. Lipídios de BCG induziram menores expressões de *IL-1β* e *IL-6*, após 12h e 24h, e menores frequências de linfócitos ativados, de memória e produtores de citocinas, quando comparada ao Mtb. **CONCLUSÃO**: Importantes deleções genômicas, em BCG, indicam a redução da síntese e do metabolismo de lipídios, que leva à atenuação da resposta imune celular, quando comparada ao Mtb.

Palavras-chave: BCG. Micobactéria. Extrato lipídico apolar. Expressão gênica. Atenuação genômica.

SANTOS, Alice Sarno Martins dos. Cellular immune response induced by lipid extracts of BCG Moreau and *Mycobacterium tuberculosis*. 2023. 112 f. Tese (Doutorado em Patologia) – Universidade Federal da Bahia. Instituto Gonçalo Moniz, Fundação Oswaldo Cruz,Salvador, 2023.

ABSTRACT

INTRODUCTION: Bacillus Calmette-Guérin (BCG) is the only licensed vaccine available against tuberculosis, despite its variable efficacy. The genomic heterogeneity among attenuated and virulent strains of *Mycobacterium tuberculosis* (Mtb), as well as the loss of key lipid antigens, might help explain the vaccine's diminished protection, considering the potential for boosting the immune response already induced by BCG. **AIM:** To compare the cellular immune response induced by lipid extracts of BCG and Mtb. **MATERIAL AND METHODS:** Non- homologous regions between Mtb and six strains of BCG were functionally categorized after alignment of whole genome sequences *in silico*. The cellular immune response induced by lipid extracts of BCG and Mtb in RAW 264.7 macrophages and peripheral mononuclear blood cells isolated from health individuals, was measured by RT-qPCR, Flow Cytometry, and Immunoenzymatic Assay (ELISA). **RESULTS:** 14 non-homologous genes in BCG, associated with lipids, indicate to a dormant-like metabolism, that might lead to reduced protection. Furthermore, BCG lipids induced lower expressions of *IL-1β* and *IL-6* after 12h and 24h, with lower frequencies of activated, memory and cytokine producing lymphocytes, when compared to Mtb. **CONCLUSION:** Key genome deletions in BCG indicate decrease in synthesis and metabolism of lipids, leading to an attenuated cellular immune response, when compared to Mtb.

Keywords: Mycobacteria. Apolar lipid extracts. Genomic attenuation. Gene expression. Cellular immune response.

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

AC2SGL	Sulfoglicolipídio diacilado
BCG	Bacille Calmette-Guérin
DAT	Diaciltrealose
PAT	Poliaciltrealose
LAM	Lipoarabinomanana
LM	Lipomanana
MA	Ácido micólico
ManLAM	Lipoarabinomanana manosilada
Mtb	Mycobacterium tuberculosis
PBMC	Células mononucleares do sangue periférico
PDIM	Dimicocerato de fitilcerol
PGL	Glicolipídio fenólico
PIM	Fosfatidilinositol manosídeo
RD	Região de diferenciação
SL-1	Sulfoglicolípide
TB	Tuberculose
TDM	Trealose dimicolato
TMM	Trealose monomicolato

SUMÁRIO

1 **INTRODUÇÃO**

1.1 TUBERCULOSE PULMONAR: ASPECTOS GERAIS E EPIDEMIOLOGIA

A tuberculose (TB) é causada a partir da inalação de bacilos de *Mycobacterium tuberculosis* (Mtb), presentes em aerossóis produzidos pela fala, tosse ou saliva de indivíduos infectados e sintomáticos (PAI et al., 2016). Dentre as diferentes formas clínicas da doença, a TB pulmonar apresenta maior prevalência e incidência: em 2021, foram estimados 10,6 milhões de novos casos e 1,6 milhão de mortes, no mundo. O Brasil figura entre os 30 países com maior incidência de TB, com 96 casos por 100.000 habitantes e 4,500 mortes anuais estimadas (WHO, 2022a)

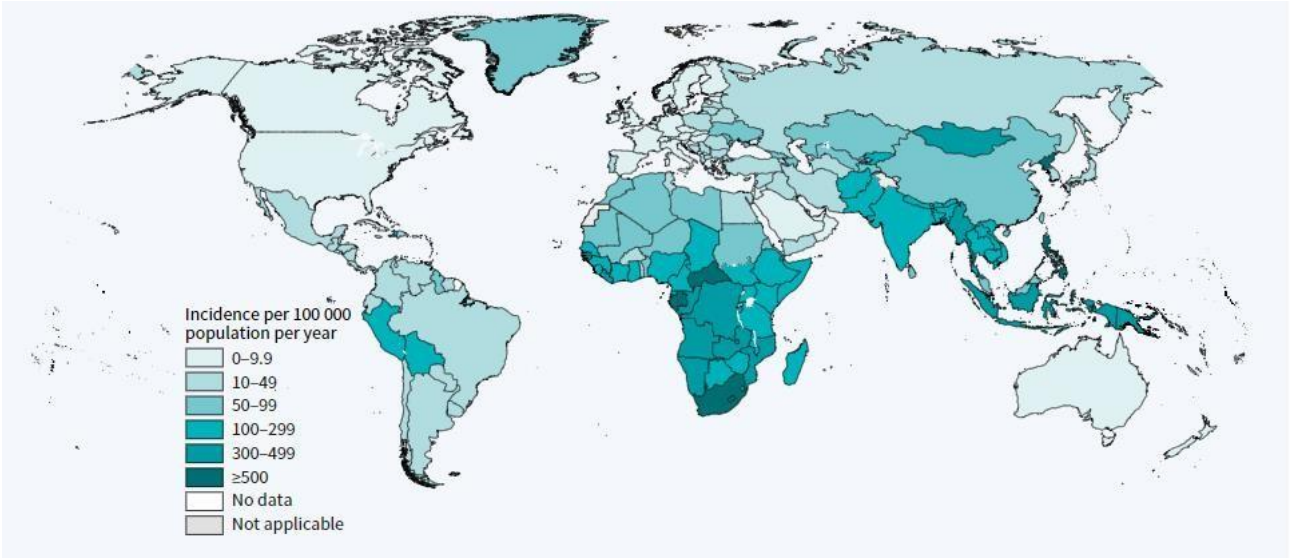
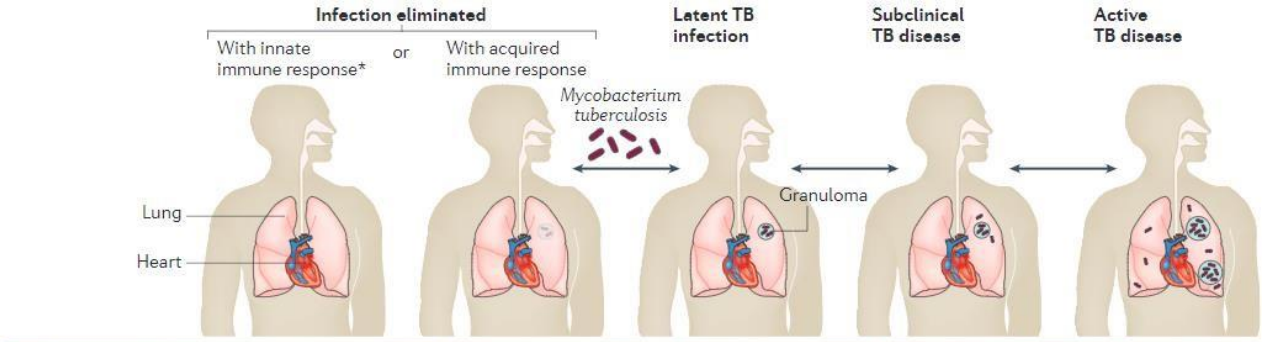


Figura 1 - Incidência dos casos de TB pulmonar no mundo em 2021.
Fonte: (WHO, 2022).

A TB pulmonar apresenta sintomas sistêmicos, como febre, perda de peso, tosse e fadiga, que podem se assemelhar a outras doenças transmitidas por vias aéreas. Assim, o diagnóstico de TB pulmonar costuma ser feito por métodos imunológicos, microbiológicos ou de imagem, sendo a cultura de amostras de escarro o padrão-ouro para determinar a infecção ativa por Mtb. O tratamento antibiótico, por seis meses, é recomendado a indivíduos infectados e assintomáticos (TB latente), como terapia preventiva (PAI et al., 2016).

As formas clínicas de TB pulmonar representam um espectro que inclui, desde a eliminação natural do bacilo, as formas latentes e subclínicas da infecção, até a TB ativa. Este amplo espectro implica diferentes perfis de respostas imune observados, bem como a apresentação de sintomas, técnicas de diagnóstico e necessidade de tratamento (BARRY et al., 2009; PAI et al., 2016). Os indivíduos com as formas subclínica e ativa da doença, possuem a infecção não- controlada e são, portanto, capazes de infectar indivíduos sadios. Já os indivíduos com a forma

latente, são capazes de controlar a infecção por Mtb, dentro de granulomas, e não apresentam sintomas ou capacidade de infecção (BARRY et al., 2009). Estima-se que um terço da população mundial esteja infectada com a forma latente da doença, com potencial reativação de 10% dos casos, o que representa, portanto, um dos maiores desafios para eliminação da TB(WHO, 2022a).



TST	Negative	Positive	Positive	Positive	Usually positive
IGRA	Negative	Positive	Positive	Positive	Usually positive
Culture	Negative	Negative	Negative	Intermittently positive	Positive
Sputum smear	Negative	Negative	Negative	Usually negative	Positive or negative
Infectious	No	No	No	Sporadically	Yes
Symptoms	None	None	None	Mild or none	Mild to severe
Preferred treatment	None	None	Preventive therapy	Multidrug therapy	Multidrug therapy

Figura 2 - Espectro de formas clínicas de TB pulmonar após infecção por Mtb.
Fonte: (PAI et al., 2016).

Neste contexto, a OMS lançou, em 2014, o programa *End TB Strategy*, que objetiva reduzir em 90% e 95%, respectivamente, o número de casos e mortes por TB até 2035 (WHO, 2016). As principais medidas deste programa incluem ações e políticas que promovam a prevenção da infecção e estimulem o desenvolvimento de pesquisa e inovação. Dados recentes, entretanto, indicam reduções de 10% e 5,9% em número de casos e mortes por TB, entre 2015 e 2021, distantes do objetivo final determinado (WHO, 2022b). O estudo das vacinas contra TB é, portanto, fundamental para esta proposta de redução acentuada de casos e mortes, e deeliminação da doença.

1.2 VACINA BCG E DIFERENÇAS GENÔMICAS ENTRE CEPAS

A imunização de crianças com a vacina BCG é uma das principais formas de prevenção contra TB. Além de ainda ser a única licenciada, BCG é a vacina mais amplamente utilizada, no mundo, com mais de 4 bilhões de indivíduos vacinados, desde sua implementação (LUCA;

MIHAESCU, 2013; WHO, 2022a). A OMS recomenda a vacinação universal em única dose, logo após o nascimento, em países com maiores incidências e prevalências de TB; ou para grupos de risco em países com menores índices. No Brasil, a BCG faz parte do Programa Nacional de Imunização e alcançou cobertura vacinal de 90% em 2022 (WHO, 2022a).

O desenvolvimento da vacina BCG se iniciou no Instituto Pasteur, em 1990, quando culturas de *Mycobacterium bovis* – agente etiológico da TB bovina – foram cultivadas por 11 anos, período no qual 230 passagens sucessivas de meio foram realizadas. Estas subculturas resultaram, então, em bacilos atenuados de *M. bovis*, que compõem hoje a vacina administrada em humanos há 100 anos (LUCA; MIHAESCU, 2013). A perda da Região de Diferenciação (RD)-1 é a principal atenuação que caracteriza todas as cepas de BCG, responsável pela codificação de antígenos micobacterianos importantes, como o Sistema de Secreção (ESX)-1 ESAT-6 e CFP-10 (BROSCH et al., 2007; LEWIS et al., 2003; MAHAIRAS et al., 1996).

Hoje, a produção de BCG é descentralizada, em laboratórios independentes, o que resultou em 14 cepas distintas atualmente utilizadas no mundo (LUCA; MIHAESCU, 2013). Estas cepas são agrupadas, filogeneticamente, em *early strains* e *late strains*, de acordo com a ausência de RDs e deleções gênicas (BROSCH et al., 2007). A cepa BCG Moreau é a única produzida e disponível no Brasil, apesar da cepa BCG Russian-I ter sido utilizada entre 2018 e 2019 (MINISTÉRIO DA SAÚDE, 2018). Além destas, outras quatro cepas representam 90% das doses de BCG administradas no mundo: Tokyo 172, Danish 1331, Glaxo 1077 e Pasteur 1173P2(WHO, 2012).

Entre as cepas virulentas de Mtb e as cepas atenuadas de *M. bovis*, existem oito grandes deleções genômicas que as diferenciam (RD1 e RD4 a RD11) (BROSCH et al., 2007). Estas diferenças influenciam a resposta imune induzida, no hospedeiro, após infecção por Mtb ou vacinação. Os principais polimorfismos, entre cepas virulentas e atenuadas, estão representados na Figura 3.

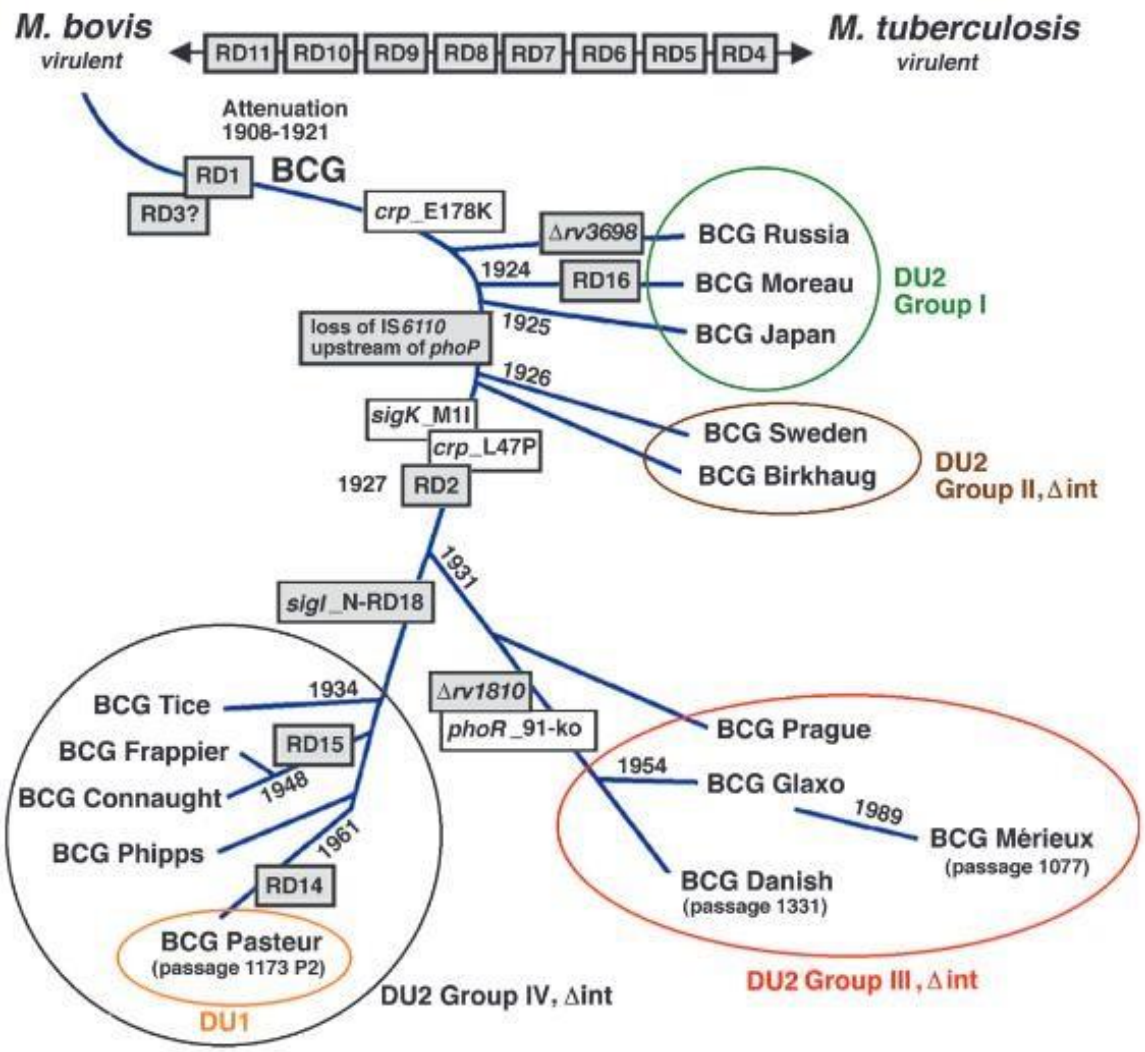


Figura 3 - Filogenia das cepas de BCG em relação às cepas virulentas de *M. bovis* e Mtb.
Fonte:(BROSCH et al., 2007).

Estudos comparativos *in vivo* indicam que as deleções observadas, entre as cepas de BCG, resultam em diferenças nos perfis de resposta imune induzidos, no controle do crescimento micobacteriano e na capacidade protetora da vacina (TRAN et al., 2016; ZHANG et al., 2016). Estas alterações são observadas tanto para a deleção de antígenos proteicos quanto para antígenos lipídicos (HAYASHI et al., 2009, 2010; LAYRE et al., 2014). Entretanto, há poucas evidências oriundas de ensaios clínicos, que indiquem que algumas cepas de BCG sejam mais eficazes que outras, de acordo com as variações genômicas entre elas (AHN et al., 2018; BEHR, 2002; BITENCOURT et al., 2021).

1.3 RESPOSTAS IMUNOLÓGICAS INDUZIDAS POR Mtb E BCG

Após inalação, os bacilos de Mtb são internalizados e fagocitados pelos macrófagos residentes, ainda no alvéolo pulmonar, através de diferentes grupos de receptores, como receptores do tipo *toll* – TLR-2 e TLR-4 – e lectina do tipo C – Mincle (*Macrophage-Inducible C-Type Lectin*), MCL (*Macrophage C-Type Lectin*) e Dectina (DUBÉ et al., 2021; ISHIKAWA; MORI;

YAMASAKI, 2017). Em caso de falha no processo fagocítico, os bacilos migram para o parênquima pulmonar ainda dentro dos macrófagos ou atravessando livremente o epitélio alveolar. Células dendríticas e macrófagos também internalizam o bacilo e migram para os linfonodos, onde antígenos micobacterianos serão apresentados aos linfócitos, via complexo principal de histocompatibilidade (MHC) e receptores CD1 (ISHIKAWA; MORI; YAMASAKI, 2017). O recrutamento de células para o parênquima pulmonar, estimulado pela migração através do epitélio alveolar e ativação linfocítica, promove a formação de granulomas, com o objetivo de conter a infecção por Mtb. Os granulomas são constituídos por diferentes tipos celulares, incluindo linfócitos T $CD4^+$ de perfil Th1 e Th17, linfócitos T $CD8^+$, fibroblastos, macrófagos, macrófagos espumosos e células epitelioides (ETNA et al., 2014; PAIet al., 2016).

Durante a infecção ativa ou reativação de uma infecção latente por Mtb, a estrutura do granuloma não é capaz de conter a proliferação dos bacilos, por motivos ainda não totalmente elucidados. O rompimento do granuloma resulta na liberação do Mtb, que pode voltar a infectar os pulmões, bem como alcançar a corrente sanguínea e outros órgãos. Antígenos micobacterianos são novamente fagocitados por macrófagos e apresentados aos linfócitos, induzindo a produção de citocinas – especialmente TNFα, INFγ, IL-1 e IL-17 – e de células T $CD4^+$ e T $CD8^+$ de memória (ETNA et al., 2014; PAI et al., 2016).

Populações celulares, semelhantes às que são observadas no processo infeccioso, estão presentes após a vacinação intradérmica por BCG. Os bacilos atenuados de *M. bovis* também são internalizados e fagocitados por macrófagos, e reconhecidos, pelas células dendríticas, para apresentação antigênica. As ações dos linfócitos T $CD4^+INF\gamma^+$ e T $CD8^+$, durante a resposta adaptativa, são as mais descritas frente ao estímulo vacinal (FLETCHER et al., 2016; SOARES et al., 2008; TANNER et al., 2019). A capacidade protetora de linfócitos B, bem como a indução de anticorpos antígeno-específicos, por sua vez, vêm sendo apenas mais recentemente exploradas. Apesar disso, não foram ainda identificados quais componentes da resposta imune induzida por BCG são responsáveis por conferir proteção (ANDERSEN; SCRIBA, 2019; MOLIVA; TURNER; TORRELLES, 2017; SATTI; MCSHANE, 2018), ou quais as deleções diminuíram a capacidade das cepas vacinais de induzir uma proteção adequada.

1.4 NOVOS CANDIDATOS VACINAIS CONTRA TB

Apesar de se desconhecer os correlatos de proteção, sabe-se que a vacinação com BCG é eficaz contra as formas disseminadas e mais graves de TB, como TB miliar e meningite tuberculosa. Entretanto, a proteção conferida por BCG contra TB pulmonar varia de 0-80% (ABUBAKAR et al., 2013; MANGTANI et al., 2014; ROY et al., 2014). Diferentes hipóteses tentam explicar esta variação, incluindo diferenças metodológicas na produção das cepas licenciadas e localização geográfica das populações estudadas. A hipótese mais aceita, entretanto, refere-se à exposição

ambiental a outras espécies de micobactéria, que teriam a capacidade de mascarar a resposta protetora induzida pela BCG (BARRETO et al., 2014).

Neste contexto de proteção variável contra TB pulmonar, associado aos altos números de casos e mortes pela doença, diferentes esquemas vacinais já foram avaliados. Em estudos realizados com crianças em idade escolar, a revacinação com BCG, apesar de induzir maiores níveis de IFNγ, se mostrou insuficiente na melhora da proteção conferida pela primeira dose da vacina, contra TB (BARBOSA et al., 2003; BARRETO et al., 2011; RODRIGUES et al., 2005). Dados mais recentes confirmam a maior produção de IFNγ e apontam para ações inespecíficas, como redução de infecções do trato respiratório, por outros agentes etiológicos, e de mortalidade infantil (DOCKRELL; BUTKEVICIUTE, 2022; DUBÉ et al., 2021; MOORLAG et al., 2022; NEMES et al., 2018).

Atualmente, novos candidatos e esquemas vacinais estão em diferentes fases de ensaio clínico (DOCKRELL; MCSHANE, 2022; WHO, 2022a) (Figura 4). Dentre estes, o MVA85A foi o primeiro a apresentar resultados mais promissores. Entretanto, a capacidade de um único antígeno proteico (Ag85A) de induzir a proliferação de linfócitos T $CD4^{+}INF\gamma^{+}$ não resultou em melhora da eficácia vacinal em humanos (TAMERIS et al., 2013a, 2013b). Apenas recentemente foram obtidos indícios iniciais de que é possível incrementar a reposta induzida por BCG, em grupo de indivíduos revacinados ou vacinados com M72/AS01E – candidato composto por antígenos proteicos (Mtb34A e Mtb39A) e sistema adjuvante lipídico (NEMES et al., 2018; TAIT et al., 2019). Estes dados reforçam, assim, a importância do estudo de novos perfis de resposta imune e esquemas vacinais nos esforços para melhor proteção vacinal contraTB pulmonar.

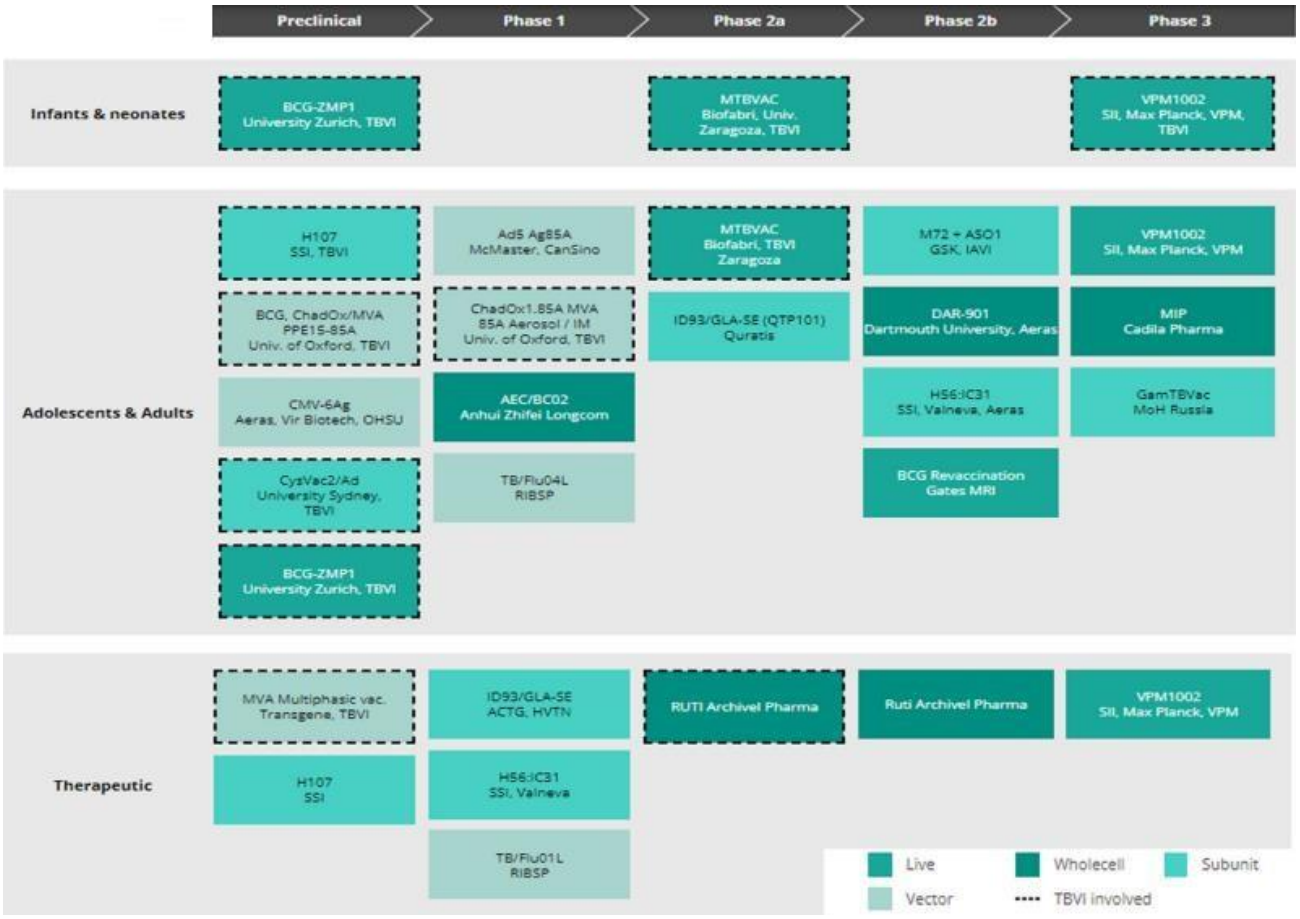


Figura 4 - Candidatos e esquemas vacinais estão em diferentes fases de ensaio clínico.
Fonte: (DOCKRELL; MCSHANE, 2022).

1.5 LIPÍDIOS DA PAREDE CELULAR DE Mtb E BCG

A parede celular de Mtb, assim como das demais micobactérias, é ricamente composta por lipídios, que representam até 40% de seu peso seco (ANDERSON, 1943). Grande parte do genoma de Mtb é, portanto, dedicado à biossíntese e degradação de mais de 5.000 espécies lipídicas (COLE; BARRELL, 1998; LAYRE et al., 2011), adaptadas durante o processo evolutivo, para promover o acúmulo de moléculas de perfil apolar, que conferem maior hidrofobicidade à parede celular, bem como melhor transmissão bacilar por aerossóis (FALKINHAM; III, 2003). Estes lipídios apresentam papel importante não apenas na persistência do patógeno, como na indução da resposta imune do hospedeiro (DULBERGER; RUBIN; BOUTTE, 2020; FORRELLAD et al., 2013; PETRILLI et al., 2020). A Tabela 1 apresenta os lipídios de Mtb mais bem descritos na literatura, seus receptores, ações associadase sua presença/ausência em BCG Moreau.

Tabela 1 - Principais lipídios expressos na parede celular de Mtb e sua presença descrita em BCG Moreau.

Nome	Sigla	Receptores	Ações associadas	BCG Moreau	Referências
Ácido micólico[a]	MA	CD1b	Proliferação micobactericana Inflamação	Presente	DULBERGER; RUBIN; BOUTTE, 2020 DUBNAU et al., 2000
Diaciltrealose[a]	DAT	CD1b Mincle	Internalização micobacteriana Inibição da proliferação de linfócitos T Imunossupressão	Ausente	KARAKOUSIS; BISHAI; DORMAN, 2004 SAAVEDRA et al., 2001 GONZALO-ASENSIO et al., 2014 REIJNEVELD et al., 2021
Dimicocerato de ftiocerol[a]	PDIM	Mac-1	Proliferação micobacteriana Inibição da fagocitose Impermeabilização da parece celular	Reduzido	CHEN et al., 2007 COX et al., 1999 CAMACHO et al., 2001 ASTARIE-DEQUEKER et al., 2009 AUGENSTREICH et al., 2020
Glicolipídio fenólico[a]	PGL	Mac-1	Proliferação micobacteriana Sobrevivência dentro dos macrófagos Inibição de citocinas pró-inflamatórias	Reduzido	LEUNG et al., 2008 OLDENBURG et al., 2018
Lipomanana[b]	LM	TLR2 SIGNR3	Manutenção da estrutura da parede celular Proliferação micobacteriana Promoção de apoptose Indução de IL-12	Presente	HARDING; BOOM, 2010 VIGNAL et al., 2003 TANNE et al., 2009 FUKUDA et al., 2013 HAITES et al., 2005 DAO et al., 2004
Lipoarabinomanana[b]	LAM	CD1b Dectina-2 TLR2 Receptor de manose	Manutenção da parede celular Proliferação micobacteriana Inibição de IL-12	Presente	HARDING; BOOM, 2010 VIGNAL et al., 2003 KANG et al., 2005 NIGOU et al., 2002

[a] Lipídio apolar/hidrofóbico. [b] Lipídio polar/hidrofílico.
Fonte: Elaborado pela autora

Tabela 1 - Principais lipídios expressos na parede celular de Mtb e sua presença descrita em BCG Moreau (continuação).

Lipoarabinomanana manosilada[b]	ManLAM	Dectina-2	Manutenção da parede celular	Presente	JÓZEFOWSKI; SOBOTA; KWIATKOWSKA, 2008
		Receptor de manose	Proliferação micobacteriana		YONEKAWA et al., 2014
		SIGNR3	Inibição de IL-12 e TNFα		KANG et al., 2005
		DC-SIGN	Indução de IL-10		TANNE et al., 2009
					GEIJTENBEEK; VAN KOOYK, 2003
					TAILLEUX et al., 2003
Poliaciltrealose[a]	PAT	Não descrito	Internalização micobacteriana	Ausente	KARAKOUSIS; BISHAI; DORMAN, 2004
					GONZALO-ASENSIO et al., 2014
Sulfoglicolípide[a]	SL-1	CD1	Inibição da fusão fagolisossoma	Ausente	DULBERGER; RUBIN; BOUTTE, 2020
			Inibição da fagocitose		PABST et al., 1988
Trealose dimicolato[a]	TDM	Mincle	Formação do granuloma	Presente	ISHIKAWA et al., 2009
		MCL	Indução de TNFα, CXCL2 e óxido nítrico		WERNINGHAUS et al., 2009
					MIYAKE et al., 2013

[a] Lipídio apolar/hidrofóbico. [b] Lipídio polar/hidrofílico.
Fonte: Elaborado pela autora

A composição lipídica da parede celular de Mtb está, portanto, associada ao perfil de resposta imune induzido no hospedeiro, em diferentes momentos da infecção. A produção de DAT e SL-1, por exemplo, resulta na inibição da resposta inflamatória, enquanto a produção de TDM promove a inflamação (DULBERGER; RUBIN; BOUTTE, 2020; QUEIROZ; RILEY, 2017).

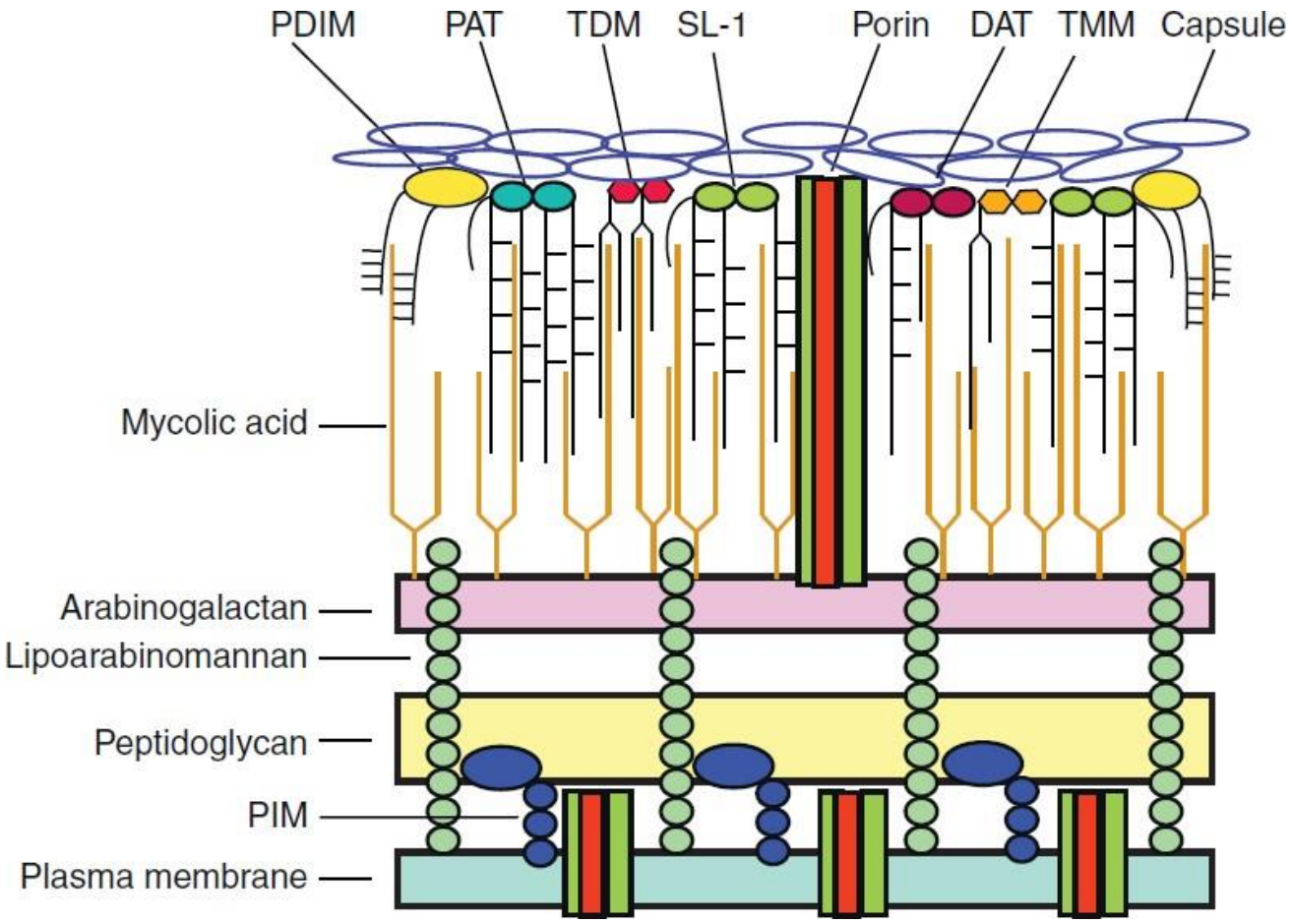


Figura 5 - Representação esquemática da parede celular de Mtb.
Fonte: (OUELLET;JOHNSTON; DE MONTELLANO, 2011).

Os *operons phoPR* e *mce1* são importantes nesta remodelagem da parede celular de Mtb, apesar deste mecanismo não estar completamente elucidado. O *operon phoPR* é um sistema de dois componentes, cuja ausência foi primeiro associada a mudanças fenotípicas, incluindo alterações morfológicas das colônias e redução no tamanho dos bacilos (BROSET; MARTÍN; GONZALO-ASENSIO, 2015). Atualmente, *phoPR* é associado à secreção de ESAT-6 e produção de lipídios compostos por trealose, como DAT, PAT, TDM e SL-1 (ASENSIO et al., 2006; WALTERS et al., 2006). Deleções e mutações em seus genes codificadores – Rv0757 (*phoP*) e Rv0758 (*phoR*) –, ainda, resultam em cepas menos virulentas: *M. africanum*, *M. bovis* e Mtb H37Ra (GONZALO-ASENSIO et al., 2014). Para BCG, particularmente, os polimorfismos observados variam entre cada cepa vacinal. BCG Danish e Glaxo, por exemplo, não produzem phoR, enquanto phoP é um pseudogene em BCG Prague (LEUNG et al., 2008). Neste contexto, o *operon phoPR* já foi utilizado como forma de ajustar a

virulência de BCG, em algumas cepas e candidatos vacinais (AHN et al., 2018; ARBUES et al., 2013).

A família de 4 *operosn mce*, por sua vez, é um grupo de transportadores transmembranares, importantes para a virulência de Mtb. O *operon mce1*, em especial, é responsável pela entrada e sobrevivência do bacilo na célula hospedeira, e sua expressão/repressão está associada a mudanças nos níveis de mais de 400 lipídios da parede celular (ARRUDA et al., 1993; QUEIROZ et al., 2015). Cepas H37Rv com deleção do *operon mce1* (Δ1) se mostraram mais virulentas *in vivo*, quando comparadas às infecções pela cepa H37Rv *wild-type* (WT) (SHIMONO et al., 2003). Cepas Δ1 apresentaram, ainda, menor capacidade de promover resposta imune celular do tipo Th1 e formação de granulomas organizados, comprometendo a contenção das cepas. A maior virulência das cepas Δ1 está também associada à maior capacidade de manutenção do estado de latência, em ambiente de hipóxia, semelhante ao observado no granuloma (HAILE; BJUNE; WIKER, 2002). Ainda, o fenótipo causado pela repressão do *operon mce1*, e descrito na cepa Δ1, foi observado na cepa H37Rv WT, durante as quatro primeiras semanas de infecção *in vivo* (UCHIDA et al., 2007). Já em BCG, os genes codificadores do *operon mce1* estão presentes, mas possíveis diferenças do papel deste *operon* nas cepas vacinais, bem como seus fenótipos ao longo do tempo, ainda não foram descritos. Sabe-se, entretanto, que há deleção de todos os genes codificadores do *operon mce3*, também associado à sobrevivência e internalização micobacteriana, bem como à produção de anticorpos (AHMAD et al., 2004; GIOFFRÉ et al., 2005; OBREGÓN-HENAO et al., 2011).

As diferenças genômicas e filogenéticas, bem como a influência destas sobre antígenos proteicos, entre BCG e as cepas virulentas de Mtb e *M. bovis*, já foram exaustivamente descritas (ABDALLAH et al., 2015; BROSCH et al., 2007; ZHANG et al., 2013). O impacto, entretanto, da heterogeneidade genômica, entre espécies, sobre o metabolismo lipídico e a composição da parede celular, nas cepas vacinais, tem sido menos explorado ou limitado à algumas espécies lipídicas. Apesar disto, seu papel na virulência e capacidade protetora da vacina já foi demonstrado. A ausência de PDIM e PGL observada em algumas cepas de BCG, por exemplo, está associada a menor virulência, atividade inflamatória e proliferação micobacteriana, nos pulmões e baço de camundongos imunodeficientes (CHEN et al., 2007; TRAN et al., 2016).

A influência dos antígenos lipídicos sobre a virulência da BCG também é refletida na capacidade de proteção vacinal. Em estudo que avaliou a inoculação de dois lipídios micobacterianos – sulfoglicolipídio diacilado (AC_2SGL) e fosfatidilinositol manosídeo (PIM_2)–, em camundongos posteriormente infectados com Mtb, foi observada redução na carga bacteriana, patogênese pulmonar mais branda e níveis de proteção comparáveis àqueles conferidos por antígenos proteicos (LARROUY-MAUMUS et al., 2017). Entretanto, um estudo comparativo mais amplo para caracterizar a resposta imune induzida pelos extratos lipídicos das cepas vacinais e de Mtb ainda não foi realizado. (MOLIVA et al., 2018).

2 **JUSTIFICATIVA E HIPÓTESE**

A TB pulmonar ainda é uma das principais causas de morte no mundo, com 1,6 milhões de óbitos estimados, em 2021, além de 10,6 milhões de casos novos. Apesar de ser a única vacina atualmente licenciada, a BCG apresenta eficácia variável – entre 0 a 80% –, de acordo com a população de estudo.

Por tratar-se de patógeno intracelular, a resposta de perfil Th1 com proliferação de células T $CD4^+$ produtoras de IFNγ, foi amplamente explorada em estudos de novos candidatos vacinais. Entretanto, em 2014, resultados de ensaio clínico do candidato MVA85A evidenciaram que o aumento da frequência de linfócitos $CD4^+IFN\gamma^+$ não resultava em maior proteção contra a TB. Apenas em 2018, foram obtidos os primeiros indícios de que é possível induzir resposta capaz de melhorar a proteção já conferida pela BCG, em ensaios clínicos que incluíram a revacinação de adolescentes e a utilização de vacinas de subunidade associadas a adjuvante lipídico.

Há potencial, portanto, para o estudo de novos candidatos vacinais que incluam grupos heterogêneos e menos explorados de antígenos, com o intuito de induzir resposta imune de maior eficácia. A atenuação de grandes regiões do genoma de BCG resultou, não apenas na perda de antígenos proteicos importante, como também antígenos lipídicos. Estes antígenos cumprem papel fundamental na interface patógeno-hospedeiro, promovendo persistência ou indução de resposta imune.

Assim, pretende-se avaliar o perfil dos lipídios expressos na parede celular de BCG, bem como a resposta imune celular por eles induzida, em comparação a Mtb. A hipótese deste trabalho é de que a perda de fatores de virulência lipídicos, pela BCG Moreau, resulta na indução de resposta imune atenuada, quando comparada à resposta induzida por Mtb.

3 **OBJETIVOS**

3.1 OBJETIVO GERAL

Comparar o perfil de resposta imune celular induzido por lipídios de BCG Moreau e Mtb.

3.2 OBJETIVOS ESPECÍFICOS

- Identificar *in silico* diferenças genômicas entre BCG e Mtb relacionadas ao conteúdo e metabolismo lipídicos;
- Comparar expressão de citocinas pró-inflamatórias e anti-inflamatória induzida por extratos lipídicos apolares de BCG Moreau e Mtb, em cultura de macrófagos murinos de linhagem;
- Comparar a ativação de linfócitos e produção de citocinas, por extratos lipídicos apolares de Mtb e BCG Moreau, em células de PBMC de indivíduos sadios.

4 **RESULTADOS**

4.1 CAPÍTULO 1: *IN SILICO* COMPARISONS OF LIPID-RELATED GENESBETWEEN *Mycobacterium tuberculosis* AND BCG VACCINE STRAINS

As diferenças genômicas, entre as cepas vacinais de BCG e de Mtb, já foram extensivamente exploradas quanto ao seu impacto sobre antígenos proteicos. Tais estudos genômicos comparativos, entretanto, foram pouco utilizados para a compreensão das diferenças na composição e metabolismo de extratos lipídicos das cepas vacinais. Este artigo, que propôs responder o primeiro objetivo específico deste trabalho, identificou genes ausentes nas seis cepas vacinais mais utilizadas no mundo, que estão relacionados à composição lipídica global de BCG.

Artigo, que segue anexado a esse documento, foi publicado na revista Genetics and Molecular Biology. SARNO, Alice; BITENCOURT, Julia; QUEIROZ, Adriano; ARRUDA, Sérgio. *In silico* comparisons of lipid-related genes between *Mycobacterium tuberculosis* and BCG vaccine strains. 2021.

Genetics and Molecular Biology, 44, 4, e20210024 (2021)

DOI: https://doi.org/10.1590/1678-4685-GMB-2021-0024

Research Article
Genomics and Bioinformatics

In silico comparisons of lipid-related genes between *Mycobacterium tuberculosis* and BCG vaccine strains

Alice Sarno[1,2], Julia Bitencourt[1], Adriano Queiroz[1] and Sergio Arruda[1,3]

[1]*Fundação Oswaldo Cruz, Instituto Gonçalo Moniz, Laboratório Avançado em Saúde Pública, Salvador, BA, Brazil.*
[2]*Universidade Federal da Bahia, Salvador, BA, Brazil.*
[3]*Universidade do Estado da Bahia, Salvador, BA, Brazil.*

Abstract

Despite highly variable efficacy, BCG (Bacillus Calmette-Guérin) is the only vaccine available to prevent the tuberculosis (TB). Genomic heterogeneity between attenuated BCG strains and virulent *Mycobacterium tuberculosis* might help to explain this vaccine's impaired capacity to induce long-term protection. Here, we investigate the lipid-related genes absent in attenuated BCG strains in order to correlate changes in both lipid metabolism and cell-wall lipid content to vaccine impairment. Whole genome sequences of *M. tuberculosis* H37Rv and the six most used BCG strains worldwide were aligned and the absent regions functionally categorized. Genomes of the BCG strains showed a total of 14 non-homologous lipid-related genes, including those belonging to *mce3* operon, as well as the gene *echaA1*, which encodes an enoyl-CoA hydratase, and the genes encoding phospholipases PlcA, PlcB and PlcC. Taken together, the depletion of these *M. tuberculosis* H37Rv genomic regions were associated with marked alterations in lipid-related genes of BCG strains. Such alterations may indicate a dormant-like state and can be determining factors to the vaccine's inability to induce long-term protection. These lipids can be further evaluated as an adjuvant to boost the current BCG-based vaccine.

Keywords: Genome comparison, BCG, lipid, cell-wall, tuberculosis.

Received: January 27, 2021; Accepted: July 22, 2021.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is a leading cause of death worldwide: In 2019 alone, 1.2 million deaths and 10 million new cases were reported. About a quarter of the world population is estimated to harbor latent TB infection and are therefore at risk of developing active disease (WHO, 2020).

BCG (Bacillus Calmette-Guérin), a live-attenuated strain of *Mycobacterium bovis*, is currently the only vaccine available to prevent TB, typically administrated in endemic countries or in populations at high risk of infection (Cernuschi *et al.,* 2018). Currently, six strains account for more than 90% of the vaccines in use worldwide: early strains BCG Moreau, BCG Russian and BCG Tokyo, and late strains BCG Danish, BCG Glaxo and BCG Pasteur (WHO, 2012, 2017). Despite its capacity to protect against disease progression and disseminated forms of TB, the efficacy of BCG against pulmonary TB in adult populations varies from 0% to 80% (Mangtani *et al.,* 2014; Roy *et al.,* 2014).

One of the hypotheses to explain the variable protectiveness of BCG posits the genomic heterogeneity between vaccine and virulent *M. bovis* and *M. tuberculosis* strains (Behr 2002; Liu *et al.,* 2009; Angelidou *et al.,* 2020). Since it was first obtained and distributed, BCG has accumulated large sequence polymorphisms and has lost several virulence factor genes, including deletion of the region RD1, which encodes antigenic proteins ESAT-6 and CFP-10 (Mahairas *et al.,* 1996; Lewis *et al.,* 2003). However, the expression of RD1 in recombinant BCG does not result in a complete restoration of protection against TB, which could indicate that other mechanisms may be involved in virulence (Pym *et al.,* 2003).

The genomic differences between BCG strains and virulent *M. bovis* and *M. tuberculosis,* as well as the remodeling of protein complexes, have been comprehensively explored through phylogenetic analysis (Brosch *et al.,* 2007; Zhang *et al.,* 2013; Abdallah *et al.,* 2015). However, the impact of genomic heterogeneity on virulence factors related to mycobacteria cell-wall lipid content and lipid metabolism has received less attention (Abdallah *et al.,* 2015). Discrepancies in lipid species in the cell walls of virulent and attenuated strains of mycobacteria might play a key role in host-pathogen interaction (Guenin-Macé *et al.,* 2009; Queiroz and Riley 2017; Mishra *et al.,* 2019). In BCG, genome polymorphisms and the absence of specific cell-wall lipid components have resulted in less-virulent strains that induce a restrained pro-inflammatory immune response and limit BCG-mediated T cell protection, with diminished immunological activity (Hayashi *et al.,* 2009; Tran *et al.,* 2016; Zhang *et al.,* 2016).

Here we compared the whole genome sequences of *M. tuberculosis* H37Rv and the six BCG strains more frequently used worldwide in an attempt to identify genomic differences related to lipid content and metabolism. By this approach, we established a comprehensive list of lipid-related genes absent in these BCG strains, in which the codified molecules may contribute to improve the BCG vaccines currently in circulation.

Send correspondence to Sergio Arruda. Universidade do Estado da Bahia, Rua Waldemar Falcão, 121, 40296-710, Salvador, BA, Brazil. E-mail: sergio.arruda@fiocruz.br.

Material and Methods

Whole genome sequence selection

The following whole genome sequences stored on GenBank were compared *in silico*: *M. tuberculosis* H37Rv (accession number NC_000962.3), early strains *M. bovis* BCG Moreau RDJ (accession number AM412059.2), *M. bovis* BCG Russian 368 (accession number CP009243.1) and *M. bovis* BCG Tokyo 172 (accession number AP010918.1), and late strains *M. bovis* BCG Danish 1331 (accession number CP039850.1), *M. bovis* BCG Glaxo (accession number NZ_CUWJ01000001.1) and *M. bovis* BCG Pasteur 1173P2 (accession number AM408590.1). The six BCG strains were selected for comparison with *M. tuberculosis* H37Rv, since these account for more than 90% of the vaccines in use worldwide.

Determination of homologous and non-homologous regions among sequences

Mauve software (Darling *et al.,* 2010) was used to align, identify and characterize homologous and non-homologous regions among the whole genomes. Regions were considered homologous if percent identity was > 60% and query cover was > 70%. After alignment, the gene annotations for homologous and non-homologous regions were obtained and exported as comma-separated values for further analysis. The number and percentage of homologous and non-homologous regions between each BCG strain and the *M. tuberculosis* H37Rv sequence were compared to measure the similarity among genomes. Finally, the gene annotations for non-homologous regions in each BCG strain were confirmed by BLASTN searches in the NCBI database (Morgulis *et al.,* 2008).

Functional category determination of non-homologous regions of early and late strains of BCG

The gene annotations in non-homologous regions confirmed by BLASTN searches were functionally categorized using the Mycobrowser database (Kapopoulou *et al.,* 2011). Early (BCG Moreau RDJ, BCG Russian and BCG Tokyo 172) and late (BCG Danish 1331, BCG Glaxo 1077 and BCG Pasteur 1173P2) BCG strains were compared to the *M. tuberculosis* H37Rv genome.

Results

Similarities among homologous and non-homologous regions

Sequence alignment was performed using Mauve software to investigate differences and similarities between the *M. tuberculosis* H37Rv and BCG strains genomes and to better visualize homology among the studied genomes. 4,034 genomic regions were identified in the *M. tuberculosis* H37Rv genome, 3,996 in BCG Danish, 3,993 in BCG Glaxo, 3,944 in BCG Moreau, 3,991 in BCG Pasteur, 4,297 in BCG Russian and 3,985 in BCG Tokyo (Figure 1).

The overlap between and the total number of homologous and non-homologous regions across *M. tuberculosis* H37Rv and all six BCG genome sequences, represented as Venn diagrams, are illustrated in Figure 1. As expected, high homogeneity was observed between most BCG strains and *M. tuberculosis* H37Rv, with up to 94.6% of homologous regions identified in the BCG Moreau genome. BCG Russian was the strain with the greater number of non-homologous regions (16.7%), when compared to *M. tuberculosis* H37Rv. Together, the data shows a comparable genomic heterogeneity between each strain and *M. tuberculosis* H37Rv, as well as the overall similarity among attenuated vaccine strains (Figure S1).

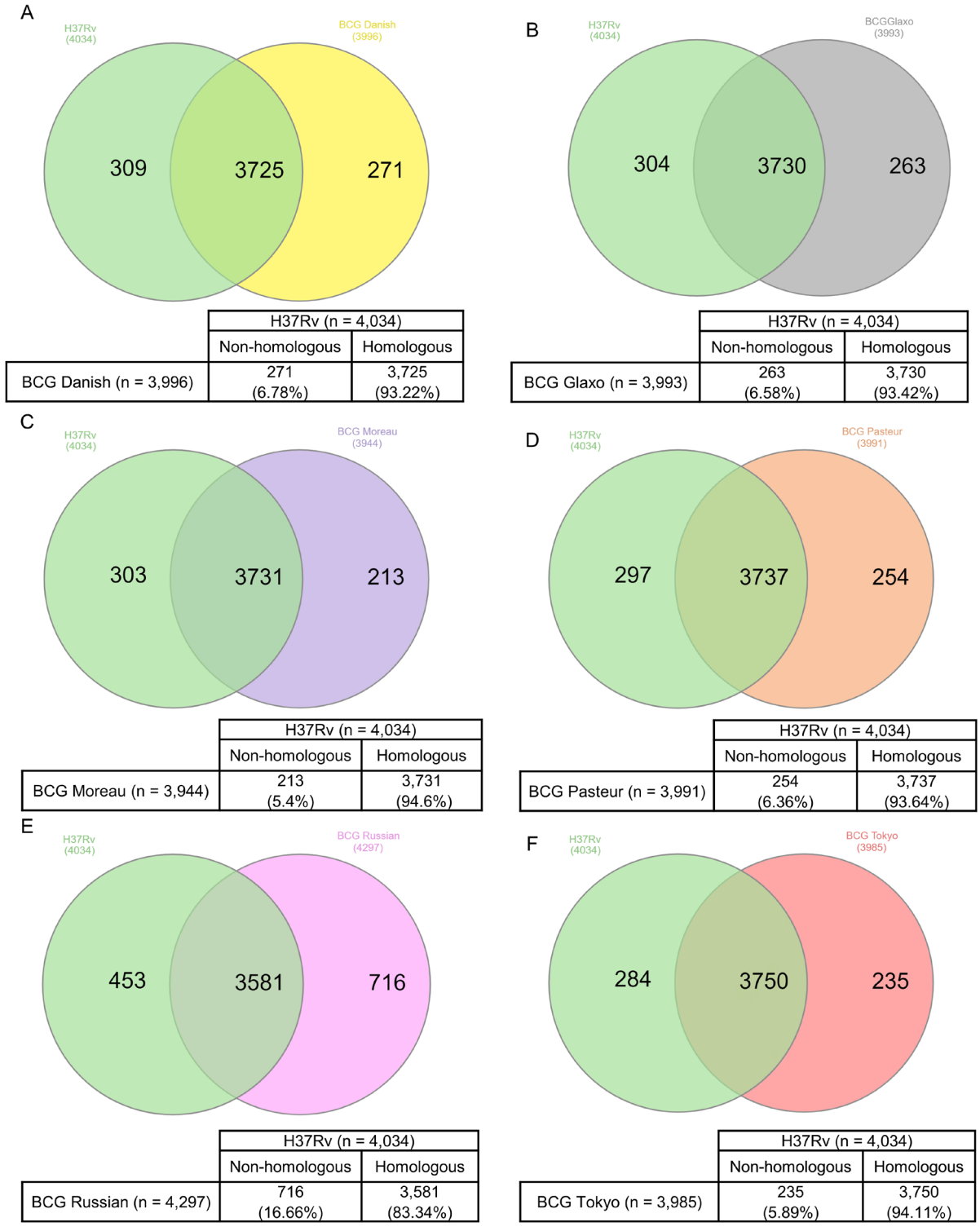
Functional category identification of genes in non-homologous regions

The genes in non-homologous regions of all BCG strains identified in the alignment were confirmed by BLASTN searches and grouped according to functional category using the Mycobrowser database (Figure 2). The distribution of non-homologous regions – with no BLASTN similarity – in each functional category was similar among strains from the same phylogenetic groups: early strains (BCG Moreau, BCG Russian and BCG Tokyo) and late strains (BCG Danish, BCG Glaxo and BCG Pasteur).

Most of the identified non-homologous regions were associated with functional category "insertion sequences and phages". In comparison to *M. tuberculosis* H37Rv, the number of non-homologous genes in this category was 28 among all BCG strains, representing between 31.11% and 37.33% of all non-homologous regions. In the early strains, the next most common identified category was "intermediary metabolism and respiration", with 11 (between 14.1% and 14.67%) non-homologous regions, followed by "cell-wall and cell processes", with 10 (between 12.82% and 13.33%) non-homologous regions. In the late strains, the opposite was identified: "cell-wall and cell processes" was the second most common identified category, with 14 (between 15.56% and 16.28%) non-homologous regions, followed by "intermediary metabolism and respiration", with 11 (between 12.22% and 12.79%) non-homologous regions. Other categories also associated with non-homologous regions included "conserved hypotheticals" and "virulence, detoxification and adaptation".

Highlighted in Table 1 are the 14 lipid-related genes absent in all six BCG vaccine strains most commonly used worldwide, when compared to *M. tuberculosis* H37Rv. The genes are associated with the functional categories "cell-wall and cell processes" (4), "virulence, detoxification and adaptation" (6), "lipid metabolism" (1) and "intermediary metabolism and respiration" (3). The complete list of absent genes, in all functional categories, is described in Table S1.

Ten of these 14 genes belonged to the *mce3* operon: four were in the "cell wall and cell processes" category (Rv1970 and Rv1972 to Rv1974) and six (Rv1965 to Rv1969 and Rv1971) in the "virulence, detoxification, adaptation" category. The gene encoding enoyl-CoA hydratase (Rv0222), which is part of the fatty acid degradation metabolism, was categorized as "lipid metabolism". Finally, three genes encoding phospholipases PlcC, PlcB and PlcA (Rv2349c to Rv2351c, respectively), related to lipid metabolism, were included in the "intermediary metabolism and respiration" category.



	H37Rv (n = 4,034)	
	Non-homologous	Homologous
BCG Danish (n = 3,996)	271 (6.78%)	3,725 (93.22%)

	H37Rv (n = 4,034)	
	Non-homologous	Homologous
BCG Glaxo (n = 3,993)	263 (6.58%)	3,730 (93.42%)

	H37Rv (n = 4,034)	
	Non-homologous	Homologous
BCG Moreau (n = 3,944)	213 (5.4%)	3,731 (94.6%)

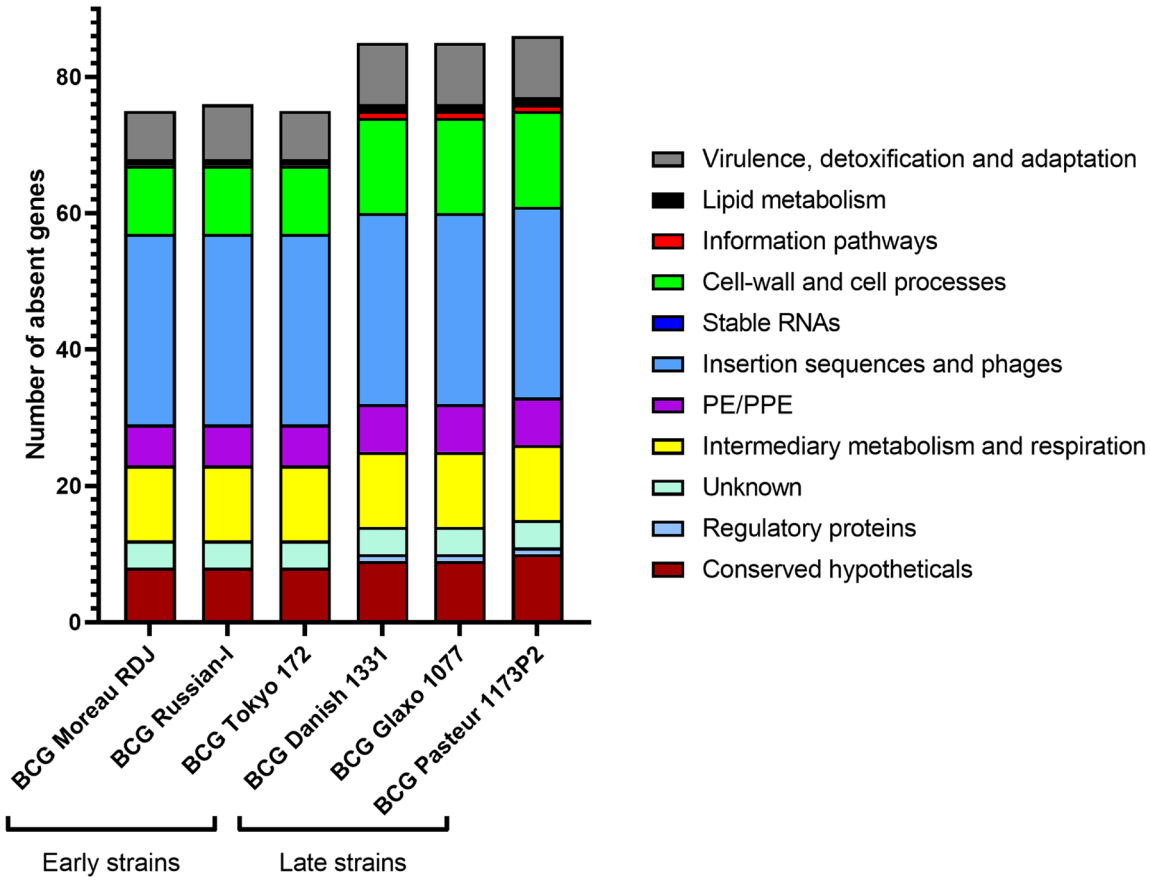
	H37Rv (n = 4,034)	
	Non-homologous	Homologous
BCG Pasteur (n = 3,991)	254 (6.36%)	3,737 (93.64%)

	H37Rv (n = 4,034)	
	Non-homologous	Homologous
BCG Russian (n = 4,297)	716 (16.66%)	3,581 (83.34%)

	H37Rv (n = 4,034)	
	Non-homologous	Homologous
BCG Tokyo (n = 3,985)	235 (5.89%)	3,750 (94.11%)

Figure 1 – Homologous and non-homologous regions between *M. tuberculosis* H37Rv, early BCG strains and late BCG strains genome sequences. Venn diagrams showing overlap between and the total number of homologous and non-homologous regions across (A) *M. tuberculosis* H37Rv and BCG Danish, (B) *M. tuberculosis* H37Rv and BCG Glaxo, (C) *M. tuberculosis* H37Rv and BCG Moreau, (D) *M. tuberculosis* H37Rv and BCG Pasteur, (E) *M. tuberculosis* H37Rv and BCG Russian and (F) *M. tuberculosis* H37Rv and BCG Tokyo.

Differences between *M. tuberculosis* H37Rv and BCG strains, previously established in the literature, were also verified. The absence of the five genes encoding the ESAT-6 secretion system-1 (ESX-1) in all six strains: Rv3874 (*esxB*), Rv3875 (*esxA*), Rv3876 (*espI*), Rv3877 (*eccD1*) and Rv3878 (*espJ*), comprised the "cell wall and cell processes" category (Table S1) (Hsu *et al.,* 2003; Lewis *et al.,* 2003). In addition, the mutation in the Rv2930 (*fadD26*) and Rv2931 (*ppsA*) loci, which impairs the biosynthesis of phthiocerol dimycocerosates (PDIMs) and phenolic glycolipids (PGLs) in BCG Moreau (Chen *et al.,* 2007; Leung *et al.,* 2008), was verified (data not shown).



	Early strains			Late strains		
	BCG Moreau	BCG Russian	BCG Tokyo	BCG Danish	BCG Glaxo	BCG Pasteur
Functional Category	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**
Virulence, detoxification and adaptation	7 (9.33)	8 (10.53)	7 (9.33)	9 (10.59)	9 (10.59)	9 (10.47)
Lipid metabolism	1 (1.33)	1 (1.32)	1 (1.33)	1 (1.18)	1 (1.18)	1 (1.16)
Information pathways	0	0	0	1 (1.18)	1 (1.18)	1 (1.16)
Cell-wall and cell processes	10 (13.33)	10 (13.16)	10 (13.33)	14 (16.47)	14 (16.47)	14 (16.28)
Stable RNAs	0	0	0	0	0	0
Insertion sequences and phages	28 (37.33)	28 (36.84)	28 (37.33)	28 (32.94)	28 (32.94)	28 (32.56)
PE/PPE	6 (8)	6 (7.89)	6 (8)	7 (8.24)	7 (8.24)	7 (8.14)
Intermediary metabolism and respiration	11 (14.67)	11 (14.47)	11 (14.67)	11 (12.94)	11 (12.94)	11 (12.79)
Unknown	4 (5.33)	4 (5.26)	4 (5.33)	4 (4.71)	4 (4.71)	4 (4.65)
Regulatory proteins	0	0	0	1 (1.18)	1 (1.18)	1 (1.16)
Conserved hypotheticals	8 (10.67)	8 (10.53)	8 (10.67)	9 (10.59)	9 (10.59)	10 (11.63)
TOTAL	75 (100)	76 (100)	75 (100)	85 (100)	90 (100)	86 (100)

Figure 2 – Functional categories of non-homologous genes in early and late BCG strains compared to *M. tuberculosis* H37Rv. n (%): number and percentage of non-homologous genes in each functional category relative to the total number of non-homologous genes per genome.

Table 1 – *M. tuberculosis* H37Rv lipid-related genes corresponding to non-homologous regions in all six BCG strains.

Functional category	H37Rv gene locus	Gene product
Cell wall and cell processes	Rv1970	mce3E
	Rv1972-Rv1974	Mce associated membrane proteins
Virulence, detoxification, adaptation	Rv1965-Rv1969	YrbE3B, Mce3A, Mce3B, Mce3C and Mce3D
	Rv1971	Mce3F
Lipid Metabolism	Rv0222	Enoyl-CoA hydratase (EchA1)
Intermediary metabolism and respiration	Rv2349c-Rv2351c	Phospholipases C (PlcC, PlcB and PlcA)

Discussion

The present study identified and categorized a comprehensive list of absent lipid-related genes shared by the most used BGC strains worldwide compared to the *M. tuberculosis* H37Rv genome. The *M. tuberculosis* H37Rv genome, and not *M. bovis*, was used as reference genomic sequence to assure comparison between vaccine strains and the best curated sequence of the causative agent of TB. The cell wall lipid content of mycobacteria plays an important role in the pathogen-host interaction and inflammation (Forrellad *et al.,* 2013; Dulberger *et al.,* 2020; Petrilli *et al.,* 2020). Thus, these genes can be further explored as common targets for virulence and efficacy improvement of the BCG vaccine currently in circulation.

Since the sequencing of the *M. tuberculosis* H37Rv genome in 1998, the *in silico* comparisons of genus, species and isolates have resulted in abundant data of mycobacterial sequences (Cole *et al.,* 1998, 2001; Fraser *et al.,* 2000; Gordon *et al.,* 2001). Furthermore, comparative genomic analyses have supported many molecular based hypotheses regarding the impact of protein virulence factors on the protection induced by BCG strains (Behr *et al.,* 1999; Lewis *et al.,* 2003; Sherman *et al.,* 2004; Zhang *et al.,* 2013). However, the role of mycobacterial cell-wall content and lipid metabolism on virulence has received less attention, with analyses often limited in number of lipid antigens and BCG strains included, as well as comparisons with *M. bovis* sequences (Rhoades *et al.,* 2005; Layre *et al.,* 2014; Abdallah *et al.,* 2015; Tran *et al.,* 2016; Gonzalo-Asensio *et al.,* 2017; Jia *et al.,* 2017; Wright *et al.,* 2017).

Genes at loci Rv1965 to Rv1974, which encode proteins from the *mce3* operon, were found to be absent in all BCG strains, accounting for 10 of the 14 non-homologous genes identified. The *mce3* operon is an important virulence factor, since *M. tuberculosis* strains disrupted on this operon displayed longer survival and lower colony-forming units (CFU) in mice and guinea pig models (Gioffré *et al.,* 2005; Obregón-Henao *et al.,* 2011). Proteins Mce3A (Rv1966), Mce3D (Rv1969) and Mce3E (Rv1970) also induced antibody response serum samples from TB patients (Ahmad *et al.,* 2004). Similar to other *mce* operons, the products of *mce3* has been shown to affect the internalization process of mycobacteria (El-Shazly *et al.,* 2007) and are possibly involved in cholesterol and fatty acids transport across the cell wall (Pandey and Sassetti 2008; Mohn *et al.,* 2008; Perkowski *et al.,* 2016). Interestingly, this intake of fatty acids seems to be greatly reduced in the BCG strains that do not produce PDIM (Nazarova *et al.,* 2017), namely BCG Moreau, Tokyo and Glaxo.

A protein involved in the degradation of fatty acid was also found to be absent in all BCG strains compared to *M. tuberculosis* H37Rv. The gene *echaA1* (Rv0222) encodes an enoyl-CoA hydratase (EchA1) involved in energy production via β-oxidation, essential for mycobacterial survival and adaptation in environments with distinct fatty acids as the only carbon sources (Muñoz-Elías and McKinney 2006; Srivastava *et al.,* 2015). Despite the gene redundancy involved in five pathways of β-oxidation, EchaA1 is secreted to the host cytosol and impairs the production of pro-inflammatory cytokines, by inhibiting TRAF6 (tumor necrosis factor (TNF)-receptor-associated factor 6) activation (Wang *et al.,* 2020).

With regard to synthesis of glycerolipids, phospholipase C (PlcC) (Rv2349c), PlcB (Rv2350c) and PlcA (Rv2351c) were absent in all BCG strains. These enzymes facilitate the hydrolysis of phosphatidylcholine, phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) to produce diacylglycerol (DAG) (Srinivas *et al.,* 2008) and their absence has been associated with reduced CFU in mice (Raynaud *et al.,* 2002). Furthermore, pre-existing DAG is used for production of triacylglycerol (TAG), which is essential for the survival of *M. tuberculosis* in the host (Garton *et al.,* 2008).

Together, these 14 non-homologous genes may signal a lipid-dependent dormant-like state in all six BCG strains. The absence of *mce3* and *echa1* indicates an overall decline of cholesterol and fatty acid intake in BCG, that could result in lower carbon sources for lipid and energy production. In addition, the absence of *plcC*, *plcB* and *plcA* seems to be associated with lower levels of lipids upstream of DAG and higher levels of TAG. This condition has been previously described in BCG Pasteur (Layre *et al.,* 2011, 2014) and related to long-term dormancy in *M. tuberculosis* (Daniel *et al.,* 2004; Galagan *et al.,* 2013). Therefore, while the lipids increased in level in *M. tuberculosis* H37Rv (such as PE, PG and trehalose-containing lipids) induce a more pro-inflammatory immune response, the accumulation of TAG could be favoring a dormant state in BCG strains.

The identification and study of genes related to cell-wall lipid content and lipid metabolism in BCG strains can contribute to elucidating the impact of attenuation on vaccine virulence and protection efficacy. We suggest that the *M. tuberculosis* lipid-related genes and its products that are absent in BCG should be explored as adjuvants alongside new vaccine candidates due to their capacity to enhance immune response.

Acknowledgments

This work was supported by Bahia State Research Support Foundation, Bahia, Brazil (grant numbers BOL 0172/2019 and BOL0264/2018).

Conflict of Interest

The authors declare no conflict of interest that could be perceived as prejudicial to the impartiality of the reported research.

Author Contributions

AS, JB and AQ were involved in the conceptualization and formal analysis of the study. AS was responsible for data curation and writing of the original draft. AQ and SA supervised the findings of the work. All authors were involved in reviewing and editing the final manuscript. All authors read and approved the final version.

References

Abdallah AM, Hill-Cawthorne GA, Otto TD, Coll F, Guerra-Assunção JA, Gao G, Naeem R, Ansari H, Malas TB, Adroub SA *et al.* (2015) Genomic expression catalogue of a global collection of BCG vaccine strains show evidence for highly diverged metabolic and cell-wall adaptations. Sci Rep 5:15443.

Ahmad S, El-Shazly S, Mustafa AS and Al-Attiyah R (2004) Mammalian cell-entry proteins encoded by the mce3 operon of *Mycobacterium tuberculosis* are expressed during natural infection in humans. Scand J Immunol 60:382–391.

Angelidou A, Conti M-G, Diray-Arce J, Benn CS, Shann F, Netea MG, Liu M, Potluri LP, Sanchez-Schmitz G, Husson R *et al.* (2020) Licensed Bacille Calmette-Guérin (BCG) formulations differ markedly in bacterial viability, RNA content and innate immune activation. Vaccine 38:2229-2240.

Behr MA (2002) BCG--different strains, different vaccines? Lancet Infect Dis 2:86–92.

Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, Rane S and Small PM (1999) Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science 284:1520–1523.

Brosch R, Gordon SV, Garnier T, Eiglmeier K, Frigui W, Valenti P, Dos Santos S, Duthoy S, Lacroix C, Garcia-Pelayo C *et al.* (2007) Genome plasticity of BCG and impact on vaccine efficacy. Proc Natl Acad Sci U S A 104:5596–5601.

Cernuschi T, Malvolti S, Nickels E and Friede M (2018) Bacillus Calmette-Guérin (BCG) vaccine: A global assessment of demand and supply balance. Vaccine 36:498–506.

Chen JM, Islam ST, Ren H and Liu J (2007) Differential productions of lipid virulence factors among BCG vaccine strains and implications on BCG safety. Vaccine 25:8114–8122.

Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE *et al.* (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393:537–544.

Cole ST, Eiglmeier K, Parkhill J, James KD, Thomson NR, Wheeler PR, Honoré N, Garnier T, Churcher C, Harris D *et al.* (2001) Massive gene decay in the leprosy bacillus. Nature 409:1007–1011.

Daniel J, Deb C, Dubey VS, Sirakova TD, Abomoelak B, Morbidoni HR and Kolattukudy PE (2004) Induction of a novel class of diacylglycerol acyltransferases and triacylglycerol accumulation in *Mycobacterium tuberculosis* as it goes into a dormancy-like state in culture. J Bacteriol 186:5017–5030.

Darling AE, Mau B and Perna NT (2010) ProgressiveMauve: Multiple genome alignment with gene gain, loss and rearrangement. PLoS One 5:e1114.

Dulberger CL, Rubin EJ and Boutte CC (2020) The mycobacterial cell envelope – a moving target. Nature 18:47–59.

El-Shazly S, Ahmad S, Mustafa AS, Al-Attiyah R and Krajci D (2007) Internalization by HeLa cells of latex beads coated with mammalian cell entry (Mce) proteins encoded by the mce3 operon of *Mycobacterium tuberculosis*. J Med Microbiol 56:1145–1151.

Forrellad MA, Klepp LI, Gioffré A, Sabio y García J, Morbidoni HR, de la Paz Santangelo M, Cataldi AA and Bigi F (2013) Virulence factors of the *Mycobacterium tuberculosis* complex. Virulence 4:3–66.

Fraser CM, Eisen J, Fleischmann RD, Ketchum KA and Peterson S (2000) Comparative genomics and understanding of microbial biology. Emerg Infect Dis 6:505–512.

Galagan JE, Minch K, Peterson M, Lyubetskaya A, Azizi E, Sweet L, Gomes A, Rustad T, Dolganov G, Glotova I *et al.* (2013) The *Mycobacterium tuberculosis* regulatory network and hypoxia. Nature 499:178–183.

Garton NJ, Waddell SJ, Sherratt AL, Lee SM, Smith RJ, Senner C, Hinds J, Rajakumar K, Adegbola RA, Besra GS *et al.* (2008) Cytological and transcript analyses reveal fat and lazy persister-like bacilli in tuberculous sputum. PLoS Med 5:0634–0645.

Gioffré A, Infante E, Aguilar D, De La Paz Santangelo M, Klepp L, Amadio A, Meikle V, Etchechoury I, Romano MI, Cataldi A *et al.* (2005) Mutation in mce operons attenuates *Mycobacterium tuberculosis* virulence. Microbes Infect 7:325–334.

Gonzalo-Asensio J, Marinova D, Martin C and Aguilo N (2017) MTBVAC: Attenuating the human pathogen of tuberculosis (TB) toward a promising vaccine against the TB epidemic. Front Immunol 8:1803.

Gordon SV, Eiglmeier K, Garnier T, Brosch R, Parkhill J, Barrell B, Cole ST and Hewinson RG (2001) Genomics of *Mycobacterium bovis*. Tuberculosis (Edinb) 81:157-163

Guenin-Macé L, Siméone R and Demangel C (2009) Lipids of Pathogenic Mycobacteria: Contributions to virulence and host immune suppression. Transbound Emerg Dis 56:255–268.

Hayashi D, Takii T, Fujiwara N, Fujita Y, Yano I, Yamamoto S, Kondo M, Yasuda E, Inagaki E, Kanai K *et al.* (2009) Comparable studies of immunostimulating activities *in vitro* among *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) substrains. FEMS Immunol Med Microbiol 56:116–128.

Hsu T, Hingley-Wilson SM, Chen B, Chen M, Dai AZ, Morin PM, Marks CB, Padiyar J, Goulding C, Gingery M *et al.* (2003) The primary mechanism of attenuation of bacillus Calmette-Guérin is a loss of secreted lytic function required for invasion of lung interstitial tissue. Proc Natl Acad Sci U S A 100:12420–12425.

Jia X, Yang L, Dong M, Chen S, Lv L, Cao D, Fu J, Yang T, Zhang J, Zhang X *et al.* (2017) The bioinformatics analysis of comparative genomics of *Mycobacterium tuberculosis* Complex (MTBC) provides insight into dissimilarities between intraspecific groups differing in host association, virulence, and epitope diversity. Front Cell Infect Microbiol 7:88.

Kapopoulou A, Lew JM and Cole ST (2011) The MycoBrowser portal: A comprehensive and manually annotated resource for mycobacterial genomes. Tuberculosis 91:8–13.

Layre E, Lee HJ, Young DC, Martinot AJ, Buter J, Minnaard AJ, Annand JW, Fortune SM, Snider BB, Matsunaga I *et al.* (2014) Molecular profiling of *Mycobacterium tuberculosis* identifies tuberculosinyl nucleoside products of the virulence-associated enzyme Rv3378c. Proc Natl Acad Sci U S A 111:2978–2983.

Layre E, Sweet L, Hong S, Madigan CA, Desjardins D, Young DC, Cheng T-YY, Annand JW, Kim K, Shamputa IC *et al.* (2011) A comparative lipidomics platform for *Mycobacterium tuberculosis* provides chemotaxonomic analysis for biomarker discovery. Chem Biol 18:1537–1549.

Leung AS, Tran V, Wu Z, Yu X, Alexander DC, Gao GF, Zhu B and Liu J (2008) Novel genome polymorphisms in BCG vaccine strains and impact on efficacy. BMC Genomics 9:413

Lewis KN, Liao R, Guinn KM, Hickey MJ, Smith S, Behr MA and Sherman DR (2003) Deletion of RD1 from *Mycobacterium tuberculosis* mimics Bacille Calmette-Guérin attenuation. J Infect Dis 187:117–123.

Liu J, Tran V, Leung AS, Alexander DC and Zhu B (2009) BCG vaccines: Their mechanisms of attenuation and impact on safety and protective efficacy. Hum Vaccin 5:70-78.

Mahairas GG, Sabo PJ, Hickey MJ, Singh DC and Stover CK (1996) Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. J Bacteriol 178:1274–1282.

Mangtani P, Abubakar I, Ariti C, Beynon R, Pimpin L, Fine PEM, Rodrigues LC, Smith PG, Lipman M, Whiting PF *et al.* (2014) Protection by BCG Vaccine against tuberculosis: A systematic review of randomized controlled trials. Clin Infect Dis 58:470–480.

Mishra M, Adhyapak P, Dadhich R and Kapoor S (2019) Dynamic remodeling of the host cell membrane by virulent mycobacterial sulfoglycolipid-1. Sci Rep 9:12844.

Mohn WW, Van Der Geize R, Stewart GR, Okamoto S, Liu J, Dijkhuizen L and Eltis LD (2008) The actinobacterial mce4 locus encodes a steroid transporter. J Biol Chem 283:35368–35374.

Morgulis A, Coulouris G, Raytselis Y, Madden TL, Agarwala R and Schäffer AA (2008) Database indexing for production MegaBLAST searches. Bioinformatics 24:1757-1764

Muñoz-Elías EJ and McKinney JD (2006) Carbon metabolism of intracellular bacteria. Cell Microbiol 8:10–22.

Nazarova EV, Montague CR, La T, Wilburn KM, Sukumar N, Lee W, Caldwell S, Russell DG and VanderVen BC (2017) Rv3723/LucA coordinates fatty acid and cholesterol uptake in *Mycobacterium tuberculosis*. eLife 6:e26969

Obregón-Henao A, Shanley C, Bianco MV, Cataldi AA, Basaraba RJ, Orme IM and Bigi F (2011) Vaccination of guinea pigs using mce operon mutants of *Mycobacterium tuberculosis*. Vaccine 29:4302–4307.

Pandey AK and Sassetti CM (2008) Mycobacterial persistence requires the utilization of host cholesterol. Proc Natl Acad Sci U S A 105:4376–4380

Perkowski EF, Miller BK, McCann JR, Sullivan JT, Malik S, Allen IC, Godfrey V, Hayden JD and Braunstein M (2016) An orphaned Mce-associated membrane protein of *Mycobacterium tuberculosis* is a virulence factor that stabilizes Mce transporters. Mol Microbiol 100:90–107

Petrilli JD, Müller I, Araújo LE, Cardoso TM, Carvalho LP, Barros BC, Teixeira M, Arruda S, Riley LW and Queiroz A (2020) Differential host pro-inflammatory response to mycobacterial cell wall lipids regulated by the Mce1 Operon. Front Immunol 11:1848.

Pym AS, Brodin P, Majlessi L, Brosch R, Demangel C, Williams A, Griffiths KE, Marchal G, Leclerc C and Cole ST (2003) Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. Nat Med 9:533–539.

Queiroz A and Riley LW (2017) Bacterial immunostat: *Mycobacterium tuberculosis* lipids and their role in the host immune response. Rev Soc Bras Med Trop 50:9–18.

Raynaud C, Guilhot C, Rauzier J, Bordat Y, Pelicic V, Manganelli R, Smith I, Gicquel B and Jackson M (2002) Phospholipases C are involved in the virulence of *Mycobacterium tuberculosis*. Mol Microbiol 45:203–217.

Rhoades ER, Geisel RE, Butcher BA, McDonough S and Russell DG (2005) Cell wall lipids from *Mycobacterium bovis* BCG are inflammatory when inoculated within a gel matrix: Characterization of a new model of the granulomatous response to mycobacterial components. Tuberculosis 85:159–176.

Roy A, Eisenhut M, Harris RJ, Rodrigues LC, Sridhar S, Habermann S, Snell L, Mangtani P, Adetifa I, Lalvani A *et al.* (2014) Effect of BCG vaccination against *Mycobacterium tuberculosis* infection in children: systematic review and meta-analysis. BMJ 349:g4643

Sherman DR, Guinn KM, Hickey MJ, Mathur SK, Zakel KL and Smith S (2004) *Mycobacterium tuberculosis* H37Rv:ΔRD1 is more virulent than *M. bovis* Bacille Calmette-Guérin in long-term murine infection. J Infect Dis 190:123–126.

Srinivas M, Rajakumari S, Narayana Y, Joshi B, Katoch VM, Rajasekharan R and Balaji KN (2008) Functional characterization of the phospholipase C activity of Rv3487c and its localization on the cell wall of *Mycobacterium tuberculosis*. J Biosci 33:221–230.

Srivastava S, Chaudhary S, Thukral L, Shi C, Gupta RD, Gupta R, Priyadarshan K, Vats A, Haque AS, Sankaranarayanan R *et al.* (2015) Unsaturated lipid assimilation by mycobacteria requires auxiliary cis-trans Enoyl CoA isomerase. Chem Biol 22:1577–1587.

Tran V, Ahn SK, Ng M, Li M and Liu J (2016) Loss of lipid virulence factors reduces the efficiency of the BCG vaccine. Sci Rep 6:29076.

Wang L, Wu J, Li J, Yang H, Tang T, Liang H, Zuo M, Wang J, Liu H, Liu F *et al.* (2020) Host-mediated ubiquitination of a mycobacterial protein suppresses immunity. Nature 577:682–688.

WHO (2012) Information Sheet - observed rate of vaccine reactions Bacille Calmette-Guérin (BCG) Vaccine. World Health Organization, Geneva.

WHO (2017) Report on BCG vaccine use for protection against mycobacterial infections including tuberculosis, leprosy, and other nontuberculous mycobacteria (NTM) infections. World Health Organization, Geneva, 77 p.

WHO (2020) Global Tuberculosis Report. World Health Organization, Geneva, 208 p.

Wright CC, Hsu FF, Arnett E, Dunaj JL, Davidson PM, Pacheco SA, Harriff MJ, Lewinsohn DM, Schlesinger LS and Purdy GE (2017) The *Mycobacterium tuberculosis* MmpL11 cell wall lipid transporter is important for biofilm formation, intracellular growth, and nonreplicating persistence. Infect Immun 85:e00131-17.

Zhang L, Ru H, Chen F, Jin C, Sun R, Fan X, Guo M, Mai J, Xu W, Lin Q *et al.* (2016) Variable virulence and efficiency of BCG vaccine strains in mice and correlation with genome polymoprhism. Mol Ther 24:398–405.

Zhang W, Zhang Y, Zheng H, Pan Y, Liu H, Du P, Wan L, Liu J, Zhu B, Zhao G *et al.* (2013) Genome sequencing and analysis of BCG vaccine strains. PLoS One 8:e71243

Internet Resources

Mauve software, http://darlinglab.org/mauve/mauve.html (acessed 12 August 2020)

Nucleotide Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi (acessed 16 November 2020)

Mycobrowser database, https://mycobrowser.epfl.ch (acessed 16 November 2020)

Supplementary material

The following online material is available for this article:

Table S1 – Complete list of H37Rv lipid-related genes corresponding to non-homologous regions in BCG-Moreau, -Danish, -Glaxo, -Pasteur, -Russian or -Tokyo.

Figure S1 – Homologous and non-homologous regions between *M. tuberculosis* H37Rv, early BCG strains and late BCG strains genome sequences.

Associate Editor: Ana Tereza R. Vasconcelos

Genetics and Molecular Biology
https://doi.org/10.1590/1678-4685-GMB-2021-0024

Suplementary Material to "*In silico* comparisons of lipid-related genes between *Mycobacterium tuberculosis* and BCG vaccine strains"

Table S1 - Complete list of H37Rv lipid-related genes corresponding to non-homologous regions in BCG-Moreau, -Danish, -Glaxo, -Pasteur, -Russian or -Tokyo.

Functional category	H37Rv gene locus	Gene product
Virulence, detoxification and adaptation	Rv1965	Integral membrane protein YrbE3B
	Rv1966	Mce-family protein Mce3A
	Rv1967	Mce-family protein Mce3B
	Rv1968	Mce-family protein Mce3C
	Rv1969	Mce-family protein Mce3D
	Rv1971	Mce-family protein Mce3F
	Rv1982c[a]	Toxin VapC36
	Rv1982A[a]	Antitoxin VapB36
	Rv3617	Epoxide hydrolase EphA
	Rv3697A[b]	Antitoxin VapB48
Lipid metabolism	Rv0222	Enoyl-CoA hydratase (EchA1)
Information pathways	Rv1981c[a]	Ribonucleoside-diphosphate reductase (NrdF1)
Cell-wall and cell processes	Rv1508c	Probable membrane protein
	Rv1970	Mce3E
	Rv1972-Rv1974	Mce associated membrane protein
	Rv1980c[a]	Immunogenic protein Mpt64
	Rv1984c[a]	Cutinase precursor CFP21
	Rv1986[a]	Conserved integral membrane protein

Functional category	H37Rv gene locus	Gene product
	Rv1987[a]	Chitinase
	Rv3874	Antigen EsxB (CFP10)
	Rv3875	Antigen EsxA (ESAT-6)
	Rv3876	ESX-1 secretion-associated protein (EspI)
	Rv3877	ESX conserved component (EccD1)
	Rv3878	ESX-1 secretion-associated (EspJ)
Insertion sequences and phages	Rv1573-Rv1586c	Probable PhiRv1 phage protein
	Rv2646	Integrase
	Rv2647	Hypothetical protein
	Rv2650c-Rv2659c	Possible PhiRv2 prophage protein
	Rv3427c-Rv3428c	Possible transposase
PE/PPE	Rv1983[a]	PE-PGRS family protein (PE_PGRS35)
	Rv2352c	PPE family protein (PPE38)
	Rv3621c	PPE family protein (PPE65)
	Rv3622c	PE family protein (PE32)
	Rv3739c	PPE family protein (PPE67)
	Rv3872	PE family-related protein (PE35)
	Rv3873	PPE family protein (PPE68)
Intermediary metabolism and respiration	Rv1256c	Cytochrome P450 130 (Cyp130)
	Rv1511	GDP-D-mannose dehydratase (GmdA)
	Rv1512	Nucleotide-sugar epimerase (EpiA)
	Rv1516c	Sugar transferase
	Rv2073c	Shortchain dehydrogenase
	Rv2074	Possible pyridoxamine 5'-phosphate oxidase (PNP/PMP oxidase)
	Rv2349c	Probable phospholipase C 3 (PlcC)
	Rv2350c	Membrane-associated phospholipase C 2 (PlcB)
	Rv2351c	Membrane-associated phospholipase C 1 (PlcA)
	Rv3119	Molybdenum cofactor biosynthesis protein E (MoaE1)
	Rv3618	Possible monooxygenase

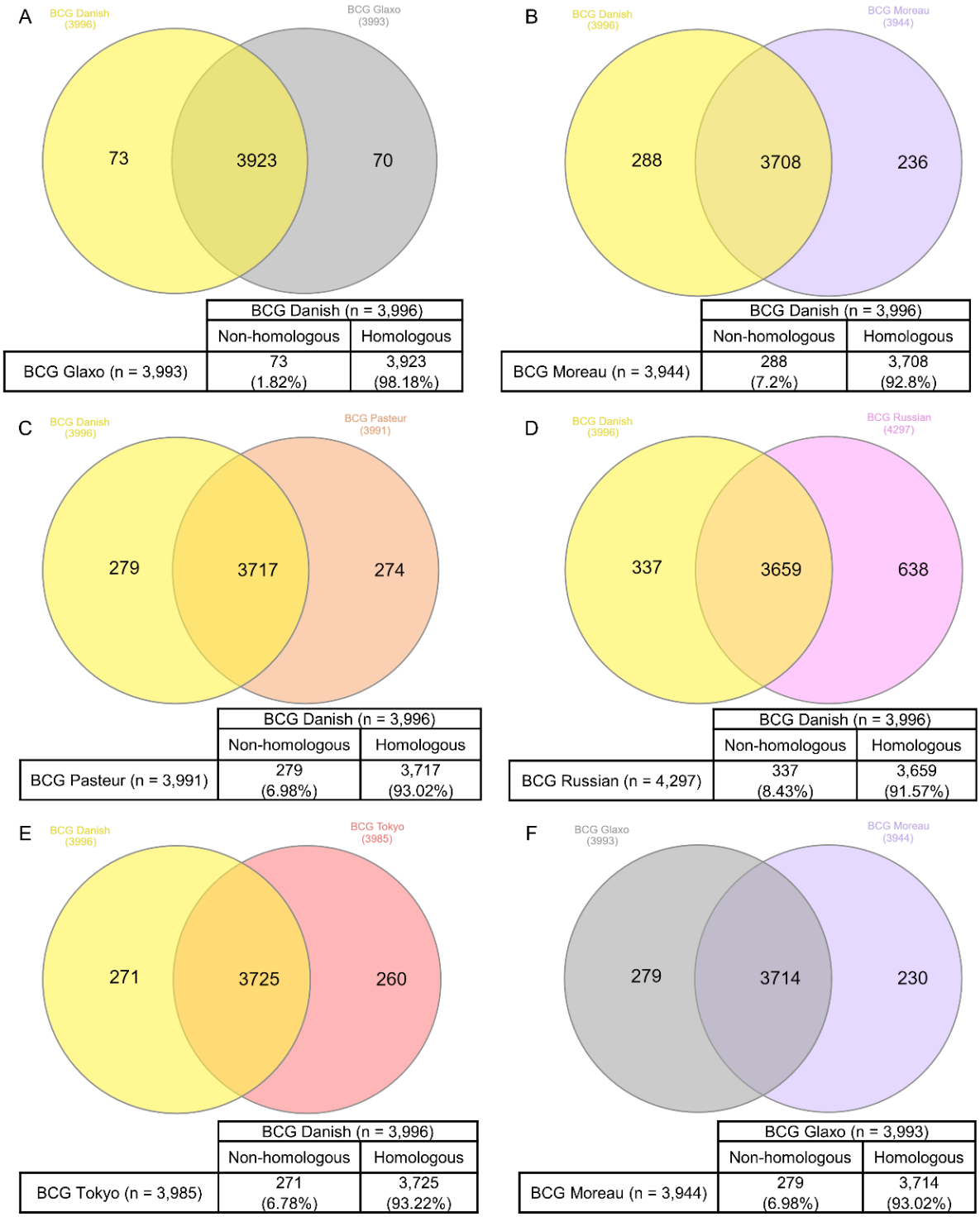
Functional category	H37Rv gene locus	Gene product
Unknown	Rv1507A	Hypothetical protein
	Rv1509	Hypothetical protein
	Rv2348c	Hypothetical protein
	Rv2645	Hypothetical protein
Regulatory proteins	Rv1985c[a]	Probable transcriptional regulatory protein (probably LysR-family)
Conserved hypotheticals	Rv1507c-Rv1508A	Conserved protein
	Rv1513-Rv1515c	Conserved protein
	Rv1769-Rv1770[c]	Conserved protein
	Rv1810[d]	Conserved protein
	Rv1975-Rv1976c	Conserved hypothetical protein
	Rv3120	Conserved hypothetical protein

[a]No BLASTN similarity in late strains (BCG Danish, BCG Glaxo and BCG Pasteur). [b]No BLASTN similarity in BCG Russia. [c]No BLASTN similarity in BCG Pasteur. [d]No BLASTN similarity in BCG Danish and BCG Glaxo.

Genetics and Molecular Biology
https://doi.org/10.1590/1678-4685-GMB-2021-0024

Suplementary Material to "*In silico* comparisons of lipid-related genes between *Mycobacterium tuberculosis* and BCG vaccine strains"

(a)



	BCG Danish (n = 3,996)	
	Non-homologous	Homologous
BCG Glaxo (n = 3,993)	73 (1.82%)	3,923 (98.18%)

	BCG Danish (n = 3,996)	
	Non-homologous	Homologous
BCG Moreau (n = 3,944)	288 (7.2%)	3,708 (92.8%)

	BCG Danish (n = 3,996)	
	Non-homologous	Homologous
BCG Pasteur (n = 3,991)	279 (6.98%)	3,717 (93.02%)

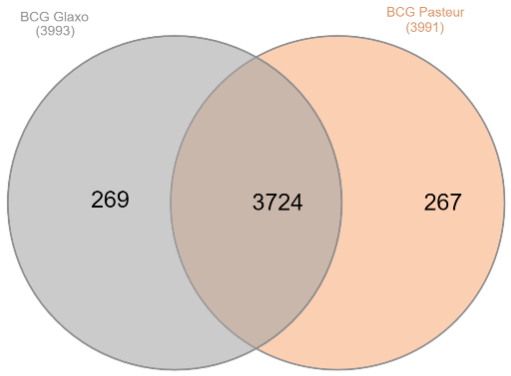
	BCG Danish (n = 3,996)	
	Non-homologous	Homologous
BCG Russian (n = 4,297)	337 (8.43%)	3,659 (91.57%)

	BCG Danish (n = 3,996)	
	Non-homologous	Homologous
BCG Tokyo (n = 3,985)	271 (6.78%)	3,725 (93.22%)

	BCG Glaxo (n = 3,993)	
	Non-homologous	Homologous
BCG Moreau (n = 3,944)	279 (6.98%)	3,714 (93.02%)

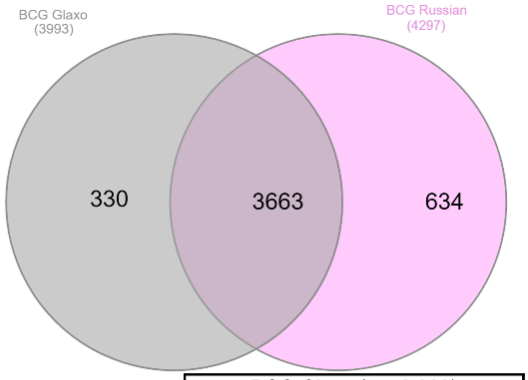
(b)

A



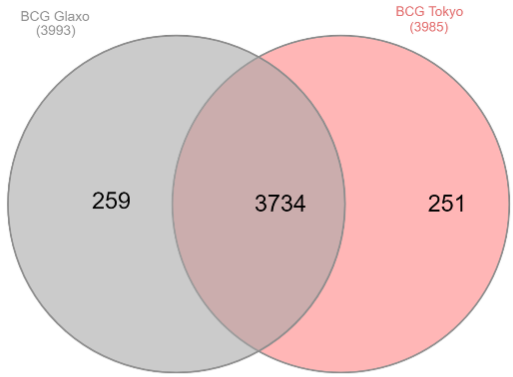
	BCG Glaxo (n = 3,993)	
	Non-homologous	Homologous
BCG Pasteur (n = 3,991)	269 (6.73%)	3,724 (93.27%)

B



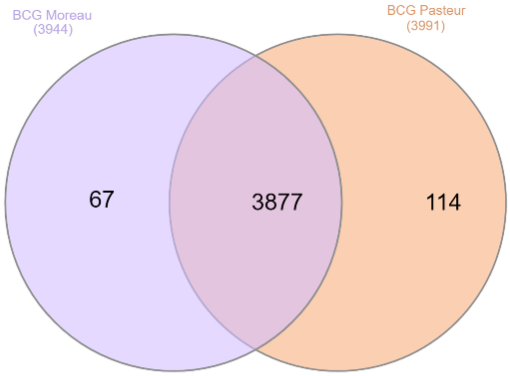
	BCG Glaxo (n = 3,993)	
	Non-homologous	Homologous
BCG Russian (n = 4,297)	330 (8.26%)	3,663 (91.74%)

C



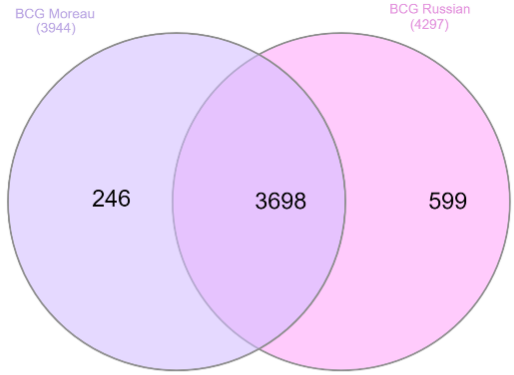
	BCG Glaxo (n = 3,993)	
	Non-homologous	Homologous
BCG Tokyo (n = 3,985)	259 (6.48%)	3,734 (93.52%)

D



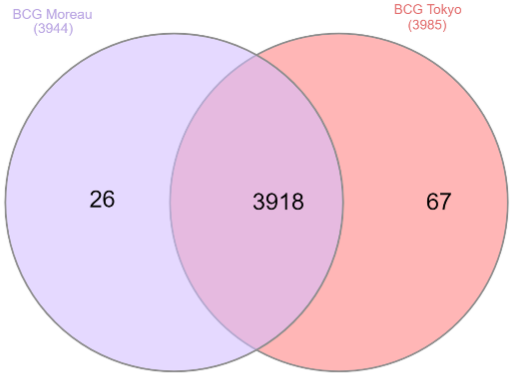
	BCG Moreau (n = 3,944)	
	Non-homologous	Homologous
BCG Pasteur (n = 3,991)	67 (1.69%)	3,877 (98.31%)

E



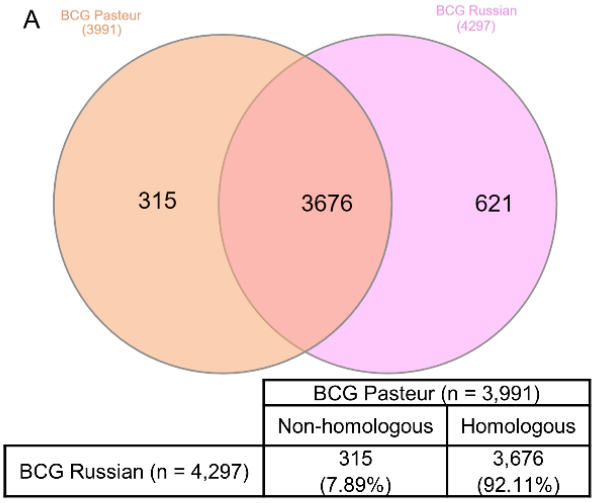
	BCG Moreau (n = 3,944)	
	Non-homologous	Homologous
BCG Russian (n = 4,297)	246 (6.23%)	3,698 (93.77%)

F

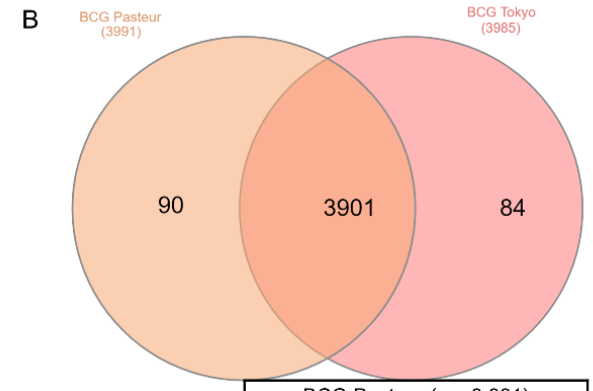


	BCG Moreau (n = 3,944)	
	Non-homologous	Homologous
BCG Tokyo (n = 3,985)	26 (0.65%)	3,918 (99.35%)

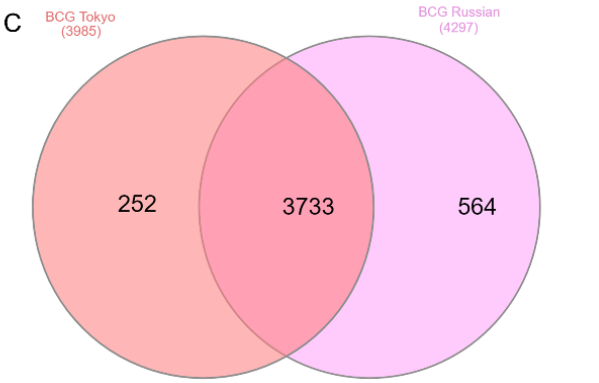
(c)



	BCG Pasteur (n = 3,991)	
	Non-homologous	Homologous
BCG Russian (n = 4,297)	315 (7.89%)	3,676 (92.11%)



	BCG Pasteur (n = 3,991)	
	Non-homologous	Homologous
BCG Tokyo (n = 3,985)	90 (2.25%)	3,901 (97.75%)



	BCG Tokyo (n = 3,985)	
	Non-homologous	Homologous
BCG Russian (n = 4,297)	252 (6.32%)	3,985 (93.68%)

Figure S1 – (a), (b), (c) Homologous and non-homologous regions between M. tuberculosis H37Rv, early BCG strains and late BCG strains genome sequences.

4.2 CAPÍTULO 2 - IMPAIRED CELL IMMUNE RESPONSES TO NONPOLAR LIPID EXTRACTED FROM BACILLUS CALMETTE-GUERIN (BCG)

A atenuação de bacilos *M. bovis* para a produção de BCG resulta não apenas na perda de importantes fatores de virulência proteicos, como também de antígenos lipídios. Estes lipídios são importantes para a indução de respostas imunes relacionadas tanto à persistência, quanto à imunogenicidade, apesar de menos explorados na literatura. Este artigo, que propôs responder os segundo e terceiros objetivos específicos, descreveu a resposta celular induzida pelo extrato de lipídios apolares de BCG, comparando-a com a resposta induzida pelos lipídios apolares deMtb.

O manuscrito está em processo de revisão para submissão. SARNO, Alice; LEITE, Avelina; AUGUSTO, Carlos; MULLER, Igor; ANGELIS, Luana de; MONTENEGRO, Lilian; QUEIROZ, Adriano; ARRUDA, Sérgio. Impaired cell immune responses to nonpolar lipid extracted from bacillus Calmette-Guerin (BCG).

Impaired macrophage and memory T-cell responses to bacillus Calmette-Guerin(BCG) nonpolar lipid extract

Alice Sarno[a,c], Avelina Leite[a], Carlos Augusto[a], Igor Müller[a], Luanna de Ângelis[b],Lilian Montenegro[b], Adriano Queiroz[a,†] and Sergio Arruda[a,d,†]

† These authors contributed equally to this work.

[a]*Advanced Laboratory of Public Health, Gonçalo Moniz Institute (IGM), Fiocruz,Salvador, Bahia 40296 710, Brazil.*
[b]*Laboratory of Immunoepidemiology, Aggeu Magalhães Institute (IAM), Fiocruz,Recife, Pernambuco 50740 465, Brazil.*
[c]*Department of Pathology and Forensic Medicine, Faculty of Medicine, FederalUniversity of Bahia, Bahia 40110 100, Brazil.*
[d]*Department of Life Sciences, State University of Bahia, Bahia 41150 000, Brazil.*

E-mail addresses: sarnoalice@gmail.com (A. Sarno), avelinalarissa218@gmail.com (A. Leite), juniorasoliveira@gmail.com (C. Oliveira), id247@cornell.edu (I. Muller), luannadeangelis@gmail.com (L. Ângelis), lilian@cpqam.fiocruz.br (L. Montenegro), adrianoqs@gmail.com (A. Queiroz), sergio.arruda@fiocruz.br (S. Arruda).

Correspondence should be addresses to Alice Sarno; sarnoalice@gmail.com

Abstract

The attenuation of BCG has led to the loss of not only immunogenic proteins but also lipid antigens. Thus, we compared the macrophage and T-cell responses to nonpolar lipid extracts harvested from BCG and *Mycobacterium tuberculosis* (Mtb) to better understand the role of BCG lipids in the already known diminished responses of the vaccine strain. Relative to Mtb, nonpolar lipid extract from BCG presented a reduced capacity to trigger the expression of the genes encoding TNF, IL-1β, IL-6 and IL-10 in RAW 264.7 macrophages. Immunophenotyping of PBMCs isolated from healthy individuals revealed that lipids from both BCG and Mtb were able to induce an increased frequency of $CD4^+$ and $CD8^+$ T cells, but only the lipid extract from Mtb enhanced the frequency of $CD4^-CD8^-$ double-negative, $\gamma\delta^+$, $CD4^+$HLA-DR^+, and $\gamma\delta^+$HLA-DR^+ T cells relative to the nonstimulated control. Interestingly, only the Mtb lipid extract was able to increase the frequency of $CD4^+$ memory ($CD45RO^+$) T cells, whereas the BCG lipid extract induced a diminished frequency of $CD4^+$ central memory ($CD45RO^+CCR7^-$) T cells after 48 h of culture compared to Mtb. These findings show that the nonpolar lipids of the BCG bacilli presented diminished ability to trigger both proinflammatory and memory responses and suggest a potential use of Mtb lipids as adjuvants to increase the BCG vaccine efficacy.

Keywords: Mycobacteria; apolar lipid extracts; genomic attenuation; gene expression; cellular immune response.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), is one of the leading infectious diseases worldwide, with 10.6 million new cases and 1.6 million deaths in 2021 [1]. BCG (Bacillus Calmette-Guérin) is currently the only licensed vaccine against pulmonary TB, despite its variable efficacy (0-80%) [2–4].

Composed of attenuated *M. bovis* bacilli, BCG has accumulated genomic polymorphisms that

account for the absence of not only protein antigens but also key lipid antigens [5–9]. A lipidomic analysis compared the lipid profiles of Mtb and BCG and revealed more than 1,000 differences between both strains [7]. Recently, we performed an *in-silico* evaluation and found 14 nonhomologous lipid-related genes absent in the six BCG strains most used worldwide relative to Mtb. Those genes were associated with the functional categories "cell wall and cell processes", "virulence, detoxification and adaptation", "lipid metabolism", and "intermediary metabolism and respiration", and together, these gene modifications may favor a dormant-like state of the BCG strains [10].

Mycobacterial lipids play a crucial role in the immunopathogenesis of TB [11]. Petrilli et al. (2020) showed differential macrophage and T-cell responses to lipids extracted from two Mtb strains, an ATP-binding cassette transporter-knockout strain and its parental strain, highlighting the role of these molecules in controlling the inflammatory response [12]. In BCG, the absence of lipid antigens has been associated with important changes in the host's immune response, with consequent decreased control of mycobacterial burden and vaccine protection *in vivo* [13–15].

Protection against TB relies on the induction of a strong cellular immune response, although correlates of protection have not been identified. The results from a phase IIb clinical trial with the candidate MVA85A did not add protection against TB, despite significant induction of T-helper type 1 (Th1) cells [16,17]. Only recent clinical trial results have shown that it is possible to boost the protection already conferred by BCG throughout the revaccination of adolescents [18] and by the immunization of adults with the candidate M72/ASO1E [19]. Other promising candidates have been proposed, including relevant findings from nonhuman primate models, that have been shown to induce not only CD4 and CD8 T cells but also polyfunctional Th17 cells and interleukin-10 production [20,21]. However, these results have not yet reached public health action.

The role of protein antigens has already been comprehensively described, whereas the importance of lipid antigens in the host's immune response has been less explored. Here, we aimed to compare the cellular immune response induced by BCG and Mtb lipid extracts to better understand the influence of lipid losses on strain attenuation. These findings could elucidate the use of this class of antigens in new vaccine candidates to promote a more effective response and protection in combination with proteins.

Materials and Methods

Bacterial Strains, Growth Conditions, and Lipid Extraction

M. bovis BCG Moreau (BCG Moreau RDJ, FAP) and *M. tuberculosis* Erdman strains were used. Both strains were cultured in Middlebrook 7H9 broth (Difco, MD) supplemented with 10% ADC (Beckton-Dickinson, MD) and incubated at 37 °C and 5% CO_2 until stationary phase.

Then, planktonic cultures of BCG Moreau and Mtb were harvested and used for extraction of nonpolar lipids [22,23]. Briefly, 5 mL of methanol with 0.3% NaCl (100:10) and 2.5 mL of petroleum ether were added to 30 mL of cultures and incubated for 30 min at room temperature. The upper petroleum ether layer containing the nonpolar lipids was collected after centrifugation and kept in glass flasks until complete solvent evaporation. Nonpolar lipid extracts of each strain were weighed and resuspended in hexane:isopropanol (1:1) at 0.02 mg/mL. The nonpolar fraction is expected to have phthiocerol dimycocerosates (PDIM), triacylglycerol (TAG), pentacyl trehalose (PAT), trehalose monomycolate (TMM), and dimycolate (TDM, the cord factor), among others [24]. Finally, 24-well tissue culture plates were layered with 0.5 mL of lipid extracts or hexane:isopropanol. Solvent evaporation was allowed, and plates were kept at -20 °C until use.

RAW Macrophage Assay

RAW 264.7 Murine Macrophage Culture

RAW 264.7 cells (ATCC TIB-71) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% FBS at 37 °C and 5% CO_2. After achieving 70% confluency, macrophages were seeded onto lipid-coated 24-well tissue culture plates at 3.7×10^5 cells/well and incubated at 37 °C and 5% CO_2 for 2 h, 12 h, 24 h or 72 h. For the control samples, wells were coated with hexane/isopropanol in the absence of lipid extracts. Staining with trypan blue (Gibco) was used to assess cell number and viability.

RNA Extraction and Purification

Total RNA was extracted from RAW cells using the TRIzol RNA extraction protocol (Invitrogen, Life Technologies) and treated with DNase (Qiagen). DNA-free RNA (500 ng) was mixed with 50 ng of random hexamers and 50 μM oligo (dT) (Invitrogen), and cDNA was synthesized by Superscript III reverse transcriptase (Invitrogen) following the manufacturer's recommendations.

RT–qPCR

The expression of the *TNFα*, *IL-1β*, *IL-6* and *IL-10* genes was measured (Table S1). Primers were designed to produce a 100–195 bp amplicon for each gene. qPCRs were performed using 25 ng of cDNA and Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher) following the manufacturer's recommendations. The expression levels of all target genes were normalized to *β-actin* and *glyceraldehyde 3-phosphate dehydrogenase* (GAPDH), and relative changes between lipid-stimulated and nonstimulated RAW cells were measured by $2^{-\Delta\Delta Ct}$ [25].

Assays with Peripheral Blood Mononuclear CellsStudy Participants

Healthy participants (n = 12) were enrolled in this study and recruited from Gonçalo Moniz Institute (FIOCRUZ). All participants had been vaccinated with BCG during infancy in accordance with national guidelines and tested negative for latent TB infection by interferon-γ release assay (QuantiFERON® TB Gold Plus) upon enrollment. The study was approved by the Research Ethics Committee at Gonçalo Moniz Institute (FIOCRUZ) (protocol number: 57273322.4.0000.0040).

PBMC isolation and culture assays

Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Paque (GE Healthcare) density gradient and cryopreserved in liquid nitrogen with inactive fetal bovine serum (FBS) and 10% DMSO before culture assays. Cryopreserved cells were then thawed, and PBMC concentrations were adjusted to 10^6 cells/mL in 1 mL of RPMI 1640 (with 2 mM L-glutamine and 30 mM HEPES) containing 1% gentamicin and 10% FBS (GIBCO). PBMCs were added to 24-well tissue culture plates previously prepared with nonpolar lipid extracts from BCG and Mtb. Phytohemagglutinin (PHA) (GIBCO) (10 μg/mL) was added as a positive control. Cells were cultured for 24 h, 48 h and 72 h at 37 °C in a 5% CO_2 humidified atmosphere, and the 48-h time-point was chosen for the analyses.

Flow Cytometry and Cytokine Analyses

Cells were first stained with CD3-FITC, CD4-PE, CD8-APC-Cy7, CD45RA PE-Cy7, CD45RO-APC, CCR7-BV510, HLA-DR-BV605, and $TCR_{\gamma\delta}$-BV421 (BD Biosciences).

For intracellular staining with IFN_{γ}-PE-Cy7, TNF-AL700, IL-2-BV421, and IL-17- BV510, cells were fixed and permeabilized using the BD Biosciences Cytofix/Cytoperm Kit. Data were acquired on BD LSRFortessa® (50,000 events), and cell frequencies, as well as median fluorescence intensity (MFI), were measured using FlowJo 10 software (Tree Star Inc.). Supernatants of PBMC cultures were collected and stored at -20 °C for cytokine assays. Concentrations of IFNγ and IL-10 were measured by ELISA (R&D Systems) according to the manufacturer's instructions.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Inc.). Normal distribution was assessed by the Shapiro–Wilk test. Statistical significance was assessed by Student's t test, one-way ANOVA followed by Tukey's posttest, or Kruskal–Wallis followed by Dunn's posttest. The results were considered significant when $p < 0.05$.

Results

Lipid extract from the BCG strain induced lower expression levels of proinflammatory genes relative to Mtb lipids.

The transcriptional expression of genes encoding pro- and anti-inflammatory cytokines was measured in macrophages cultured with nonpolar lipid extracts harvested from both BCG and Mtb strains.

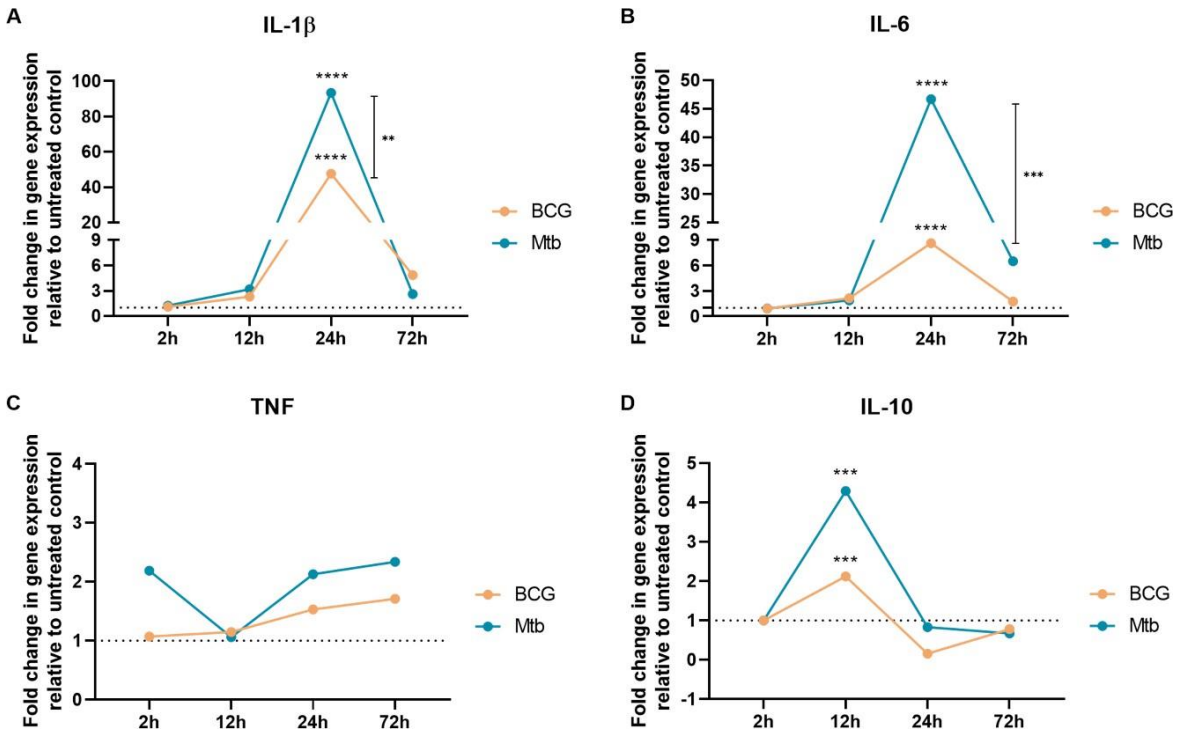


Figure 1. RT–qPCR analyses of **(A)** *IL-1β*, **(B)** *IL-6*, **(C)** *TNF,* and **(D)** *IL-10* after 2 h, 12 h, 24 h, and 72 h of cell exposure to BCG (orange) and Mtb (blue) lipid extracts. Data represent the mean fold-change difference between BCG and Mtb relative to untreated control. Gene expression was normalized to *glyceraldehyde 3-phosphate dehydrogenase* (GAPDH) and *β-actin* genes. *p values* were calculated by t test, with***p <0.001; ****p <0.0001.

Lipids from the BCG strain induced lower transcript production than Mtb for all evaluated genes at most time points (Figure 1). Relative to the nonstimulated control, there was increased expression of *IL-1β* and *IL*-6 at 24 h of culture in macrophages cultured with both BCG and Mtb lipid extracts (p <0.0001) (Figures 1A and 1B). The expression of *IL-1β* and *IL-6* increased by 48- and 9-fold in macrophages stimulated with BCG lipid extract and by 93- and 47-fold in Mtb lipid-induced cells, respectively (Table S2). In addition, BCG lipids induced lower expression of *TNF* across the 2 h, 24 h and 72 h time points when compared with the stimulus triggered by Mtb lipids (Figure 1C). Whereas Mtb lipids sustained a 2-fold upregulation of this gene, the $2^{-\Delta\Delta Ct}$ of

TNF in BCG lipid-stimulated macrophages varied from 1.1 to 1.7 over the 2 h, 24 h and 72 h time points (Table S2). After 12 h of incubation, the expression of *IL-10* was 2- and 4- fold in cultures with lipids extracted from BCG and Mtb, respectively (p <0.001)(Figure 1D).

Both BCG and Mtb nonpolar lipid extracts increased the frequency of $CD4^+$ and $CD8^+$ T cells

To evaluate whether lipids from BCG could also elicit a lymphocyte response, PBMCs obtained from healthy individuals were cultured with lipid extracts and stained for immunophenotyping (Figure S1). Relative to nonstimulated controls, lipids from both strains enhanced the frequencies of $CD4^+$ and $CD8^+$ T cells (p <0.05) (Figures 2A and 2B), whereas only Mtb significantly increased the frequencies of $CD4^-CD8^-$ double- negative (DN) and $\gamma\delta^+$ T cells (p <0.05 and p <0.01, respectively) (Figures 2C and 2D). Similarly, Mtb but not BCG lipids induced the proliferation of HLA-DR-positive $CD4^+$ and $\gamma\delta^+$ T cells (p <0.05) (Figures 2E and 2H).

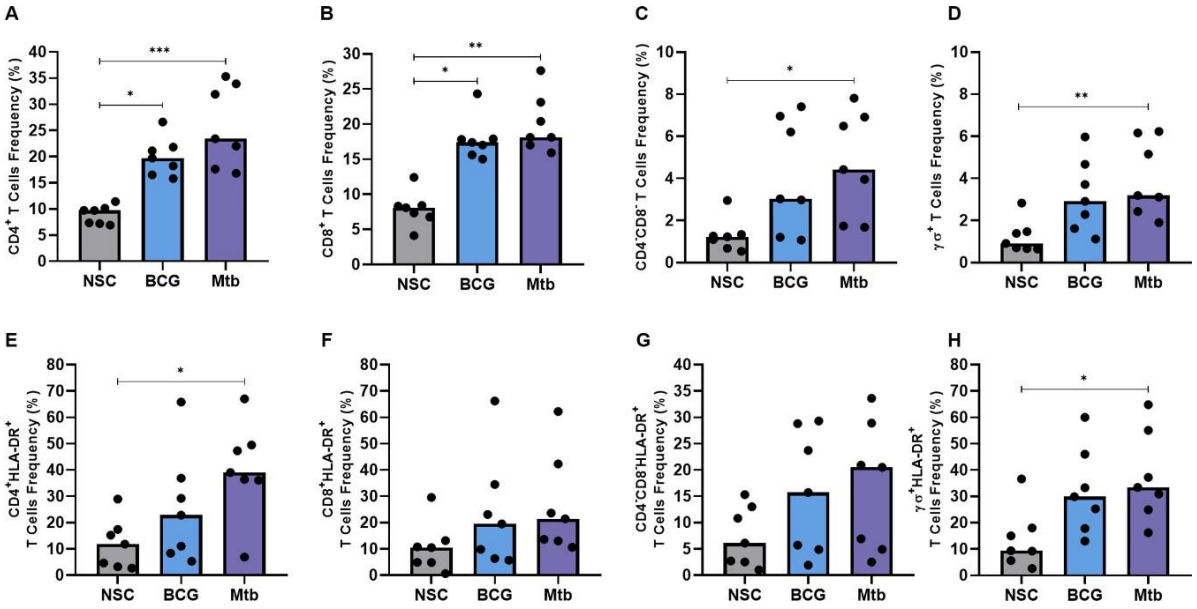


Figure 2. Flow cytometry of conventional and nonconventional T cells after 48 h of *in vitro* culture of PBMCs from healthy individuals with lipid extracts of BCG and Mtb.

(A) and **(E)** Frequencies of $CD4^+$ and $CD4^+$HLA-DR^+ T cells. **(B)** and **(F)** Frequencies of $CD8^+$ and $CD8^+$HLA-DR^+ T cells. **(C)** and **(G)** Frequencies of $CD4^-CD8^-$ DN and $CD4^-CD8^-$ DN HLA-DR^+ T cells. **(D)** and **(H)** Frequencies of $\gamma\delta^+$ and $\gamma\delta^+$HLA-DR^+ T cells. Normal distribution was determined by the Shapiro–Wilk test. *p values* for normal distributions were calculated by one-way ANOVA, and *p values* for nonnormal distributions were calculated by the Kruskal–Wallis test.*p<0.05;**p<0.01;***p<0.001.Nonstimulated control (negative control); Phytohemagglutinin, PHA (positive control).

BCG lipids present a reduced capacity to induce CD4$^+$ memory and central memory T-cell proliferation.

Neither BCG nor Mtb lipid extract stimulation resulted in significant changes in the frequencies of CD4$^+$ and CD8$^+$ naïve T cells (Figure 3A and 3B). Conversely, Mtb lipids induced both CD4$^+$ and CD8$^+$ memory T cells (p <0.0001), whereas lipid extract from BCG only increased the frequencies of the latter (p <0.01) when compared to the nonstimulated controls (Figures 3C and 3D). BCG lipids were not only unable to increase the frequency of CD4$^+$ memory T cells but also induced significantly lower proliferation of this population than Mtb lipids (p <0.001) (Figure 3C).

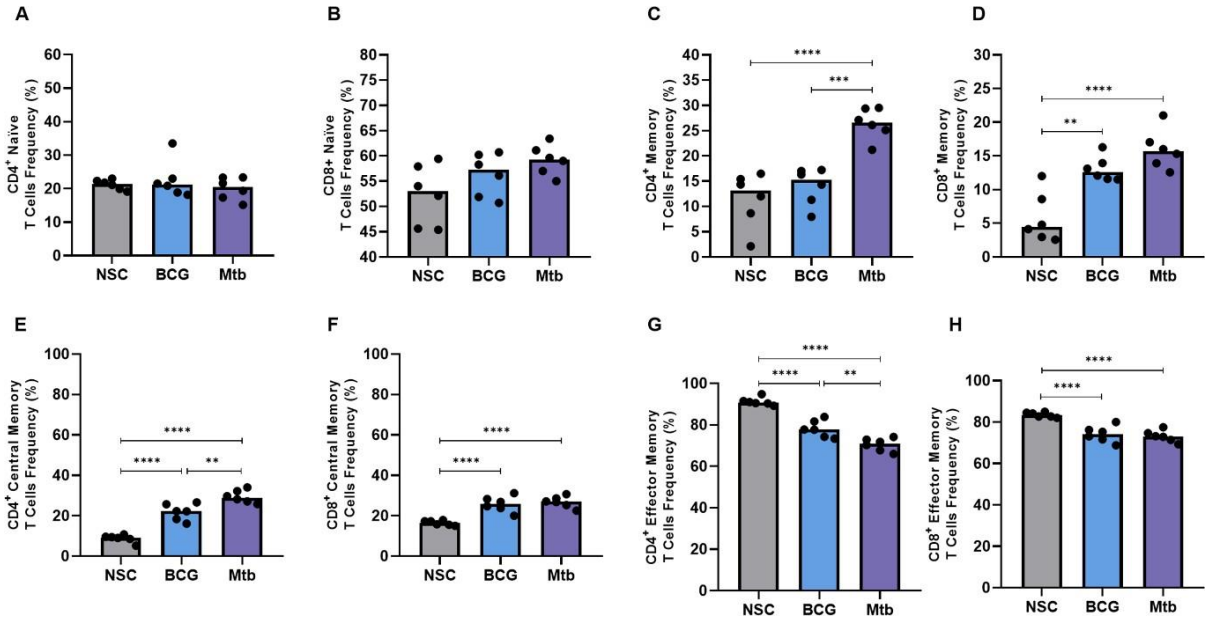


Figure 3. Flow cytometry of memory T cells after 48 h of *in vitro* culture of PBMCs from healthy individuals with lipid extracts of BCG and Mtb. (A) and (B) Frequencies of CD4$^+$ and CD8$^+$ naïve T cells (CD45RA$^+$). (C) and (D) Frequencies of CD4$^+$ and CD8$^+$ memory T cells (CD45RO$^+$). (E) and (F) Frequencies of CD4$^+$ and CD8$^+$ central memory T cells (CD45RO$^+$CCR7$^+$). (G) and (H) Frequencies of CD4$^+$ and CD8$^+$ effector memory T cells (CD45RO$^+$CCR7$^-$). Normal distribution was determined by the Shapiro–Wilk test. *p values* for normal distributions were calculated by one-way ANOVA, and p values for nonnormal distributions were calculated by the Kruskal–Wallis test. **p <0.01; ***p <0.001; ****p <0.0001. Nonstimulated control (negative control); Phytohemagglutinin, PHA (positive control).

Nonpolar lipid extracts from both BCG and Mtb strains enhanced the frequency of CD4$^+$ and CD8$^+$ central memory T cells (p <0.0001) (Figures 3E and 3F) but induced lower proliferation of CD4$^+$ and CD8$^+$ effector memory T cells (p <0.0001) (Figures 3G and 3H) when compared to nonstimulated controls. Furthermore, compared to Mtb, BCG lipid extract induced significantly lower frequencies of central memory in CD4$^+$ T-cell populations (p <0.01) (Figure 3E).

Distinct level of cytokine synthesis between Mtb- and BCG-lipid-induced T cells

Intracellular cytokine levels in $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ DN T cells in lipid-inducedPBMCs from healthy individuals were also assessed (Figure 4 and Table S3). Relativeto nonstimulated cells, Mtb lipid extracts significantly increased the intracellular levelsof TNF and IFNγ in all evaluated cell populations (Figures 4A to 4F). Lipids from Mtbalso triggered the synthesis of IL-2 in $CD4^+$ (Figure 4G) (p <0.05) and IL-17 in $CD4^-CD8^-$ DN T cells (Figure 4L) (p <0.01). BCG lipid extract stimuli were able to onlyenhance the MFI of TNF and $IFN\gamma^+$ in $CD4^-CD8^-$ DN T cells (p <0.0001) (Figures 4Cand 4F) and IL-17 in CD8+ T cells (Figure 4K) (p <0.05). Compared to Mtb, the BCGlipid extract induced significantly lower expression of TNF in $CD4^-CD8^-$ DN cells (p<0.001) (Figure 4F).

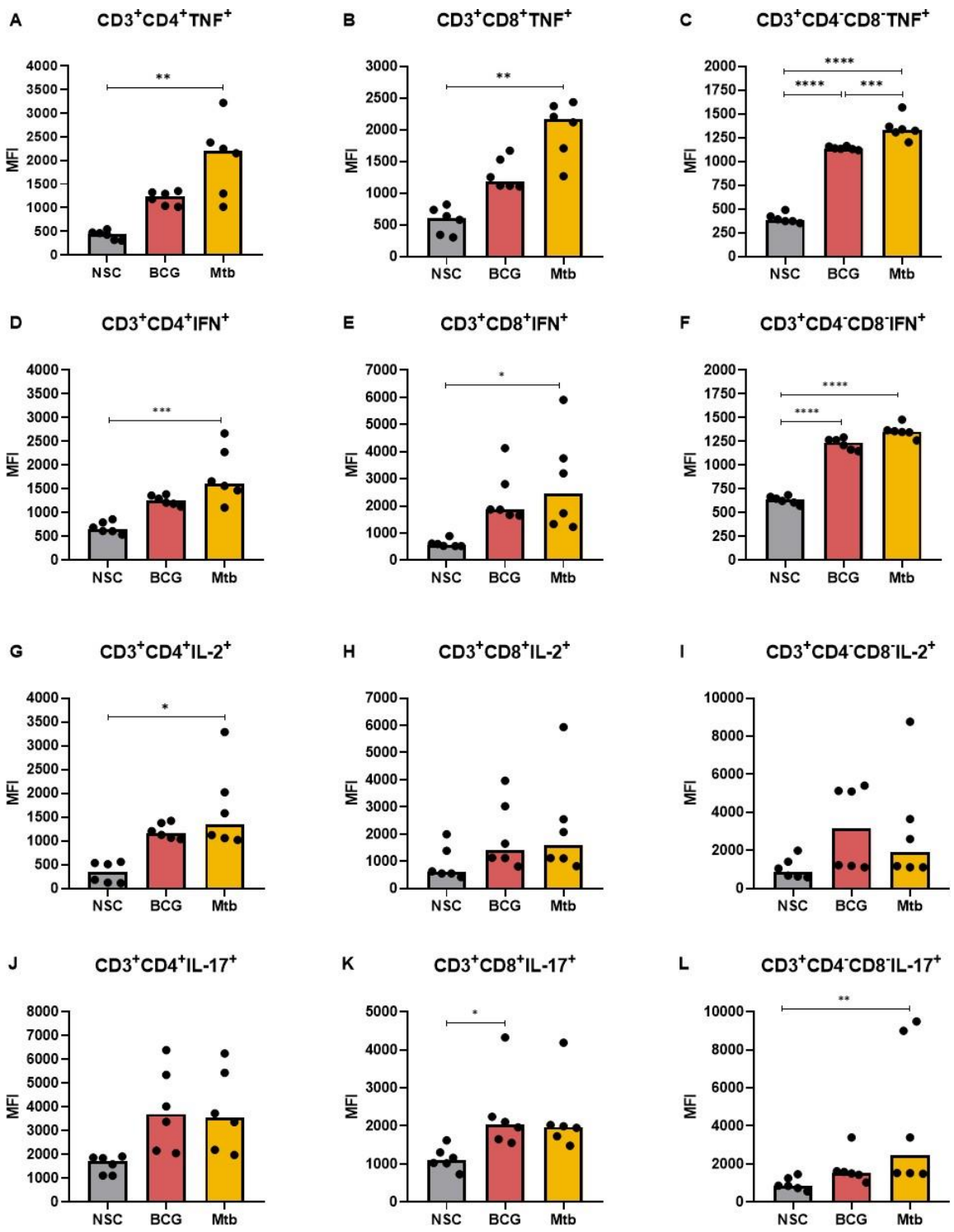


Figure 4. Flow cytometry of $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ DN T cells producing TNF, IFNγ, IL-2, and IL-17 after 48 h of *in vitro* culture of PBMCs from healthy individuals with lipid extracts of BCG and Mtb. **(A)**, **(B),** and **(C)** MFI of $CD4^+$, $CD8^+$ and $CD4^-$ $CD8^-$ DN T cells producing TNF. **(D)**, **(E),** and **(F)** MFI of $CD4^+$, $CD8^+$ and $CD4^-CD8^-$

DN T cells producing IFNγ. **(G)**, **(H),** and **(I)** MFI of $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ DN T cells producing IL-2. (J), (K), and (L) MFI of $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ DN T cells producing IL-17. Normal distribution was determined by the Shapiro–Wilk test. *p values* for normal distributions were calculated by one-way ANOVA, and *p* values for nonnormal distributions were calculated by the Kruskal–Wallis test. **p* <0.05; ***p* <0.01; ****p* <0.001; *****p* <0.0001. MFI: Median fluorescence intensity. Nonstimulated control (negative control); Phytohemagglutinin, PHA (positive control).

The concentrations of IFNγ and IL-10 in culture supernatants were measured at 48 h. Only Mtb lipid extract increased the concentration of both cytokines (*p* <0.01) (Figures 5A and 5B; Table S5).

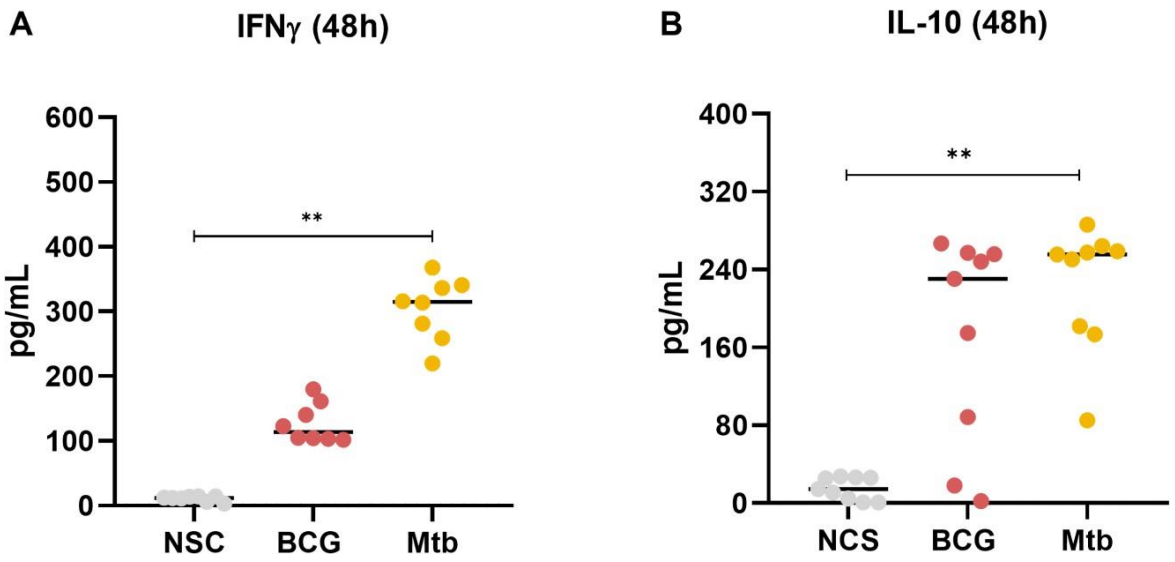


Figure 5. Concentrations of **(A)** IFNγ and **(B)** IL-10 production after 48 h of *in vitro* culture of PBMCs from healthy individuals with lipid extracts of BCG and Mtb. Normal distribution was determined by the Shapiro–Wilk test. *p values* for normal distributions were calculated by one-way ANOVA, and *p* values for nonnormal distributions were calculated by the Kruskal–Wallis test. ***p* <0.01. Nonstimulated control (negative control); Phytohemagglutinin, PHA (positive control).

Discussion

Consecutive *in vitro* passages of *M. bovis* gave rise to BCG, an attenuated strain with depletion of at least nine regions of difference (RD), including RD1, which encodes the proteins from the ESX-1 secretion system ESX-1, such as ESAT-6 and CFP-10 (9). Although these proteins are important virulence factors, their deletion does not completely explain the reduced ability of BCG to induce a protective immune response after vaccination [14,15,26]. The lipid profiles of Mtb

and BCG differ from each other in more than 1,000 species [7], whereas an *in silico* analysis showed that lipid-related gene deletions hinder the production of mce3, enoyl-CoA hydratase, and phospholipase C in BCG. Thus, we reasoned that losses of nonpolar lipid antigens in BCG would contribute to impairing host cell functions and might determine whether the vaccine can be improved by adding Mtb-derived lipid adjuvants.

Compared to Mtb, we observed that BCG lipids predominantly induced diminished macrophage expression of the pro-inflammatory markers *IL-1β*, *IL-6,* and *TNF*. This diminished ability of BCG nonpolar lipids to induce inflammatory responses was also evidenced by the synthesis of TNF and IFNγ in both $CD4^+$ and $CD8^+$ T cells. Conversely, the difference between each stimulus was less evident in TNF- and IFNγ- producing $CD4^-CD8^-$ DN T cells. Considering that the frequencies of these populations are relatively low compared to conventional T cells, we can assume that the BCG strain also has an impaired ability to induce inflammation at the adaptive immune response level.

Lipid extracts from Mtb and BCG strains were able to, directly or indirectly, activate several T-cell subpopulations. Interestingly, there was no difference in the frequencies of $CD4^+$ and $CD8^+$ T cells in cultures with both lipid extracts. In addition, although there was a significant increase in the frequencies of $CD4^-CD8^-$ DN and $\gamma\delta^+$ T cells in cultures with Mtb lipid extract, relative to the untreated control, there were no distinguishable differences in the proportions of these cells between cultures with BCG and Mtb lipid extracts. These data suggest that despite the reduced capacity of BCG nonpolar lipids to induce inflammation, depletions in the genes related to lipid metabolism did not alter the ability of this strain to increase the frequency of some subsets of T cells.

Furthermore, different TB vaccine candidates and inoculation routes have been associated with the activation of distinct memory T-cell subsets, but there is no consensus about which cell subtype is responsible for providing protection. Here, Mtb lipid extracts induced a greater response from memory T cells than BCG, especially $CD4^+$ T cells, which have been associated with vaccine candidates that use lipid formulations [27,28]. In addition, lipid extracts from both BCG and Mtb strains prompted higher and lower frequencies of effector and central memory T cells, respectively. In particular, effector memory cells are induced by BCG vaccination in humans and represent the predominant cell population in the lungs of vaccinated mice, which has been associated with stronger and more efficient protection against infection [29–32]. Conversely, smaller populations of central memory T cells result in poorer memory responses [33–36]. Notably, Mtb lipids were associated with a greater increase of central memory and decrease of effector memory T cells, whereas BCG lipids induced a similar yet diminished dynamic between these populations. This finding

might indicate that Mtb lipids have a better chance to induce a long-lasting T-cell memory response than BCG lipids.

The question that arises from these analyses is whether the nonpolar lipids from Mtb could in fact induce long lasting memory during a BCG vaccination and, if that is the case, which lipid species are responsible for such activation. Although our study could not provide evidence of long-term protection induced by mycobacterial lipids, our results support the possibility of improvement of the BCG vaccine by including Mtb lipid molecules as adjuvants in the vaccination scheme against TB.

Funding

This study received financial support from the Bahia State Research Support Foundation, Bahia, Brazil (grant numbers 5303/2017, BOL0172/2019 and BOL1421/2021).

Conflict of Interest Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] WHO. Global tuberculosis report 2022. 2022.

[2] Abubakar I, Pimpin L, Ariti C, Beynon R, Mangtani P, Sterne J, et al. Systematicreview and meta-analysis of the current evidence on the duration of protection bybacillus Calmette–Guérin vaccination against tuberculosis. vol. 17. 2013. https://doi.org/10.3310/hta17370.

[3] Mangtani P, Abubakar I, Ariti C, Beynon R, Pimpin L, Fine PEM, et al. Protection by BCG Vaccine Against Tuberculosis: A Systematic Review of Randomized Controlled Trials. Clinical Infectious Diseases 2014;58:470–80.https://doi.org/10.1093/cid/cit790.

[4] Roy A, Eisenhut M, Harris RJ, Rodrigues LC, Sridhar S, Habermann S, et al.Effect of BCG vaccination against Mycobacterium tuberculosis infection in children: systematic review and meta-analysis. BMJ 2014;349:1–11. https://doi.org/10.1136/BMJ.G4643.

[5] Hayashi D, Takii T, Fujiwara N, Fujita Y, Yano I, Yamamoto S, et al. Comparable studies of immunostimulating activities in vitro among Mycobacterium bovis bacillus Calmette-Guérin (BCG) substrains. FEMSImmunol Med Microbiol 2009;56:116–28. https://doi.org/10.1111/j.1574-695X.2009.00559.x.

[6] Hayashi D, Takii T, Mukai T, Makino M, Yasuda E, Horita Y, et al. Biochemicalcharacteristics among Mycobacterium bovis BCG substrains. FEMS Microbiol Lett 2010;306:103–9. https://doi.org/10.1111/j.1574-6968.2010.01947.x.

[7] Layre E, Lee HJ, Young DC, Jezek Martinot A, Buter J, Minnaard AJ, et al. Molecular profiling of Mycobacterium tuberculosis identifies tuberculosinyl nucleoside products of the virulence-

associated enzyme Rv3378c. Proceedings ofthe National Academy of Sciences 2014;111:2978–83. https://doi.org/10.1073/pnas.1315883111.

[8] Bottai D, Brosch R. The BCG Strain Pool: Diversity Matters. Mol Ther2016;24:201–3. https://doi.org/10.1038/mt.2016.18.

[9] Brosch R, Gordon S V, Garnier T, Eiglmeier K, Frigui W, Valenti P, et al. Genome plasticity of BCG and impact on vaccine efficacy. Proceedings of theNational Academy of Sciences 2007;104:5596–601. https://doi.org/10.1073/pnas.0700869104.

[10] Sarno A, Bitencourt J, Queiroz A, Arruda S. In silico comparisons of lipid- related genes between Mycobacterium tuberculosis and BCG vaccine strains. Genet Mol Biol 2021;44. https://doi.org/10.1590/1678-4685-GMB-2021-0024.

[11] Queiroz A, Riley LW. Bacterial immunostat: Mycobacterium tuberculosis lipids and their role in the host immune response. Rev Soc Bras Med Trop 2017;50:9– 18. https://doi.org/10.1590/0037-8682-0230-2016.

[12] Petrilli JD, Müller I, Araújo LE, Cardoso TM, Carvalho LP, Barros BC, et al. Differential Host Pro-Inflammatory Response to Mycobacterial Cell Wall LipidsRegulated by the Mce1 Operon. Front Immunol 2020;11:1848. https://doi.org/10.3389/fimmu.2020.01848.

[13] Chen JM, Islam ST, Ren H, Liu J. Differential productions of lipid virulence factors among BCG vaccine strains and implications on BCG safety. Vaccine2007;25:8114–22. https://doi.org/10.1016/j.vaccine.2007.09.041.

[14] Tran V, Ahn SK, Ng M, Li M, Liu J. Loss of Lipid Virulence Factors Reducesthe Efficacy of the BCG Vaccine. Sci Rep 2016;6:29076. https://doi.org/10.1038/srep29076.

[15] Zhang L, Ru H, Chen F, Jin C, Sun R, Fan X, et al. Variable Virulence andEfficacy of BCG Vaccine Strains in Mice and Correlation With Genome Polymorphisms. Molecular Therapy 2016;24:398–405. https://doi.org/10.1038/MT.2015.216.

[16] Tameris MD, Hatherill M, Landry BS, Scriba TJ, Snowden MA, Lockhart S, etal. Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: A randomised, placebo-controlled phase 2b trial. The Lancet 2013;381:1021–8. https://doi.org/10.1016/S0140- 6736(13)60177-4.

[17] Tameris M, McShane H, McClain JB, Landry B, Lockhart S, Luabeya AKK, et al. Lessons learnt from the first efficacy trial of a new infant tuberculosis vaccinesince BCG. Tuberculosis 2013;93:143–9. https://doi.org/10.1016/j.tube.2013.01.003.

[18] Nemes E, Geldenhuys H, Rozot V, Rutkowski KT, Ratangee F, Bilek N, et al. Prevention of Infection with Mycobacterium tuberculosis by H4:IC31® Vaccination or BCG Revaccination in Adolescents. N Engl J Med 2018;379:138.https://doi.org/10.1056/NEJMOA1714021.

[19] Van Der Meeren O, Hatherill M, Nduba V, Wilkinson RJ, Muyoyeta M, Van Brakel E, et al. Phase 2b Controlled Trial of M72/AS01 E Vaccine to Prevent Tuberculosis . New England ournal of Medicine 2018;379:1621–34.

https://doi.org/10.1056/NEJMOA1803484/SUPPL_FILE/NEJMOA1803484_DISCLOSURES.PDF.

[20] Darrah PA, Zeppa JJ, Hackney JA, Wadsworth MHI, Hughes TK, Pokkali S, et al. Prevention of tuberculosis in nonhuman primates following intravenous BCG immunization. Nature in Press 2019;577:95–102. https://doi.org/10.1038/s41586-019-1817-8.

[21] Dijkman K, Sombroek CC, Vervenne RAW, Hofman SO, Boot C, Remarque EJ,et al. Prevention of tuberculosis infection and disease by local BCG in repeatedlyexposed rhesus macaques. Nature Medicine 2019 25:2 2019;25:255–62. https://doi.org/10.1038/s41591-018-0319-9.

[22] Reed MB, Domenech P, Manca C, Su H, Barczak AK, Kreiswirth BN, et al. Aglycolipid of hypervirulent tuberculosis strains that inhibits the innate immuneresponse. Nature 2004 431:7004 2004;431:84–7. https://doi.org/10.1038/nature02837.

[23] Layre E, Sweet L, Hong S, Madigan CA, Desjardins D, Young DC, et al. A comparative lipidomics platform for Mycobacterium tuberculosis provides chemotaxonomic analysis for biomarker discovery. Chem Biol 2011;18:1537–49. https://doi.org/10.1016/j.chembiol.2011.10.013.

[24] Pirson C, Jones GJ, Steinbach S, Besra GS, Vordermeier HM. Differential effectsof Mycobacterium bovis - Derived polar and apolar lipid fractions on bovine innate immune cells. Vet Res 2012;43:54. https://doi.org/10.1186/1297-9716-43-54.

[25] Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods 2001;25:402–8. https://doi.org/10.1006/METH.2001.1262.

[26] Pym AS, Brodin P, Majlessi L, Brosch R, Demangel C, Williams A, et al. Recombinant BCG exporting ESAT-6 confers enhanced protection againsttuberculosis. Nat Med 2003;9:533–9. https://doi.org/10.1038/nm859.

[27] Rai PK, Chodisetti SB, Zeng W, Nadeem S, Maurya SK, Pahari S, et al. A lipidated peptide of Mycobacterium tuberculosis resuscitates the protective efficacy of BCG vaccine by evoking memory T cell immunity. J Transl Med2017;15:201. https://doi.org/10.1186/s12967-017-1301-x.

[28] Ancelet LR, Aldwell FE, Rich FJ, Kirman JR. Oral Vaccination with Lipid- Formulated BCG Induces a Long-lived, Multifunctional CD4+ T Cell MemoryImmune Response. PLoS One 2012;7:e45888. https://doi.org/10.1371/JOURNAL.PONE.0045888.

[29] Warr AJ, Anterasian C, Shah JA, De Rosa SC, Nguyen FK, Maleche-Obimbo E,et al. A CD4+ TNF+ monofunctional memory T-cell response to BCG vaccination is associated with Mycobacterium tuberculosis infection in infants exposed to HIV. EBioMedicine 2022;80. https://doi.org/10.1016/j.ebiom.2022.104023.

[30] Kumar NP, Padmapriyadarsini C, Rajamanickam A, Bhavani PK, Nancy A, Jayadeepa B, et al. BCG vaccination induces enhanced frequencies of memory Tcells and altered plasma levels of common γc cytokines in elderly individuals. PLoS One 2021;16.

https://doi.org/10.1371/JOURNAL.PONE.0258743.

[31] Li L, Qiao D, Zhang X, Liu Z, Wu C. The immune responses of central and effector memory BCG-specific CD4+ T cells in BCG-vaccinated PPD+ donorswere modulated by Treg cells. Immunobiology 2011;216:477–84. https://doi.org/10.1016/J.IMBIO.2010.09.003.

[32] Henao-Tamayo MI, Ordway DJ, Irwin SM, Shang S, Shanley C, Orme IM. Phenotypic Definition of Effector and Memory T-Lymphocyte Subsets in Mice Chronically Infected with Mycobacterium tuberculosis. Clin Vaccine Immunol2010;17:618. https://doi.org/10.1128/CVI.00368-09.

[33] Basile JI, Liu R, Mou W, Gao Y, Carow B, Rottenberg ME. Mycobacteria- Specific T Cells Are Generated in the Lung During Mucosal BCG Immunizationor Infection With Mycobacterium tuberculosis. Front Immunol 2020;11:2551. https://doi.org/10.3389/FIMMU.2020.566319/BIBTEX.

[34] Kaushal D, Foreman TW, Gautam US, Alvarez X, Adekambi T, Rangel-Moreno J, et al. Mucosal vaccination with attenuated Mycobacterium tuberculosis inducesstrong central memory responses and protects against tuberculosis. Nat Commun 2015;6. https://doi.org/10.1038/NCOMMS9533.

[35] Vogelzang A, Perdomo C, Zedler U, Kuhlmann S, Hurwitz R, Gengenbacher M,et al. Central Memory CD4+ T Cells Are Responsible for the Recombinant Bacillus Calmette-Guérin ΔureC::hly Vaccine's Superior Protection Against Tuberculosis. J Infect Dis 2014;210:1928. https://doi.org/10.1093/INFDIS/JIU347.

[36] Nambiar JK, Pinto R, Aguilo JI, Takatsu K, Martin C, Britton WJ, et al. Protective immunity afforded by attenuated, PhoP-deficient Mycobacterium tuberculosis is associated with sustained generation of CD4+ T-cell memory. EurJ Immunol 2012;42:385–92. https://doi.org/10.1002/EJI.201141903.

Supplementary Table 1. List of primers used for RT-qPCR.

Gene	Nucleotide Sequence (5'3')
TNF Forward	CTACCTTGTTGCCTCCTCTTT
TNF Reverse	GAGCAGAGGTTCAGTGATGTAG
IL-1β Forward	TCGTGCTGTCGGACCCATAT
IL-1β Reverse	GTCGTTGCTTGGTTCTCCTTGT
IL-6 Forward	CGAGAGTCCTTCAGAGAGATACA
IL-6 Reverse	CCTTCTGTGACTCCAGCTTATC
IL-10 Forward	CTGTAAAACAAGAGCAAGGC
IL-10 Reverse	GAAGCTTCTGTTGGCTCCC
GAPDH Forward	TCAACGGCACAGTCAAGG
GAPDH Reverse	ACTCCACGACATACTCAGC
β-actina Forward	GAGGTATCCTGACCCTGAAGTA
β-actina Reverse	CACACGCAGCTCATTGTAGA

Supplementary Table 2. Mean, upper limit and lower limit of $2^{-\Delta\Delta Ct}$ values from RT-qPCR analyses.

		2h			12 h			24 h			72 h		
		Mean	**Upper Limit**	**Lower Limit**	**Mean**	**Upper Limit**	**Lower Limit**	**Mean**	**Upper Limit**	**Lower Limit**	**Mean**	**Upper Limit**	**Lower Limit**
BCG	**TNF**	1.07	1.32	0.86	1.15	1.32	1.01	1.53	1.89	1.25	1.71	3.74	0.78
	IL-1	1.10	1.26	0.96	2.31	2.87	1.85	47.67	51.99	43.71	4.86	7.40	3.19
	IL-6	0.91	1.01	0.82	2.14	2.67	1.71	8.64	10.37	7.19	1.74	2.47	1.23
	IL-10	1.00	1.00	1.00	2.12	4.26	1.06	0.16	0.26	0.10	0.78	2.02	0.30
Mtb	**TNF**	2.19	2.69	1.78	1.06	1.20	0.93	2.13	2.74	1.65	2.34	2.39	2.29
	IL-1	1.23	1.53	0.99	3.19	3.93	2.60	93.37	106.88	81.58	2.61	2.65	2.57
	IL-6	0.93	1.12	0.77	1.88	2.68	1.32	46.70	53.87	40.48	6.51	10.04	4.23
	IL-10	1.00	1.00	1.00	4.29	7.96	2.31	0.83	1.26	0.55	0.67	0.86	0.52

Supplementary Table 3. Frequency values from Flow Cytometry analyses.

	T CD4+					T CD8+					T CD4-CD8-					T γδ+			
	CN	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**
A07	9.7	21.8	23.4	x	**A07**	12.4	24.3	27.6	x	**A07**	2.955	6.95	6.91	x	**A07**	2.825	5.97	6.23	x
A01	9.75	19.7	17.6	x	**A01**	8.3	17.8	20.4	x	**A01**	1.34	3.03	3.95	x	**A01**	0.915	2.91	3.11	x
A02	6.90	15.8	31.90	x	**A02**	7.35	15.0	18.10	x	**A02**	0.68	1.1	1.74	x	**A02**	0.66	1.1	1.90	x
A04	7.2	16.5	16.8	x	**A04**	8.1	17.9	18.1	x	**A04**	0.54	1.2	1.68	x	**A04**	0.645	2.29	3.19	x
A06	7.35	18.2	22	61.1	**A06**	6.75	17.4	17	42.7	**A06**	1.10	2.98	4.42	9.7	**A06**	1.47	3.71	5.15	15.6
A08	10.15	26.6	35.3	57.90	**A08**	8.4	17	23.1	47.90	**A08**	1.23	6.2	6.49	13	**A08**	1.385	4.67	6.16	12.7
A09	11.43	21.1	33.9	59.2	**A09**	4.105	15.6	15.9	51.8	**A09**	1.265	7.4	7.81	9.96	**A09**	0.705	1.62	2.43	14.1
Mean	8.93	19.96	25.84	59.40	**Mean**	7.92	17.86	20.03	47.47	**Mean**	1.30	4.12	4.71	10.89	**Mean**	1.23	3.18	4.02	14.13
	T CD4+HLA-DR+					**T CD8+HLA-DR+**					**T CD4-CD8-HLA-DR+**					**T γδ+HLA-DR+**			
	CN	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**
A07	28.95	65.8	67	x	**A07**	29.55	66.2	62.2	x	**A07**	15.3	23.7	20.5	X	**A07**	36.65	60	64,8	x
A01	4.62	8.35	6.93	x	**A01**	13.15	34.5	23.6	x	**A01**	2.51	4.88	4.91	X	**A01**	9.35	17.8	24,9	x
A02	3.22	5.3	49.5	x	**A02**	10.5	19.5	21.4	x	**A02**	10.8	29.3	33.6	X	**A02**	5.65	33.3	37,2	x
A04	17.4	369	47.3	x	**A04**	10.75	23.1	42.3	x	**A04**	13	28.8	28.9	X	**A04**	15	29.9	33,4	x
A06	11.85	22.8	39	56.7	**A06**	4.81	9.8	13.6	41	**A06**	6.1	15.7	20.9	38.8	**A06**	18.05	46	55,1	65
A08	15.2	29.2	36.4	55.9	**A08**	4.88	6.38	10.6	50.3	**A08**	2.725	5.71	6.9	34.4	**A08**	9.2	25.3	30,9	63.7
A09	2.7	11	36.1	58.9	**A09**	0.6	5.71	13	56.3	**A09**	1	1.9	2.52	34.9	**A09**	2.6	13	16.2	68.4
Mean	11.99	25.62	40.32	57.17	**Mean**	10.61	23.60	26.67	49.20	**Mean**	7.35	15.71	16.89	36.03	**Mean**	13.79	32.19	37.5	65.7
	T CD4+CD45RA+					**T CD4+CD45RO+**					**T CD4+CD45RO+CCR7+**					**T CD4+CD45RO+CCR7-**			
	CN	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**
A01	19.95	18.9	17.4	x	**A01**	12	17	29.4	x	**A01**	10.8	18.4	28.2	x	**A01**	89.2	81.6	71.8	X
A02	19.1	18.2	15.2	x	**A02**	8.7	14.3	21.2	x	**A02**	9.5	16.1	27.1	x	**A02**	90.6	83.9	72.9	X
A04	22.3	33.5	19.4	x	**A04**	2.1	17.2	25.1	x	**A04**	9	25.7	32,2	x	**A04**	91	74.3	67.8	X
A06	23	23	23.4	49	**A06**	14.4	11.3	27.1	42.7	**A06**	8.5	22.3	34	41.1	**A06**	91.5	77.7	66	68.9

Supplementary Table 3 (cont.). Frequency values from Flow Cytometry analyses.

	T CD4+CD45RA+					T CD4+CD45RO+					T CD4+CD45RO+CCR7+					T CD4+CD45RO+CCR7-			
	CN	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**
A08	21.1	20.9	21.6	51.3	**A08**	15.4	16.3	26.1	45.8	**A08**	9.6	26.6	25.8	43.3	**A08**	90.4	73.4	74.2	66.7
A09	21.8	21.5	23.3	44.5	**A09**	16.5	8	29.5	45.3	**A09**	5.2	22.2	29.7	48.3	**A09**	94.8	77.8	70.3	61.7
Mean	21.18	22.67	20.05	48.27	**Mean**	11.5	14.02	26.4	44.6	**Mean**	8.76	21.88	29.51	44.23	**Mean**	91.24	78.12	70.5	65.77
	T CD8+CD45RA+					**T CD8+CD45RO+**					**T CD8+CD45RO+CCR7+**					**T CD8+CD45RO+CCR7-**			
	CN	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**
A01	59.4	50.7	59	x	**A01**	8.6	14	12.6	x	**A01**	17.3	28.3	27.1	x	**A01**	82.7	71.7	72.9	x
A02	57.9	58.3	57	x	**A02**	4.1	12.1	15.3	x	**A02**	15.8	31.2	25.3	x	**A02**	84.2	68.8	74.7	x
A04	45.6	60.2	61.1	x	**A04**	4.8	11.5	21	x	**A04**	15	20	22.5	x	**A04**	85	80	77.5	x
A06	54.0	56.1	55	72.3	**A06**	3.0	11.6	16	25.6	**A06**	15.5	24.7	26.8	70.8	**A06**	84	75.3	73.2	29.2
A08	45.4	60.7	59.6	77	**A08**	2.6	13.1	13.9	28.4	**A08**	17.9	23.8	30.7	67.5	**A08**	82.1	76.2	69.3	32.5
A09	52.1	51.9	63.4	74.6	**A09**	12.0	16.3	17	22.3	**A09**	17.3	26.9	28.6	73.3	**A09**	82.7	73.1	71.4	26.7
Mean	52.38	56.32	59.18	74.63	**Mean**	5.85	13.09	15.97	25.43	**Mean**	16.47	25.82	26.84	70.53	**Mean**	83.53	74.18	73.16	29.47

Supplementary Table 4. Median Fluorescence Intensity (MFI) values from Flow Cytometry analyses.

	T CD4+TNF+					T CD4+IFN+					T CD4+IL-2+					T CD4+IL-17+			
	CN	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**
A01	318	1295	2248	x	**A01**	613	1129	1660	x	**A01**	559	1124	1121	x	**A01**	1111	2166	2194	x
A02	429	1181	3218	x	**A02**	688	1388	2275	x	**A02**	507	1034	1017	x	**A02**	1919	2054	1983	x
A04	462	1353	2152	x	**A04**	537	1210	1109	x	**A04**	536	1066	1058	x	**A04**	1589	6387	5438	x
A06	474	1017	1017	13352	**A06**	794	1296	1470	18019	**A06**	111	1375	3289	12715	**A06**	1114	5350	6248	37930
A08	546	1039	1297	13391	**A08**	615	1361	1565	17582	**A08**	120	1204	1580	12040	**A08**	1849	3374	3732	37432
A09	303	1326	2379	13960	**A09**	861	1189	2669	14101	**A09**	181	1420	2020	14401	**A09**	1871	4020	3364	29975
	T CD8+TNF+					**T CD8+IFN+**					**T CD8+IL-2+**					**T CD8+IL-17+**			
	CN	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**
A01	735	1529	2116	x	**A01**	521	1674	1226	x	**A01**	417	809	820	x	**A01**	1019	1964	1993	x
A02	342	1115	2204	x	**A02**	613	4129	5907	x	**A02**	554	1118	1106	x	**A02**	1306	2107	2029	x
A04	579	1104	2370	x	**A04**	524	2802	3756	x	**A04**	549	1130	1118	x	**A04**	1166	4329	4189	x
A06	818	1250	1707	11688	**A06**	887	1867	1333	15986	**A06**	1383	3026	5935	37651	**A06**	1623	2243	1953	17994
A08	636	1119	2434	13393	**A08**	530	1861	1727	14697	**A08**	625	1647	2545	22760	**A08**	732	1558	1732	16520
A09	300	1669	1266	14012	**A09**	596	1647	3198	13653	**A09**	1990	3966	2075	24583	**A09**	1026	1652	1483	15402
	T CD4-CD8-TNF+					**T CD4-CD8-IFN+**					**T CD4-CD8-IL-2+**					**T CD4-CD8-IL-17+**			
	CN	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**		**CN**	**BCG**	**Mtb**	**PHA**
A01	351	1117	1201	x	**A01**	686	1147	1345	x	**A01**	586	1115	1172	x	**A01**	741	1441	1489	x
A02	372	1129	1568	x	**A02**	648	1164	1357	x	**A02**	673	1223	1115	x	**A02**	855	1504	1522	x
A04	372	1138	1337	x	**A04**	624	1266	1349	x	**A04**	632	1189	1109	x	**A04**	863	1585	1529	x
A06	390	1133	1322	12470	**A06**	608	1264	1479	11221	**A06**	1048	5403	8761	26070	**A06**	562	3394	3394	19470
A08	420	1158	1306	11245	**A08**	667	1293	1367	11545	**A08**	1986	5125	3650	25640	**A08**	1461	1623	9494	15446
A09	489	1163	1366	12132	**A09**	573	1210	1261	11571	**A09**	1407	5093	2598	30808	**A09**	1258	1026	9005	19243

Supplementary Table 5. Concentration (pg/mL) levels of IFNγ and IL-10 from ELISA analyses.

IFNγ	24 h (Lipids)	Samples	pg/mL	48 h (Lipids)	Samples	pg/mL	72 h (Lipids)	Samples	pg/mL
		CNA06	19.88		CNA01	5.58		CNA04	105.46
		CNA15	34.01		CNA02	14.31		CNA05	105.06
		CNA17	103.49		CNA03	14.11		CNA06	114.48
		BCGA06	52.85		CNA04	3.15		BCGA04	46.18
		BCGA15	134.11		CNA06	12.08		BCGA05	201.24
		BCGA17	138.43		CNA07	13.50		BCGA06	161.20
		MtbA06	288.77		CNA08	11.88		MtbA04	228.32
		MtbA15	278.57		CNA09	11.07		MtbA05	223.61
		MtbA17	222.04		BCGA01	179.75		MtbA06	222.04
		PHAA06	1,159.83		BCGA02	122.92		PHAA04	713.11
		PHAA15	1,560.62		BCGA03	161.28		PHAA05	623.61
		PHAA17	1,182.20		BCGA04	140.37		PHAA06	642.46
		-	-		BCGA06	103.22		-	-
		-	-		BCGA07	104.85		-	-
		-	-		BCGA08	104.04		-	-
		-	-		BCGA09	101.60		-	-
		-	-		MtbA01	281.25		-	-
		-	-		MtbA02	367.73		-	-
		-	-		MtbA03	219.95		-	-
		-	-		MtbA04	258.72		-	-
		-	-		MtbA06	336.47		-	-
		-	-		MtbA07	340.53		-	-
		-	-		MtbA08	313.73		-	-
		-	-		MtbA09	315.96		-	-

Supplementary Table 5 (cont.). Concentration (pg/mL) levels of IFNγ and IL-10 from ELISA analyses.

IFNγ	24h (Lipids)	Samples	pg/mL	48h (Lipids)	Samples	pg/mL	72h (Lipids)	Samples	pg/mL
		-	-		PHAA06	529.31		-	-
		-	-		PHAA08	544.53		-	-
		-	-		PHAA09	531.75		-	-
IL-10	**24h (Lipids)**	**Samples**	**pg/mL**	**48h (Lipids)**	**Samples**	**pg/mL**	**72h (Lipids)**	**Samples**	**pg/mL**
		CNA06	38.37		CNA01	25.41		CNA04	2.86
		CNA15	70.93		CNA02	26.46		CNA05	12.99
		CNA17	25.76		CNA03	14.56		CNA06	13.41
		BCGA06	56.92		CNA04	4.76		BCGA04	136.69
		BCGA15	199.42		CNA05	27.51		BCGA05	108.83
		BCGA17	243.88		CNA06	26.11		BCGA06	113.68
		MtbA06	412.99		CNA07	10.71		MTBA04	169.41
		MtbA15	589.09		CNA08	0.91		MTBA05	127.62
		MtbA17	339.46		CNA09	0.91		MTBA06	147.25
		PHAA06	1,215.0		BCGA01	257.19		PHAA04	206.99
		PHAA15	1,378.9		BCGA02	255.79		PHAA05	202.35
		PHAA17	1,042.8		BCGA03	174.91		PHAA06	205.3
					BCGA04	230.58			
					BCGA05	266.99			
					BCGA06	248.08			
					BCGA07	88.43			
					BCGA08	18.06			
					BCGA09	2.31			

Supplementary Table 5 (cont.). Concentration (pg/mL) levels of IFNγ and IL-10 from ELISA analyses.

IL-10	24h (Lipids)	Samples	pg/mL	48h (Lipids)	Samples	pg/mL	72h (Lipids)	Samples	pg/mL
		-	-		MtbA01	264.19		-	-
		-	-		MtbA02	255.44		-	-
		-	-		MtbA03	181.91		-	-
		-	-		MtbA04	257.54		-	-
		-	-		MtbA05	258.94		-	-
		-	-		MtbA06	250.54		-	-
		-	-		MtbA07	85.28		-	-
		-	-		MtbA08	286.25		-	-
		-	-		MtbA09	173.51		-	-
		-	-		PHAA06	341.56		-	-
		-	-		PHAA08	358.37		-	-
		-	-		PHAA09	365.72		-	-

5 **DISCUSSÃO**

A deleção de genes codificadores de antígenos proteicos, apesar de extensamente estudada, não elucida por completo a redução de eficácia da vacina BCG (PYM et al., 2003; TRAN et al., 2016; ZHANG et al., 2016). O conteúdo lipídico da parede celular micobacteriana cumpre papel fundamental na interação patógeno-hospedeiro, culminando em persistência do bacilo ou indução de resposta imune (DULBERGER; RUBIN; BOUTTE, 2020; FORRELLAD et al., 2013; PETRILLI et al., 2020; QUEIROZ; RILEY, 2017). Entretanto, o estudo de antígenos lipídicos é frequentemente limitado à resposta de lipídios isolados de Mtb, com poucas avaliações dedicadas à influência destes antígenos no metabolismo e virulência de BCG (ABDALLAH et al., 2015; GONZALO-ASENSIO et al., 2017; JIA et al., 2017; LAYRE et al., 2014; RHOADES et al., 2005; TRAN et al., 2016; WRIGHT et al., 2017).

O presente estudo comparou sequências de genoma completo de cepas de BCG e Mtb, identificando genes não-homólogos relacionados à lipídios, que pudessem esclarecer o impacto da atenuação das cepas vacinais na síntese de lipídios e estado metabólico de BCG. Frente às diferenças genômicas observadas, extratos de lipídios apolares de BCG Moreau e Mtb foram comparados, para avaliação da resposta imune de macrófagos murinos e PBMCs de indivíduos sadios induzida *in vitro*.

A deleção de genes codificadores do *operon mce3* (*loci* Rv1965 a Rv1974) e das enzimas echaA1 (*locus* Rv0222) e fosfolipases C (*loci* Rv2349c a Rv2351c) apontam para um estado de dormência, dependente de lipídios, em todas as seis cepas de BCG estudadas. Estes 14 genes não-homólogos estão relacionados à sobrevivência do bacilo no hospedeiro, e suas deleções acarretam redução do número de unidades formadoras de colônia (GARTON et al., 2008; GIOFFRÉ et al., 2005; MUÑOZ-ELÍAS; MCKINNEY, 2006; OBREGÓN-HENAO et al., 2011; RAYNAUD et al., 2002; SRIVASTAVA et al., 2015). Em específico, o *operon mce3* é um importante fator de virulência associado à entrada do bacilo na célula hospedeira; transporte de colesterol e ácidos graxos; e produção de anticorpos, em pacientes com TB ativa (AHMAD et al., 2004; EL-SHAZLY et al., 2007; MOHN et al., 2008; PANDEY; SASSETTI, 2008; PERKOWSKI et al., 2016). As enzimas EchA1 e fosfolipases C, por sua vez, cumprem importante papel metabólico durante a β-oxidação e produção de DAG, respectivamente (MUÑOZ-ELÍAS; MCKINNEY, 2006; SRINIVAS et al., 2008; SRIVASTAVA et al., 2015).

Em conjunto, a ausência do *operon mce3* e de *echa1* indicam menor internalização de colesterol e ácidos graxos, em BCG, e consequente redução de fontes de carbono disponíveis para produção de energia. Além disso, a ausência de *plcC*, *plcB* e *plcA* parece estar associada com níveis menores de lipídios envolvidos na síntese de DAG, o que resulta consequentemente

no acúmulo dos níveis de TAG. Esta condição já foi descrita anteriormente *in vitro*, para BCG Pasteur, e está relacionada à dormência prolongada de Mtb (DANIEL et al., 2004; GALAGAN et al., 2013; LAYRE et al., 2011, 2014). Assim, as alterações genômicas observadas, nas cepas de BCG, culminam no acúmulo de lipídios associados à persistência do bacilo (TAG) e redução de lipídios capazes de induzir a resposta pró-inflamatória do hospedeiro (fosfatidiletanolamina, fosfatidilglicerol e lipídios compostos por trealose).

A resposta imune celular induzida pelo extrato de lipídios apolares de BCG *in vitro* também se mostrou atenuada, quando comparada ao extrato lipídico de Mtb. Em cultivo de macrófagos RAW 264.7 com lipídios apolares de BCG, foram observadas expressões de *IL-1β*, *IL-6* até cinco vezes menores às expressões induzidas pelos lipídios apolares de Mtb. As maiores diferenças, entre as expressões dos genes de citocinas, foram observadas 12 horas e 24 horas após o cultivo, tempo que parece importante para a cinética de infecção por Mtb, quando são observados *up*-regulação de genes envolvidos no transporte de lipídios, produção de citocinas e regulação da resposta imune do hospedeiro (CHACÓN-SALINAS et al., 2005; ROY et al., 2018).

Macrófagos de linhagem foram utilizados, nesses experimentos, por esta se tratar da primeira tentativa de avaliação da capacidade do extrato de lipídios apolares de BCG de induzirem resposta imune *in vitro*. Além disso, comparações realizadas entre células de linhagem e primárias, após infecção com cepas de BCG, Mtb e *M. bovis*, apontam diferenças restritas ao perfil cinético das respostas (ANDREU et al., 2017; GUO et al., 2015; JORDAO et al., 2008). Portanto, células de linhagem apresentam respostas mais lentas *in vitro*, mas com perfis de expressão gênica e capacidade de controle da proliferação micobacteriana comparáveis às células primárias, cerca de 24 horas após a infecção (ANDREU et al., 2017; BLISCHAK et al., 2015; KOO; SUBBIAN; KAPLAN, 2012; NALPAS et al., 2015; TAILLEUX et al., 2008).

A indução – direta ou indireta – de frequências menores de linfócitos não-convencionais ($CD4^-CD8^-$ e $\gamma\delta^+$) e convencionais ($CD4^+$ e $CD8^+$) bem como da produção de citocinas intracelulares e no sobrenadante das culturas, também evidenciou a atenuação de BCG observada anteriormente. Após cultivo com PBMCs de indivíduos sadios, os lipídios apolares de BCG induziram maiores intensidades de fluorescência de linfócitos T $CD4^-CD8^-$ produtores de IFNγ e TNF, o que não se repetiu com os linfócitos T $CD4^+$ e $CD8^+$. Considerando a importância tanto das células duplo-negativas no reconhecimento de antígenos lipídicos, quanto da resposta de perfil Th1 durante a infecção e resposta vacinal, a atenuação da parede celular

de BCG parece promover uma resposta imune celular limitada, no hospedeiro (ABDALLAH et al., 2015; SARNO et al., 2021; TRAN et al., 2016; ZHANG et al., 2016).

Apesar de não existir consenso quanto aos *subsets* de células T de memória importantes para proteção vacinal, a presença de lipídios apolares de BCG foi associada à redução de células de memória central ($CD45RO^{+}CCR7^{+}$) e aumento de células de memória efetora ($CD45RO^{+}CCR7$), quando o oposto aconteceu após cultivo com lipídios de Mtb. A indução de células de memória já foi relacionada a candidatos vacinais de composição lipídica (KUMAR et al., 2021; WARR et al., 2022), enquanto as células de memória efetora, em específico, são induzidas pela vacina BCG, em humano, e representam a população de células predominante no pulmão de camundongos (BASILE et al., 2020; HENAO-TAMAYO et al., 2010; KAUSHAL et al., 2015; LI et al., 2011). Entretanto, a menor população de células de memória central, induzida por lipídios de BCG, parece indicar uma resposta de memória prolongada inferior àquela induzida por Mtb (ANDREU et al., 2017; GUO et al., 2015; NAMBIAR et al., 2012; VOGELZANG et al., 2014).

A identificação *in silico* de genes relacionados ao conteúdo da parede celular e do metabolismo de lipídios auxiliou no esclarecimento da potencial influência de antígenos lipídicos na redução da virulência e proteção de BCG. Ainda, foi observado *in vitro* a indução de resposta imune celular atenuada, de menor intensidade e abrangência, pelos lipídios apolares de BCG, quando comparados a Mtb. Apesar destes resultados não confirmarem a promoção de resposta imune protetora prolongada, há indícios favoráveis à utilização de lipídios de Mtb, na indução de resposta celular de memória mais efetiva pela BCG. Estes lipídios apolares – em extrato ou isolados – podem ser, então, utilizados como adjuvantes em novos candidatos ou esquemas vacinais que incluam a vacina BCG.

6 **CONSIDERAÇÕES FINAIS**

- As principais cepas de BCG possuem deleções comuns de genes envolvidos no metabolismo lipídico e composição da parede celular, e associados à manutenção de características associadas à dormência micobacteriana.
- Lipídios apolares de BCG induzem predominantemente expressão de genes de citocinas de menor intensidade que Mtb, em macrófagos de linhagem.
- Lipídios apolares de BCG apresentam menor capacidade de induzir linfócitos convencionais e não-convencionais, ao mesmo tempo que queapresentam maiores porcentuais de células T de memória efetora e menores porcentagens de células T de memória central, que PBMCs cultivadas com lipídios apolares de Mtb.
- Lipídios apolares de BCG estão associados a menores concentrações de citocinas pró e anti-inflamatórias, quando comparados aos lipídios apolares de Mtb.

REFERÊNCIAS

ABDALLAH, A. M. et al. Genomic expression catalogue of a global collection of BCG vaccine strains show evidence for highly diverged metabolic and cell-wall adaptations. **Scientific Reports**, v. 5, n. 1, p. 15443, 2015.

ABUBAKAR, I. et al. **Systematic review and meta-analysis of the current evidence on the duration of protection by bacillus Calmette–Guérin vaccination against tuberculosis**. [s.l: s.n.]. v. 17

AHMAD, S. et al. Mammalian Cell-Entry Proteins Encoded by the mce3 Operon of Mycobacterium tuberculosis are Expressed During Natural Infection in Humans. **Scandinavian Journal of Immunology**, v. 60, n. 4, p. 382–391, 1 out. 2004.

AHN, S. K. et al. Recombinant BCG Overexpressing phoP-phoR Confers Enhanced Protection against Tuberculosis. **Molecular Therapy**, v. 26, n. 12, p. 2863–2874, 5 dez. 2018.

ANDERSEN, P.; SCRIBA, T. J. Moving tuberculosis vaccines from theory to practice. **Nature Reviews Immunology**, p. 1, 21 maio 2019.

ANDERSON, R. J. The Chemistry of the Lipids of the Tubercle Bacillus. **The Yale Journal of Biology and Medicine**, v. 15, n. 3, p. 311, jan. 1943.

ANDREU, N. et al. Primary macrophages and J774 cells respond differently to infection with Mycobacterium tuberculosis. **Scientific Reports 2017 7:1**, v. 7, n. 1, p. 1–12, 8 fev. 2017.

ARBUES et al. Construction, characterization and preclinical evaluation of MTBVAC, the first live-attenuated M. tuberculosis-based vaccine to enter clinical trials. **Vaccine**, v. 31, n. 42, p. 4867–4873, 1 out. 2013.

ARRUDA, S. et al. Cloning of an M. tuberculosis DNA fragment associated with entry and survival inside cells. **Science**, v. 261, n. 5127, p. 1454–1457, set. 1993.

ASENSIO, J. G. et al. The virulence-associated two-component PhoP-PhoR system controls the biosynthesis of polyketide-derived lipids in Mycobacterium tuberculosis. **Journal of Biological Chemistry**, v. 281, n. 3, p. 1313–1316, 2006.

ASTARIE-DEQUEKER, C. et al. Phthiocerol dimycocerosates of M. tuberculosis participate in macrophage invasion by inducing changes in the organization of plasma membrane lipids. **PLoS pathogens**, v. 5, n. 2, fev. 2009.

AUGENSTREICH, J. et al. Phthiocerol Dimycocerosates from Mycobacterium tuberculosis Increase the Membrane Activity of Bacterial Effectors and Host Receptors. **Frontiers in Cellular and Infection Microbiology**, v. 0, p. 420, 14 ago. 2020.

BARBOSA, T. et al. BCG (Bacille of Calmette–Guérin) revaccination leads to improved in vitro IFN-γ response to mycobacterial antigen independent of tuberculin sensitization in Brazilian school-age children. **Vaccine**, v. 21, n. 17–18, p. 2152–2160, 16 maio 2003.

BARRETO, M. L. et al. Evidence of an effect of BCG revaccination on incidence of tuberculosis in school-aged children in Brazil: Second report of the BCG-REVAC cluster-randomised trial. **Vaccine**, v. 29, n. 31, p. 4875–4877, 12 jul. 2011.

BARRETO, M. L. et al. Causes of variation in BCG vaccine efficacy: Examining evidence from the BCG REVAC cluster randomized trial to explore the masking and the blocking hypotheses. **Vaccine**, v. 32, n. 30, p. 3759–3764, 24 jun. 2014.

BARRY, C. E. et al. **The spectrum of latent tuberculosis: Rethinking the biology and intervention strategies**. **Nature Reviews Microbiology**Nature Publishing Group, , 26 out. 2009.

BASILE, J. I. et al. Mycobacteria-Specific T Cells Are Generated in the Lung During Mucosal BCG Immunization or Infection with Mycobacterium tuberculosis. **Frontiers in Immunology**, v. 11, p. 2551, 22 out. 2020.

BEHR, M. A. BCG--different strains, different vaccines? **The Lancet infectious diseases**, v. 2, n. 2, p. 86–92, 2002.

BITENCOURT, J. et al. Comparing cytokine production and clinical response following vaccination with BCG Moreau and BCG Russia strains in a Brazilian infant population. **Vaccine**, 5 maio 2021.

BLISCHAK, J. et al. Mycobacterial infection induces a specific human innate immune response. **Scientific reports**, v. 5, 20 nov. 2015.

BROSCH, R. et al. Genome plasticity of BCG and impact on vaccine efficacy. **Proceedings of the National Academy of Sciences**, v. 104, n. 13, p. 5596–5601, 2007.

BROSET, E.; MARTÍN, C.; GONZALO-ASENSIO, J. Evolutionary landscape of the mycobacterium tuberculosis complex from the viewpoint of phoPR: Implications for virulence regulation and application to vaccine development. **mBio**, v. 6, n. 5, 2015.

CAMACHO, L. et al. Analysis of the phthiocerol dimycocerosate locus of Mycobacterium tuberculosis. Evidence that this lipid is involved in the cell wall permeability barrier. **The Journal of biological chemistry**, v. 276, n. 23, p. 19845–19854, 8 jun. 2001.

CHACÓN-SALINAS, R. et al. Differential pattern of cytokine expression by macrophages infected in vitro with different Mycobacterium tuberculosis genotypes. **Clinical and experimental immunology**, v. 140, n. 3, p. 443–449, jun. 2005.

CHEN, J. M. et al. Differential productions of lipid virulence factors among BCG vaccine strains and implications on BCG safety. **Vaccine**, v. 25, n. 48, p. 8114–8122, 2007.

COLE, S. T.; BARRELL, B. G. Analysis of the genome of Mycobacterium tuberculosis H37Rv. **Novartis Found Symp**, v. 217, p. 160–167, 1998.

COX, J. et al. Complex lipid determines tissue-specific replication of Mycobacterium tuberculosis in mice. **Nature**, v. 402, n. 6757, p. 79–83, 4 nov. 1999.

DANIEL, J. et al. Induction of a novel class of diacylglycerol acyltransferases and triacylglycerol accumulation in Mycobacterium tuberculosis as it goes into a dormancy-like state in culture. **Journal of Bacteriology**, v. 186, n. 15, p. 5017–5030, 2004.

DAO, D. N. et al. Mycobacterium tuberculosis Lipomannan Induces Apoptosis and Interleukin-12 Production in Macrophages. **Infection and Immunity**, v. 72, n. 4, p. 2067, abr.2004.

DOCKRELL, H. M.; BUTKEVICIUTE, E. Can what have we learnt about BCG vaccination in the last 20 years help us to design a better tuberculosis vaccine? **Vaccine**, v. 40, n. 11, p. 1525–1533, 8 mar. 2022.

DOCKRELL, H. M.; MCSHANE, H. Tuberculosis vaccines in the era of Covid-19 – what is taking us so long? **eBioMedicine**, v. 79, 1 maio 2022.

DUBÉ, J. Y. et al. Underwhelming or misunderstood? Genetic Variability of Pattern Recognition Receptors in Immune Responses and Resistance to Mycobacterium tuberculosis. **Frontiers in Immunology**, v. 12, p. 2631, 30 jun. 2021.

DUBNAU et al. Oxygenated mycolic acids are necessary for virulence of Mycobacterium tuberculosis in mice. **Molecular microbiology**, v. 36, n. 3, p. 630–637, 2000.

DULBERGER, C. L.; RUBIN, E. J.; BOUTTE, C. C. The mycobacterial cell envelope — a moving target. **Nature**, v. 18, p. 47–59, 2020.

EL-SHAZLY, S. et al. Internalization by HeLa cells of latex beads coated with mammalian cell entry (Mce) proteins encoded by the mce3 operon of Mycobacterium tuberculosis. **Journal of Medical Microbiology**, v. 56, n. 9, p. 1145–1151, 2007.

ETNA, M. P. et al. Pro-and anti-inflammatory cytokines in tuberculosis: A two-edged sword in TB pathogenesis. **Seminars in Immunology**, v. 26, n. 6, p. 543–551, 1 dez. 2014.

FALKINHAM, J. O.; III. Mycobacterial Aerosols and Respiratory Disease. **Emerging Infectious Diseases**, v. 9, n. 7, p. 763, 1 jul. 2003.

FLETCHER, H. A. et al. T-cell activation is an immune correlate of risk in BCG vaccinated infants. **Nature Co**, 2016.

FORRELLAD, M. A. et al. Virulence factors of the Mycobacterium tuberculosis complex. **Virulence**, v. 4, n. 1, p. 3–66, 2013.

FUKUDA, T. et al. Critical roles for lipomannan and lipoarabinomannan in cell wall integrity of mycobacteria and pathogenesis of tuberculosis. **mBio**, v. 4, n. 1, 2013.

GALAGAN, J. E. et al. The Mycobacterium tuberculosis regulatory network and hypoxia. **Nature**, v. 499, n. 7457, p. 178–183, 2013.

GARTON, N. J. et al. Cytological and transcript analyses reveal fat and lazy persister-like bacilli in tuberculous sputum. **PLoS Medicine**, v. 5, n. 4, p. 0634–0645, 1 abr. 2008.

GEIJTENBEEK, T.; VAN KOOYK, Y. Pathogens target DC-SIGN to influence their fate DC-SIGN functions as a pathogen receptor with broad specificity. **APMIS : acta pathologica, microbiologica, et immunologica Scandinavica**, v. 111, n. 7–8, p. 698–714,jul. 2003.

GIOFFRÉ, A. et al. Mutation in mce operons attenuates Mycobacterium tuberculosis virulence. **Microbes and Infection**, v. 7, n. 3, p. 325–334, 2005.

GONZALO-ASENSIO, J. et al. Evolutionary history of tuberculosis shaped by conserved mutations in the PhoPR virulence regulator. **Proceedings of the National Academy of Sciences**, v. 111, n. 31, p. 11491–11496, 5 ago. 2014.

GONZALO-ASENSIO, J. et al. **MTBVAC**: Attenuating the human pathogen of tuberculosis

(TB) toward a promising vaccine against the TB epidemic. Frontiers in Immunology, 2017. Disponível em: <www.frontiersin.org>. Acesso em: 21 ago. 2018

GUO, M. et al. High-resolution quantitative proteome analysis reveals substantial differences between phagosomes of RAW 264.7 and bone marrow derived macrophages. **Proteomics**, v. 15, n. 18, p. 3169, 1 set. 2015.

HAILE, Y.; BJUNE, G.; WIKER, H. Expression of the mceA, esat-6 and hspX genes in Mycobacterium tuberculosis and their responses to aerobic conditions and to restricted oxygen supply. **Microbiology (Reading, England)**, v. 148, n. Pt 12, p. 3881–3886, 1 dez.2002.

HAITES, R. et al. Function of phosphatidylinositol in mycobacteria. **The Journal of biological chemistry**, v. 280, n. 12, p. 10981–10987, 25 mar. 2005.

HARDING, C. V.; BOOM, W. H. Regulation of antigen presentation by Mycobacterium tuberculosis: a role for Toll-like receptors. **Nature Reviews Microbiology**, v. 8, n. 4, p. 296–307, 1 abr. 2010.

HAYASHI, D. et al. Comparable studies of immunostimulating activities in vitro among Mycobacterium bovis bacillus Calmette-Guérin (BCG) substrains. **FEMS Immunology and Medical Microbiology**, v. 56, n. 2, p. 116–128, 2009.

HAYASHI, D. et al. Biochemical characteristics among Mycobacterium bovis BCG substrains. **FEMS Microbiology Letters**, v. 306, n. 2, p. 103–109, 1 maio 2010.
HENAO-TAMAYO, M. I. et al. Phenotypic Definition of Effector and Memory T- Lymphocyte Subsets in Mice Chronically Infected with Mycobacterium tuberculosis. **Clinical and Vaccine Immunology: CVI**, v. 17, n. 4, p. 618, abr. 2010.

ISHIKAWA, E. et al. Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. **The Journal of Experimental Medicine**, v. 206, n. 13, p. 2879–2888, 2009.

ISHIKAWA, E.; MORI, D.; YAMASAKI, S. **Recognition of Mycobacterial Lipids by Immune Receptors**. **Trends in Immunology**Elsevier Current Trends, 1 jan. 2017.
Disponível em:
<https://www.sciencedirect.com/science/article/pii/S1471490616301806?via%3Dihub>. Acesso em: 30 out. 2018

JIA, X. et al. The Bioinformatics Analysis of Comparative Genomics of Mycobacterium tuberculosis Complex (MTBC) Provides Insight into Dissimilarities between Intraspecific Groups Differing in Host Association, Virulence, and Epitope Diversity. **Frontiers in Cellular and Infection Microbiology**, v. 7, n. MAR, p. 88, 21 mar. 2017.

JORDAO, L. et al. On the killing of mycobacteria by macrophages. **Cellular microbiology**, v. 10, n. 2, p. 529–548, fev. 2008.

JÓZEFOWSKI, S.; SOBOTA, A.; KWIATKOWSKA, K. How Mycobacterium tuberculosis subverts host immune responses. **BioEssays: news and reviews in molecular, cellular and developmental biology**, v. 30, n. 10, p. 943–954, 2008.

KANG, P. et al. The human macrophage mannose receptor directs Mycobacterium tuberculosis lipoarabinomannan-mediated phagosome biogenesis. **The Journal of experimental medicine**, v. 202, n. 7, p. 987–999, 3 out. 2005.

KARAKOUSIS, P.; BISHAI, W.; DORMAN, S. Mycobacterium tuberculosis cell envelope lipids and the host immune response. **Cellular microbiology**, v. 6, n. 2, p. 105–116, fev. 2004.

KAUSHAL, D. et al. Mucosal vaccination with attenuated Mycobacterium tuberculosis induces strong central memory responses and protects against tuberculosis. **Nature communications**, v. 6, 13 out. 2015.

KOO, M.; SUBBIAN, S.; KAPLAN, G. Strain specific transcriptional response in Mycobacterium tuberculosis infected macrophages. **Cell communication and signaling: CCS**, v. 10, n. 1, 2012.

KUMAR, N. P. et al. BCG vaccination induces enhanced frequencies of memory T cells and altered plasma levels of common γc cytokines in elderly individuals. **PLoS ONE**, v. 16, n. 11, 1 nov. 2021.

LARROUY-MAUMUS, G. et al. Protective efficacy of a lipid antigen vaccine in a guinea pig model of tuberculosis. **Vaccine**, v. 35, n. 10, p. 1395–1402, 7 mar. 2017.

LAYRE, E. et al. A comparative lipidomics platform for Mycobacterium tuberculosis provides chemotaxonomic analysis for biomarker discovery. **Chemistry and Biology**, v. 18, n. 12, p. 1537–1549, 2011.

LAYRE, E. et al. Molecular profiling of Mycobacterium tuberculosis identifies tuberculosinyl nucleoside products of the virulence-associated enzyme Rv3378c. **Proceedings of the National Academy of Sciences**, v. 111, n. 8, p. 2978–2983, 2014.

LEUNG, A. S. et al. Novel genome polymorphisms in BCG vaccine strains and impact on efficacy. **BMC Genomics**, v. 9, 2008.

LEWIS, K. N. et al. Deletion of RD1 from Mycobacterium tuberculosis Mimics Bacille Calmette-Guérin Attenuation. **The Journal of Infectious Diseases**, v. 187, n. 1, p. 117–123, 2 jan. 2003.

LI, L. et al. The immune responses of central and effector memory BCG-specific CD4+ T cells in BCG-vaccinated PPD+ donors were modulated by Treg cells. **Immunobiology**, v. 216, n. 4, p. 477–484, abr. 2011.

LUCA, S.; MIHAESCU, T. History of BCG Vaccine. **Maedica**, v. 8, n. 1, p. 53–8, mar. 2013.

MAHAIRAS, G. G. et al. Molecular analysis of genetic differences between Mycobacterium bovis BCG and virulent M. bovis. **Journal of Bacteriology**, v. 178, n. 5, p. 1274–1282, 1996.

MANGTANI, P. et al. Protection by BCG Vaccine Against Tuberculosis: A Systematic Review of Randomized Controlled Trials. **Clinical Infectious Diseases**, v. 58, n. 4, p. 470–480, 2014.

MINISTÉRIO DA SAÚDE, B. **Nota Informativa n°18/2018-CGPNI/DEVIT/SVS/MS**. [s.l: s.n.]. . Acesso em: 13 set. 2021.

MOHN, W. W. et al. The actinobacterial mce4 locus encodes a steroid transporter. **Journal of Biological Chemistry**, v. 283, n. 51, p. 35368–35374, 19 dez. 2008.

MOLIVA, J. I.; TURNER, J.; TORRELLES, J. B. Immune Responses to Bacillus Calmette–Guérin Vaccination: Why Do They Fail to Protect against Mycobacterium tuberculosis?

Frontiers in Immunology, v. 8, p. 407, 5 abr. 2017.
MOORLAG, S. J. C. F. M. et al. Efficacy of BCG Vaccination Against Respiratory Tract Infections in Older Adults During the Coronavirus Disease 2019 Pandemic. **Clinical Infectious Diseases**, v. 75, n. 1, p. e938–e946, 24 ago. 2022.

MUÑOZ-ELÍAS, E. J.; MCKINNEY, J. D. **Carbon metabolism of intracellular bacteria**. **Cellular Microbiology**, jan. 2006. Acesso em: 12 jan. 2020

NALPAS, N. C. et al. RNA sequencing provides exquisite insight into the manipulation of the alveolar macrophage by tubercle bacilli. **Scientific Reports 2015 5:1**, v. 5, n. 1, p. 1–12, 8 set. 2015.

NAMBIAR, J. K. et al. Protective immunity afforded by attenuated, PhoP-deficient Mycobacterium tuberculosis is associated with sustained generation of CD4+ T-cell memory. **European journal of immunology**, v. 42, n. 2, p. 385–392, fev. 2012.

NEMES, E. et al. Prevention of Infection with Mycobacterium tuberculosis by H4:IC31® Vaccination or BCG Revaccination in Adolescents. **The New England Journal of Medicine**, v. 379, n. 2, p. 138, 2018.

NIGOU, J. et al. Mycobacterial lipoarabinomannans: modulators of dendritic cell function and the apoptotic response. **Microbes and Infection**, v. 4, n. 9, p. 945–953, 1 jul. 2002.

OBREGÓN-HENAO, A. et al. Vaccination of guinea pigs using mce operon mutants of Mycobacterium tuberculosis. **Vaccine**, v. 29, n. 26, p. 4302–4307, 2011.

OLDENBURG, R. et al. Mycobacterial Phenolic Glycolipids Selectively Disable TRIF-Dependent TLR4 Signaling in Macrophages. **Frontiers in Immunology**, v. 0, n. JAN, p. 2, 19 jan. 2018.

OUELLET, H.; JOHNSTON, J.; DE MONTELLANO, P. Cholesterol catabolism as a therapeutic target in Mycobacterium tuberculosis. **Trends in microbiology**, v. 19, n. 11, p. 530–539, nov. 2011.

PABST, M. J. et al. Inhibition of macrophage priming by sulfatide from Mycobacterium tuberculosis. **The Journal of Immunology**, v. 140, n. 2, 1988.
PAI, M. et al. Tuberculosis. **Nature Reviews Disease Primers**, v. 2, p. 1–23, 2016.

PANDEY, A. K.; SASSETTI, C. M. Mycobacterial persistence requires the utilization of host cholesterol. **Proceedings of the National Academy of Sciences of the United States of America**, v. 105, n. 11, p. 4376–4380, 18 mar. 2008.

PERKOWSKI, E. F. et al. An orphaned Mce-associated membrane protein of *M ycobacterium tuberculosis* is a virulence factor that stabilizes Mce transporters. **Molecular Microbiology**, v. 100, n. 1, p. 90–107, 1 abr. 2016.

PETRILLI, J. D. et al. Differential Host Pro-Inflammatory Response to Mycobacterial Cell Wall Lipids Regulated by the Mce1 Operon. **Frontiers in Immunology**, v. 11, p. 1848, 18ago. 2020.
PYM, A. S. et al. Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. **Nature Medicine**, v. 9, n. 5, p. 533–539, 2003.

QUEIROZ, A. et al. Comparative metabolic profiling of mce1 operon mutant vs wild-type Mycobacterium tuberculosis strains. **Pathogens and disease**, v. 73, n. 8, p. ftv066, 1 nov. 2015.
QUEIROZ, A.; RILEY, L. W. Bacterial immunostat: Mycobacterium tuberculosis lipids and

their role in the host immune response. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 50, n. 1, p. 9–18, 2017.

RAYNAUD, C. et al. Phospholipases C are involved in the virulence of Mycobacterium tuberculosis. **Molecular Microbiology**, v. 45, n. 1, p. 203–217, 2002.

REIJNEVELD, J. F. et al. Synthetic mycobacterial diacyl trehaloses reveal differential recognition by human T cell receptors and the C-type lectin Mincle. **Scientific Reports 2021 11:1**, v. 11, n. 1, p. 1–10, 21 jan. 2021.

RHOADES, E. R. et al. Cell wall lipids from Mycobacterium bovis BCG are inflammatory when inoculated within a gel matrix: Characterization of a new model of the granulomatous response to mycobacterial components. **Tuberculosis**, v. 85, n. 3, p. 159–176, maio 2005.

RODRIGUES, L. C. et al. Effect of BCG revaccination on incidence of tuberculosis in school-aged children in Brazil: the BCG-REVAC cluster-randomised trial. **Lancet (London, England)**, v. 366, n. 9493, p. 1290–1295, 8 out. 2005.

ROY, A. et al. Effect of BCG vaccination against Mycobacterium tuberculosis infection in children: systematic review and meta-analysis. **BMJ (Clinical research ed.)**, v. 349, n. 1, p. 1–11, 2014.

ROY, S. et al. Transcriptional landscape of Mycobacterium tuberculosis infection in macrophages. **Scientific reports**, v. 8, n. 1, 1 dez. 2018.

SAAVEDRA, R. et al. Mycobacterial di-O-acyl-trehalose inhibits mitogen- and antigen-induced proliferation of murine T cells in vitro. **Clinical and diagnostic laboratory immunology**, v. 8, n. 6, p. 1081–1088, 2001.

SARNO, A. et al. In silico comparisons of lipid-related genes between Mycobacterium tuberculosis and BCG vaccine strains. **Genetics and Molecular Biology**, v. 44, n. 4, 22 out. 2021.

SATTI, I.; MCSHANE, H. Current approaches toward identifying a correlate of immune protection from tuberculosis. **Expert Review of Vaccines**, v. 18, n. 1, 2018.

SHIMONO, N. et al. Hypervirulent mutant of Mycobacterium tuberculosis resulting from disruption of the mce1 operon. **Proceedings of the National Academy of Sciences**, v. 100, n. 26, p. 15918–15923, 23 dez. 2003.

SOARES, A. P. et al. Bacillus Calmette-Guérin vaccination of human newborns induces T cells with complex cytokine and phenotypic profiles. **Journal of immunology (Baltimore, Md.: 1950)**, v. 180, n. 5, p. 3569–77, 1 mar. 2008.

SRINIVAS, M. et al. Functional characterization of the phospholipase C activity of Rv3487c and its localization on the cell wall of Mycobacterium tuberculosis. **Journal of Biosciences**, v. 33, n. 2, p. 221–230, jun. 2008.

SRIVASTAVA, S. et al. Unsaturated Lipid Assimilation by Mycobacteria Requires Auxiliary cis-trans Enoyl CoA Isomerase. **Chemistry and Biology**, v. 22, n. 12, p. 1577–1587, 2015.

TAILLEUX, L. et al. DC-SIGN is the major Mycobacterium tuberculosis receptor on human dendritic cells. **The Journal of experimental medicine**, v. 197, n. 1, p. 121–127, jan. 2003.

TAILLEUX, L. et al. Probing Host Pathogen Crosstalk by Transcriptional Profiling of Both Mycobacterium tuberculosis and Infected Human Dendritic Cells and Macrophages. **PLOS ONE**, v. 3, n. 1, p. e1403, 2 jan. 2008.

TAIT, D. R. et al. Final Analysis of a Trial of M72/AS01 E Vaccine to Prevent Tuberculosis. **New England Journal of Medicine**, p. NEJMoa1909953, 28 out. 2019.

TAMERIS, M. et al. Lessons learnt from the first efficacy trial of a new infant tuberculosis vaccine since BCG. **Tuberculosis**, v. 93, n. 2, p. 143–9, 2013a.

TAMERIS, M. D. et al. Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: A randomised, placebo-controlled phase 2b trial. **The Lancet**, v. 381, n. 9871, p. 1021–1028, 23 mar. 2013b.

TANNE, A. et al. A murine DC-SIGN homologue contributes to early host defense against Mycobacterium tuberculosis. **Journal of Experimental Medicine**, v. 206, n. 10, p. 2205– 2220, 28 set. 2009.

TANNER, R. et al. The Humoral Immune Response to BCG Vaccination. **Frontiers in Immunology**, v. 10, p. 1317–1335, 2019.

TRAN, V. et al. Loss of Lipid Virulence Factors Reduces the Efficacy of the BCG Vaccine. **Scientific Reports**, v. 6, n. 1, p. 29076, 2016.

UCHIDA, Y. et al. Accelerated immunopathological response of mice infected with Mycobacterium tuberculosis disrupted in the mce1 operon negative transcriptional regulator. **Cellular Microbiology**, v. 9, n. 5, p. 1275–1283, 1 maio 2007.

VIGNAL, C. et al. Lipomannans, but not lipoarabinomannans, purified from Mycobacterium chelonae and Mycobacterium kansasii induce TNF-alpha and IL-8 secretion by a CD14-toll-like receptor 2-dependent mechanism. **Journal of immunology (Baltimore, Md. : 1950)**, v. 171, n. 4, p. 2014–2023, 15 ago. 2003.

VOGELZANG, A. et al. Central Memory CD4+ T Cells Are Responsible for the Recombinant Bacillus Calmette-Guérin ΔureC::hly Vaccine's Superior Protection Against Tuberculosis. **The Journal of Infectious Diseases**, v. 210, n. 12, p. 1928, 12 dez. 2014.

WALTERS et al. The Mycobacterium tuberculosis PhoPR two-component system regulates genes essential for virulence and complex lipid biosynthesis. **Molecular microbiology**, v. 60, n. 2, p. 312–330, abr. 2006.

WARR, A. J. et al. A CD4+ TNF+ monofunctional memory T-cell response to BCG vaccination is associated with Mycobacterium tuberculosis infection in infants exposed to HIV. **eBioMedicine**, v. 80, 1 jun. 2022.

WERNINGHAUS, K. et al. Adjuvanticity of a synthetic cord factor analogue for subunit Mycobacterium tuberculosis vaccination requires FcRgamma-Syk-Card9-dependent innate immune activation. **The Journal of experimental medicine**, v. 206, n. 1, p. 89–97, 16 jan. 2009.

WHO. **Information Sheet - Observed Rate of Vaccine Reactions Bacille Calmette-Guérin (BCG) Vaccine**. [s.l: s.n.].

WHO. **End TB Strategy**. [s.l: s.n.]. Disponível em:

<http://www.who.int/tb/End_TB_brochure.pdf>. Acesso em: 26 fev. 2018. WHO. **Global Tuberculosis Report**. [s.l: s.n.].

WHO. **Global tuberculosis report 2022**. [s.l: s.n.]. Disponível em: <https://www.who.int/publications/i/item/9789240061729>. Acesso em: 31 out. 2022a.

WHO. **Implementing the end TB strategy: the essentials, 2022 update**. [s.l: s.n.]. Disponível em: <https://www.who.int/publications/i/item/9789240065093>. Acesso em: 25maio. 2023b.

WRIGHT, C. C. et al. The Mycobacterium tuberculosis MmpL11 cell wall lipid transporter is important for biofilm formation, intracellular growth, and nonreplicating persistence. **Infection and Immunity**, v. 85, n. 8, p. 1–17, 2017.

Y, M. Y. et al. C-type lectin MCL is an FcRγ-coupled receptor that mediates the adjuvanticity of mycobacterial cord factor. **Immunity**, v. 38, n. 5, p. 1050–1062, 23 maio 2013.

YONEKAWA, A. et al. Dectin-2 is a direct receptor for mannose-capped lipoarabinomannan of mycobacteria. **Immunity**, v. 41, n. 3, p. 402–413, 18 set. 2014.

ZHANG, L. et al. Variable Virulence and Efficacy of BCG Vaccine Strains in Mice and Correlation with Genome Polymorphisms. **Molecular Therapy**, v. 24, n. 2, p. 398–405,2016.

ZHANG, X. et al. Mycobacterium bovis and BCG induce different patterns of cytokine and chemokine production in dendritic cells and differentiation patterns in CD4+T cells. **Microbiology (United Kingdom)**, v. 159, n. 2, p. 366–379, fev. 2013.

Apêndice A - Figuras Suplementares

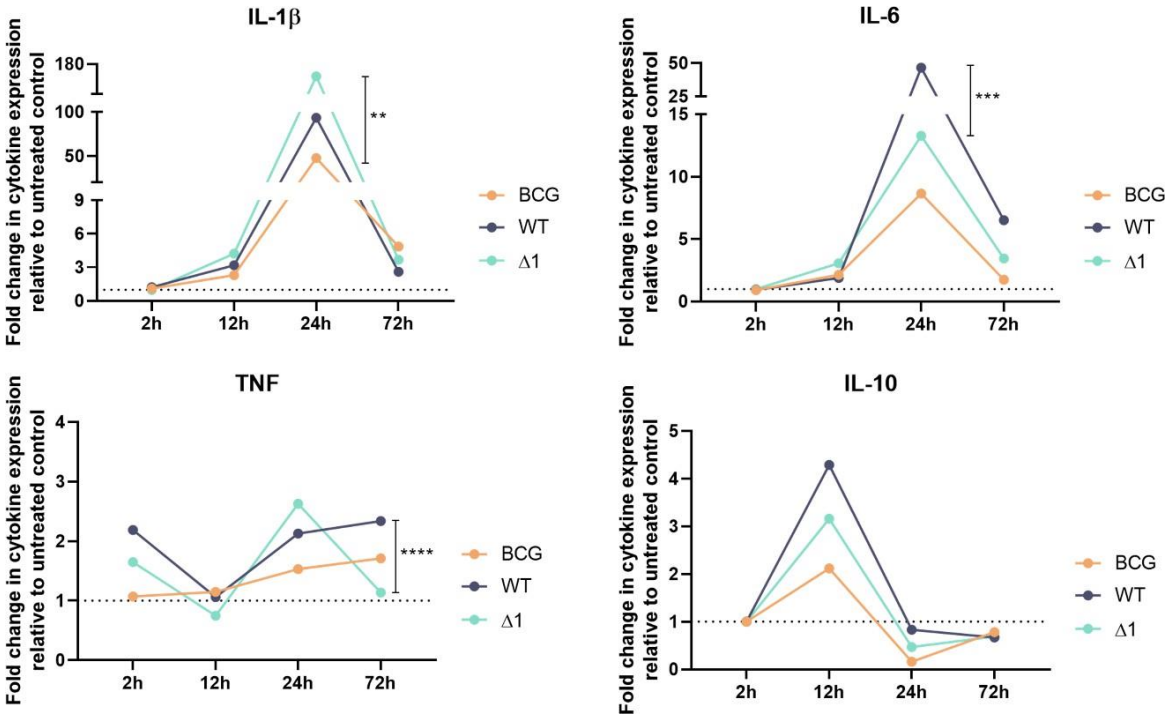


Figura 1. Análises de expressão de *IL-1β*, *IL-6*, *TNF* e *IL-10*, por RT-qPCR, após 2h, 12h, 24h e 72h de cultivo de macrófagos RAW 264.7, com extratos lipídicos de BCG, Mtb WT e Mtb Δ1. Valores representam a média de *fold-change* entre BCG, Mtb WT e Mtb Δ1 em relação ao controle negativo. Expressão gênica normalizada utilizando os genes *gliceraldeído 3-fosfato desidrogenase* (GAPDH) e *β-actina*. Valores de *p* foram calculados utilizando t-Test (**$p <$ 0,001; ***$p <$ 0,001; ****$p <$ 0,0001).

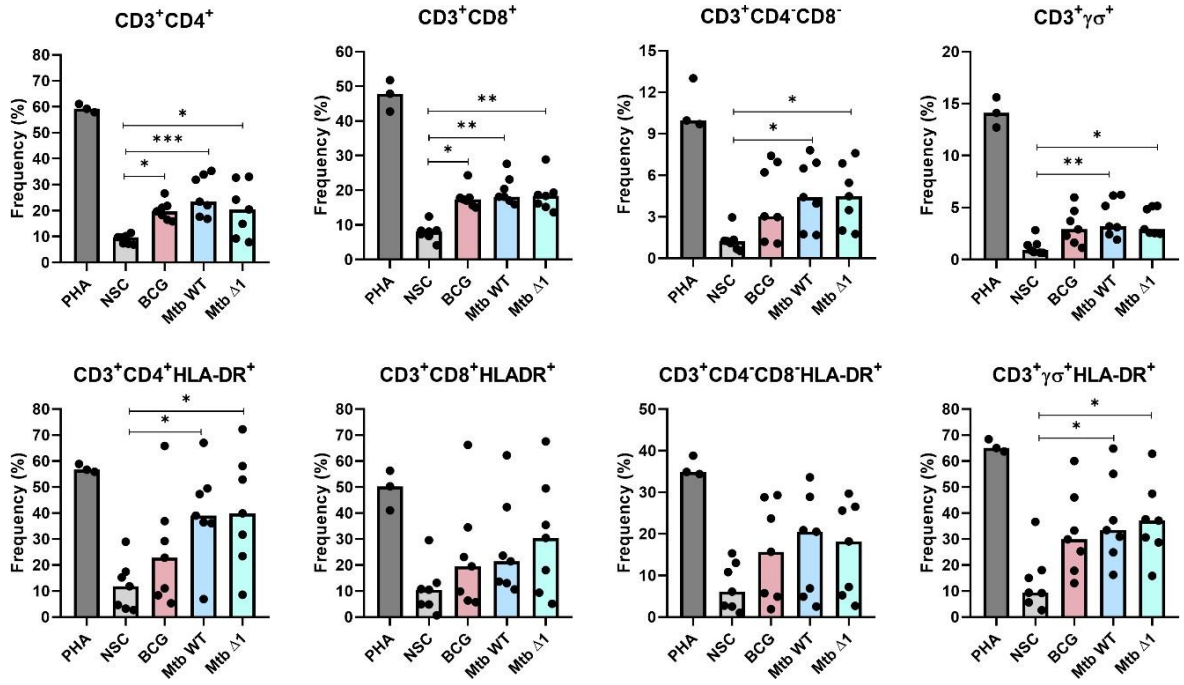


Figura 2. Citometria de Fluxo de células T convencionais e não-convencionais, após 48 horas de cultivo *in vitro* de células PBMC de indivíduos sadios, com extratos lipídicos de BCG, Mtb WT e Mtb Δ1. Distribuição normal dos dados foi determinada pelo teste Shapiro-Wilk. Para distribuição paramétrica, valores de *p* foram calculados pelo teste One-Way ANOVA. Para distribuição não-paramétrica, valores de *p* foram calculados pelo teste Kruskal-Wallis. **p* < 0,05; ***p* < 0,01; ****p* < 0,001. NSC (controle negative); PHA (fitohemaglutinina, controle positivo).

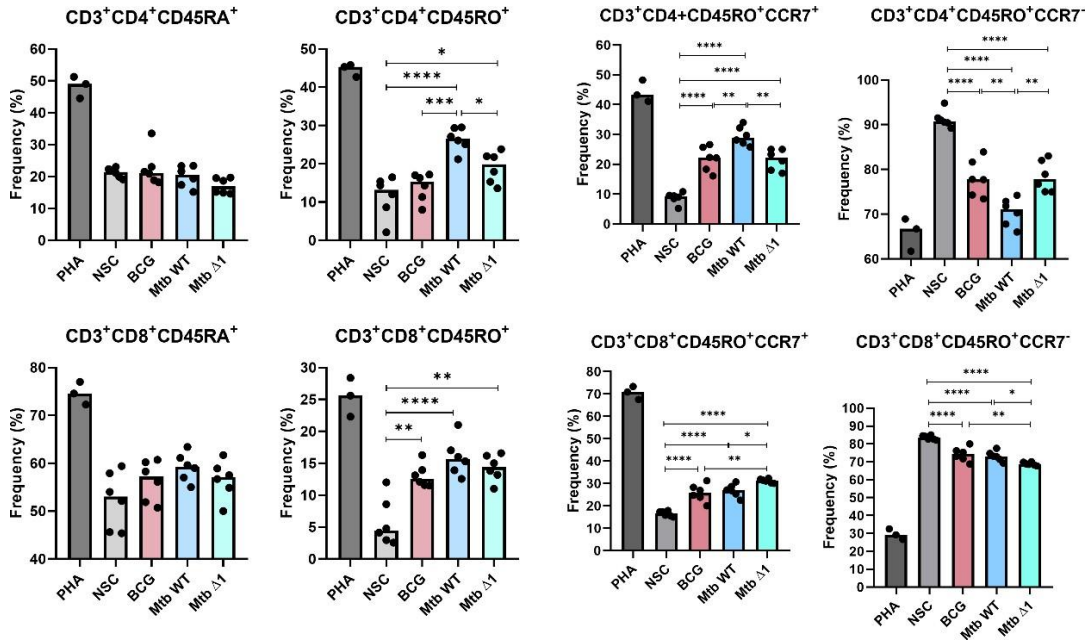


Figura 3. Citometria de Fluxo de células T de memória, após 48 horas de cultivo *in vitro* de células PBMC de indivíduos sadios, com extratos lipídicos de BCG, Mtb WT e Mtb Δ1. Naïve ($CD45RA^+$), memória ($CD45RO^+$), memória central ($CD45RO^+CCR7^+$) e memória efetora ($CD45RO^+CCR7^-$). Distribuição normal dos dados foi determinada pelo teste Shapiro-Wilk. Para distribuição paramétrica, valores de *p* foram calculados pelo teste One-Way ANOVA. Para distribuição não-paramétrica, valores de *p* foram calculados pelo teste Kruskal-Wallis. $^{**}p < 0{,}01$; $^{***}p < 0{,}001$; $^{****}p < 0{,}0001$. NSC (controle negative); PHA (fitohemaglutinina, controle positivo).

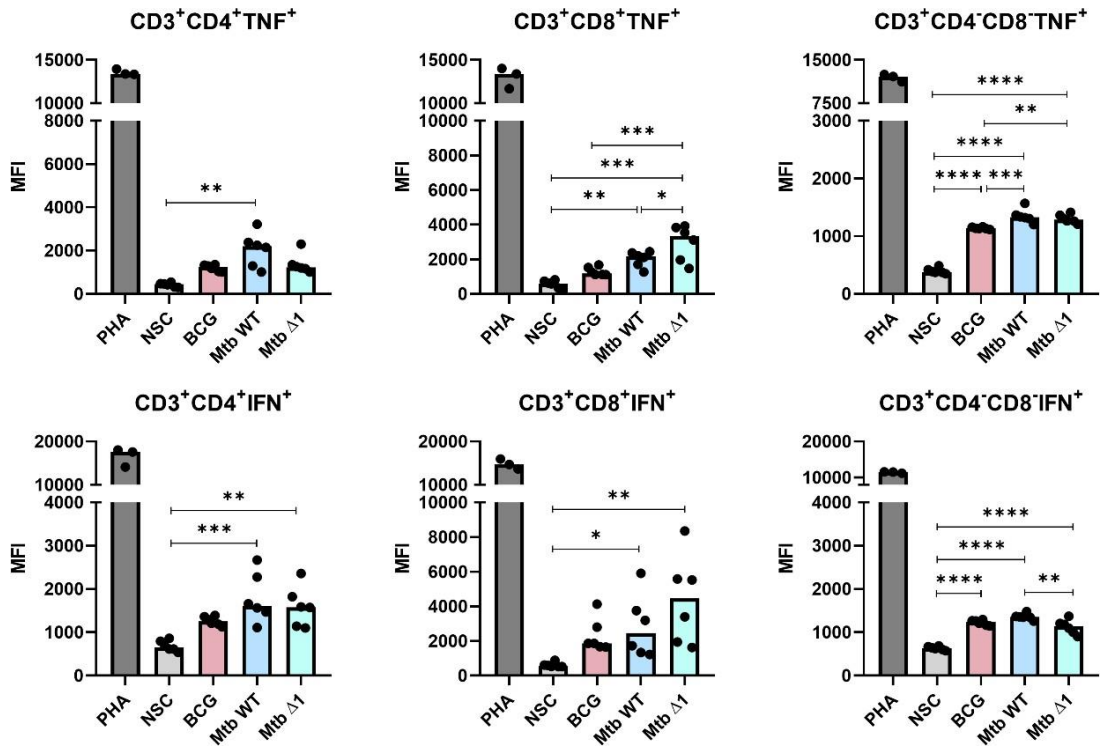


Figura 4. Citometria de Fluxo de células T $CD4^+$, $CD8^+$ e Duplo-Negativas ($CD4^-CD8^-$) produtoras de TNF e IFNγ, após 48 horas de cultivo *in vitro* de células PBMC de indivíduos sadios, com extratos lipídicos de BCG, Mtb WT e Mtb Δ1. Distribuição normal dos dados foi determinada pelo teste Shapiro-Wilk. Para distribuição paramétrica, valores de *p* foram calculados pelo teste One-Way ANOVA. Para distribuição não-paramétrica, valores de *p* foram calculados pelo teste Kruskal-Wallis. $*p < 0{,}05$; $**p < 0{,}01$; $***p < 0{,}001$; $****p < 0{,}0001$. NSC (controle negative); PHA (fitohemaglutinina, controle positivo).

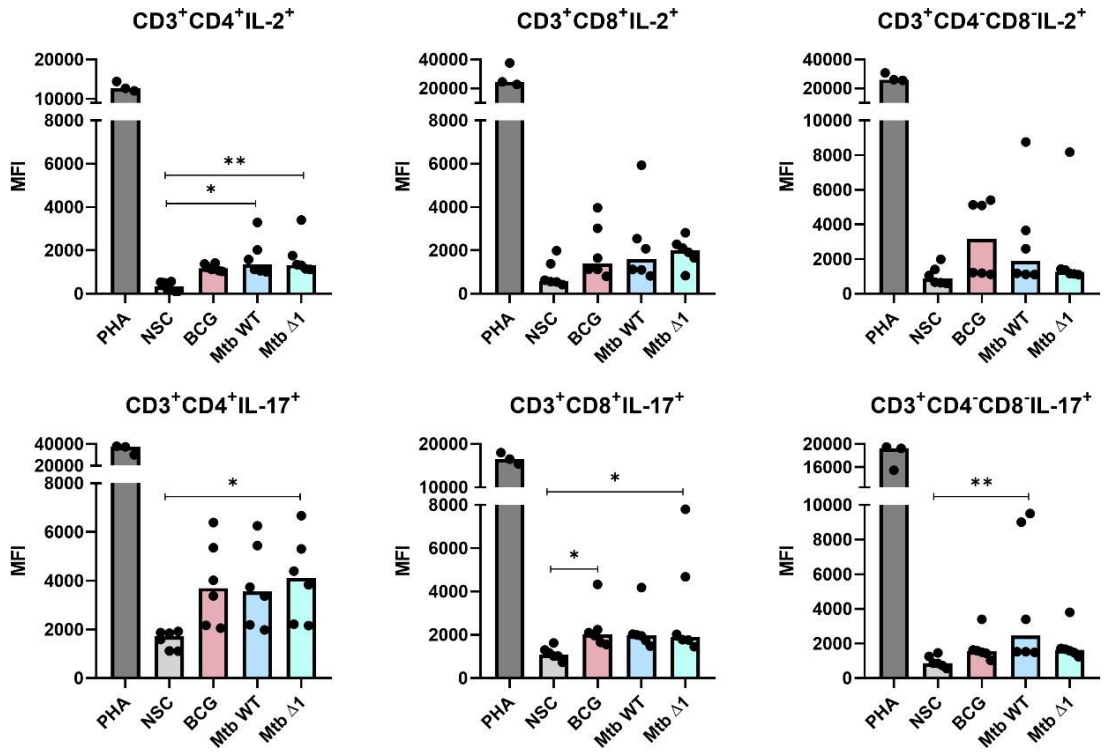


Figura 5. Citometria de Fluxo de células T $CD4^+$, $CD8^+$ e Duplo-Negativas ($CD4^-CD8^-$) produtoras de IL-2 e IL-17, após 48 horas de cultivo *in vitro* de células PBMC de indivíduos sadios, com extratos lipídicos de BCG, Mtb WT e Mtb Δ1. Distribuição normal dos dados foi determinada pelo teste Shapiro-Wilk. Para distribuição paramétrica, valores de *p* foram calculados pelo teste One-Way ANOVA. Para distribuição não-paramétrica, valores de *p* foram calculados pelo teste Kruskal-Wallis. $*p < 0{,}05$; $**p < 0{,}01$. NSC (controle negative); PHA (fitohemaglutinina, controle positivo).

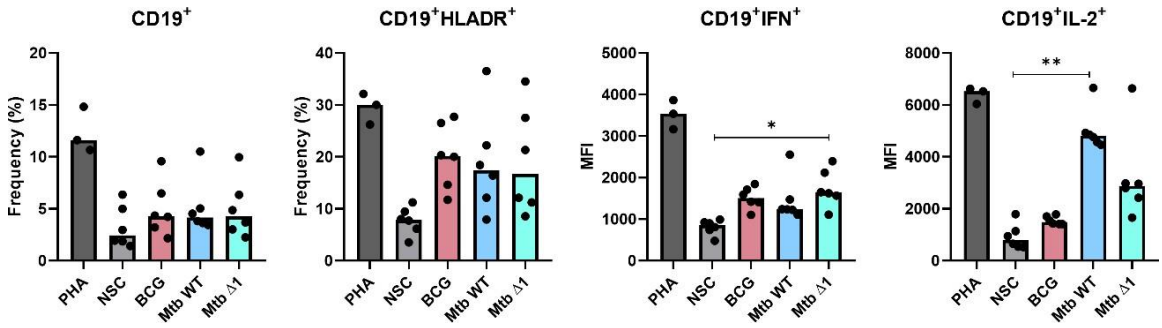


Figura 6. Citometria de Fluxo de células B, após 48 horas de cultivo *in vitro* de células PBMC de indivíduos sadios, com extratos lipídicos de BCG, Mtb WT e Mtb Δ1. Distribuição normal dos dados foi determinada pelo teste Shapiro-Wilk. Para distribuição paramétrica, valores de *p* foram calculados pelo teste One-Way ANOVA. Para distribuição não-paramétrica, valores de *p* foram calculados pelo teste Kruskal-Wallis. $**p < 0{,}01$. NSC (controle negative); PHA (fitohemaglutinina, controle positivo).

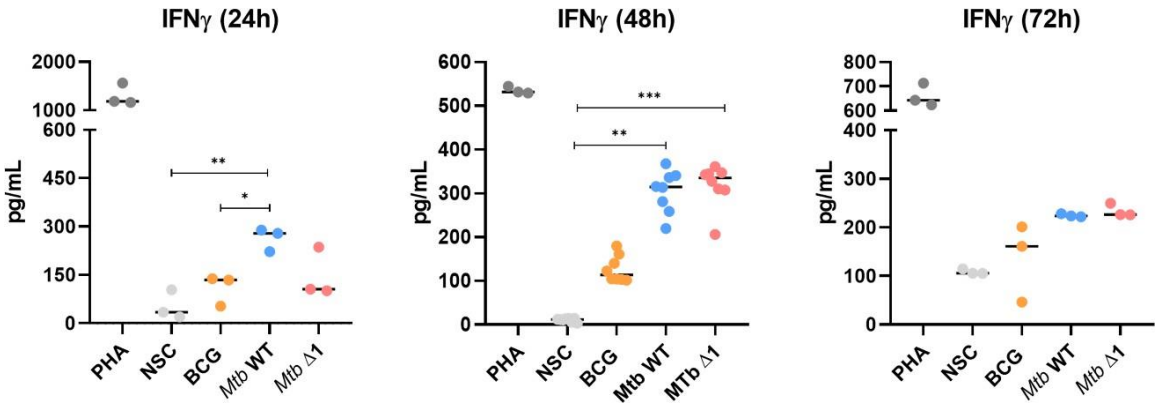


Figura 7. Produção de IFNγ após 24 horas, 48 horas e 72 horas de cultivo *in vitro* de células PBMC de indivíduos sadios, com extratos lipídicos de BCG, Mtb WT e Mtb Δ1. Distribuição normal dos dados foi determinada pelo teste Shapiro-Wilk. Para distribuição paramétrica, valores de *p* foram calculados pelo teste One-Way ANOVA. Para distribuição não-paramétrica, valores de *p* foram calculados pelo teste Kruskal-Wallis. **p* < 0,05; ***p* < 0,01; ****p* < 0,001. NSC (controle negative); PHA (fitohemaglutinina, controle positivo).

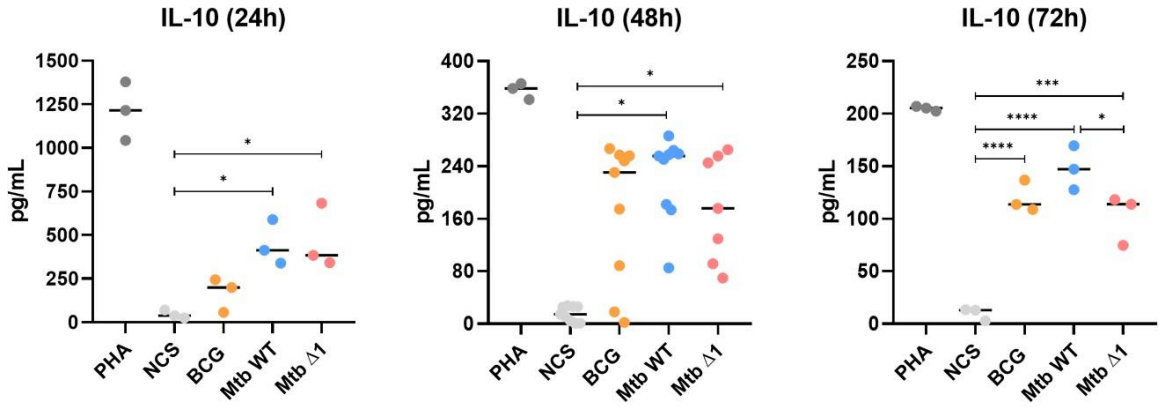


Figura 8. Produção de IL-10 após 24 horas, 48 horas e 72 horas de cultivo *in vitro* de células PBMC de indivíduos sadios, com extratos lipídicos de BCG, Mtb WT e Mtb Δ1. Para distribuição paramétrica, valores de *p* foram calculados pelo teste One-Way ANOVA. Para distribuição não-paramétrica, valores de *p* foram calculados pelo teste Kruskal-Wallis. **p* < 0,05; ****p* < 0,001; *****p* < 0,0001. NSC (controle negative); PHA (fitohemaglutinina, controle positivo).

Anexo A - Artigos publicados

1. Artigo publicado em Journal of the Brazilian Society of Tropical Medicine. SARNO, Alice; BORGES, Cleidiane Daltro; MENDES, Carlos Mauricio Cardeal; BARBOSA, Theolis. Distribution of HLA-DRB1 alleles in BRICS countries with a high tuberculosis burden: a systematic review and meta-analysis. 2021.
2. Artigo publicado em Vaccine. BITENCOURT, Julia; SARNO, Alice; OLIVEIRA, Carlos; DE SOUZA, Ramon Andrade; LIMA, Carla Cristina; TAKENAMI, Iukary; PEREIRA, Susan M; ARRUDA, Sergio. Distribution of HLA-DRB1 alleles in BRICS countries with a high tuberculosis burden: a systematic review and meta-analysis. 2021.

Revista da Sociedade Brasileira de Medicina Tropical
Journal of the Brazilian Society of Tropical Medicine
Vol.:54 | (e0017-2021) | 2021
https://doi.org/10.1590/0037-8682-0017-2021

Major Article

Distribution of HLA-DRB1 alleles in BRICS countries with a high tuberculosis burden: a systematic review and meta-analysis

***Alice Sarno*[1], *Cleidiane Borges Daltro*[1], *Carlos Mauricio Cardeal Mendes*[2] *and Theolis Barbosa*[1],[3]**

[1]. Fundação Oswaldo Cruz, Instituto Gonçalo Moniz, Salvador, BA, Brasil.
[2]. Universidade Federal da Bahia, Instituto de Ciências da Saúde, Salvador, BA, Brasil.
[3]. Rede Brasileira de Pesquisas em Tuberculose, Rio de Janeiro, RJ, Brasil.

Abstract

Introduction: Tuberculosis (TB) is the leading cause of death worldwide caused by a single infectious disease agent. Brazil, Russia, India, China, and South Africa (BRICS) account for more than half of the world's TB cases. Bacillus Calmette-Guérin (BCG) remains the only vaccine available despite its variable efficacy. Promising antigen-based vaccines have been proposed as prophylactic and/or immunotherapeutic approaches to boost BCG vaccination. Relevant antigens must interact with the range of human leukocyte antigen (HLA) molecules present in target populations; yet this information is currently not available. **Methods:** MEDLINE and EMBASE were systematically searched for articles published during 2013-2020 to measure the allelic frequencies of HLA-DRB1 in the BRICS. **Results:** In total, 67 articles involving 3,207,861 healthy individuals were included in the meta-analysis. HLA-DRB1 alleles *03, *04, *07, *11, *13, and *15 were consistently identified at high frequencies across the BRICS, with a combined estimated frequency varying from 52% to 80%. HLA-DRB1 alleles *01, *08, *09, *10, *12, and *14 were found to be relevant in only one or two BRICS populations. **Conclusions:** By combining these alleles, it is possible to ensure at least 80% coverage throughout the BRICS populations.

Keywords: Tuberculosis. Epitope-based vaccine. Rational vaccine design. Vaccine candidate. Major histocompatibility complex. Immunogenicity.

INTRODUCTION

Tuberculosis (TB), an infectious disease caused by *Mycobacterium tuberculosis*, is the most fatal infectious disease in the world that is caused by a single agent. The emerging countries Brazil, Russia, India, China, and South Africa, all members of a group known as the BRICS, currently account for approximately 50% of all TB cases worldwide as well as 38% of all disease-associated deaths[1].

The only available vaccine against TB, i.e., BCG, refers in fact to different attenuated strains of *Mycobacterium bovis*, used as part of the immunization schedule in countries and populations where the disease is highly prevalent[2,3]. Meta-analyses have shown that BCG offers protection against infection and the development of active TB, when comparing among vaccinated and unvaccinated children[4]. However, the efficacy of BCG varies greatly according to the age and clinical form of TB. While the vaccine is highly protective in children with tuberculous meningitis and miliary TB, its effectiveness varies from 0% to 80% with regard to pulmonary TB[5], which is responsible for disease transmission, hampering the ability to control disease.

While the immune response has a role in hampering bacilli multiplication and avoiding disease development, the relative roles of innate and adaptive mechanisms are difficult to weight. Currently, there is a lack of a reliable biomarker to guide the development and evaluation of new vaccine strategies. Nevertheless, progress has been made using strategies that rely on arrays of antigens selected on the basis of inducing powerful Th1 responses[6,7]. There is accumulating evidence that points to $CD4^+$ T cell involvement and, possibly, the role of these cells in interferon (IFN)-γ production

Corresponding author: Dr. Theolis Barbosa.
e-mail: theolis.bessa@fiocruz.br
http://orcid.org/0000-0003-1928-0404
Received 4 February 2021
Accepted 21 May 2021

as well as the building of antibody responses as a component of the protective anti-TB immunity[8-11]. This is highlighted by the fact that individuals infected with human immunodeficiency virus have a high increase in the risk of developing TB, which is dependent on the grade of deterioration of the $CD4^+$ T cell compartment[12-13].

Antigen presentation to $CD4^+$ T lymphocytes triggers the activation and proliferation of these cells, followed by differentiation into effector cytokine-producing cells that migrate to the infected tissue to amplify the bactericidal action of the infected macrophages[14].

Mycobacterial antigens are presented to $CD4^+$ T cells in the context of human leukocyte antigen (HLA) class II molecules. These proteins are encoded by a set of highly polymorphic genes located on the short arm of chromosome 6 and are expressed on the membranes of antigen-presenting cells[15]. Among these genes, HLA-DRB1 alleles are among the most frequently studied. HLA-DRB1 alleles are highly variable, some of which have been associated with susceptibility to active TB disease development. Moreover, the proportions of these alleles can differ greatly among populations[16].

New vaccine candidates have been proposed to prevent active disease and transmission, given as a booster to the primary vaccination with BCG. Currently, several candidates are undergoing different phases of clinical trials aimed at protecting newborns and children from infection or protecting adults with latent TB[7]. Among these, promising candidates include epitope-based vaccines that use HLA class II peptide ligands recognized by T cells to generate effective cellular immunity and protection. The impact of HLA class II binding efficiency to relevant epitopes in the immune response against infection at a population level has recently been addressed[17]. To provide high coverage in endemic settings, the present study performed a series of systematic reviews, followed by a meta-analysis, to identify the most relevant HLA alleles for targeting by effective epitopes in the BRICS populations. We could retrieve sufficient literature to describe the proportions of HLA-DRB1 allelic groups in the BRICS countries.

Systematic reviews and meta-analyses allow for a comprehensive overview of findings from a field of research to avoid bias and to produce a synthesis of comparable studies. Systematic reviews are used to identify studies that are both relevant and of good quality, according to pre-established inclusion, exclusion, and quality grading criteria, while meta-analyses are used to estimate the overall effect or outcome from the findings of the studies selected from a systematic review. Meta-analyses are widely employed in evidence-based medicine to measure the main effect of an intervention or hypothetical causal association of a condition. They are also powerful in achieving the optimized estimate of a given measure across larger numbers of study outcomes, thus reaching broad generalizations that are more robust than those that can be obtained by examining a single study[18,19]. Using a systematic review followed by a meta-analysis, we aimed to achieve optimized estimates of HLA-DRB1 allelic frequencies in disease-free individuals in the context of the BRICS, to determine the most relevant alleles to target by epitope-based vaccines.

METHODS

Search strategy

Throughout the article search and analysis steps, two investigators (AS and CBD) independently assessed each article, and the results were then compared and validated by consensus.

A literature search for articles published during 2013-2020 was conducted using two databases: MEDLINE and EMBASE. MEDLINE is the United States National Library of Medicine database, containing more than 24 million references in biomedicine and life sciences from more than 5,000 worldwide journals and books. EMBASE is an Elsevier database, containing more than 29 million references from 8,500 journals, which is focused on the biomedical literature regarding drug, disease, and device information and which includes more than 2,900 peer-reviewed journals not available in MEDLINE.

For each of the five BRICS countries, the following search terms were used: "HLA [All fields] AND frequency [All fields] AND Brazil", "HLA [All fields] AND frequency [All fields] AND Russia", "HLA [All fields] AND frequency [All fields] AND India", "HLA [All fields] AND frequency [All fields] AND China" and "HLA [All fields] AND frequency [All fields] AND South Africa". Because of the excessive number of articles originating from China retrieved for analysis (2,083 in total), we arbitrarily excluded 50%, that is, only the most recent articles were maintained. Pre-specified inclusion and exclusion criteria were applied in accordance with our study protocol (registered in PROSPERO, CRD # 42018092979).

Inclusion criteria

The titles and summary sections of the articles retrieved by each search string were accessed, and the following inclusion criteria were considered: articles describing the frequency of HLA-DRB1 alleles (except literature reviews) in healthy individuals from the BRICS, with access provided free of charge, or made available by institutional subscription through the Capes Portal de Periodicos, or by the authors themselves.

Exclusion criteria

Full-text articles were analyzed, and any studies restricted to patients, i.e., those that did not employ healthy controls, were excluded to avoid the possibility of selection bias, which could correlate the most frequent alleles with the disease studied in the case group. Studies involving individuals from the same family and/or members of tribes, villages, or castes, those that incompletely analyzed the 13 HLA-DRB1 subtypes (HLA-DRB1 *01, *03, *04, *07, *08, *09, *10, *11, *12, *13, *14, *15, and *16), and those reporting only HLA-DQ and/or HLA-DP frequencies were also excluded. The corresponding authors of three studies that analyzed the 13 HLA-DRB1 alleles but did not provide the explicit proportions thereof were contacted by e-mail to request this information. We received one reply from the three authors contacted. This study was maintained in the analysis, and the other two studies were excluded.

Quality assessment

The methodological quality of the articles was evaluated using a scale adapted from the Newcastle-Ottawa Scale[20] to preserve applicability in cross-sectional studies **(supplementary material File S1)**. Only the allelic frequencies of the control groups (healthy individuals) in each study were collected. To be selected for the meta-analysis, studies were required to have a minimum score of 4.

Article search and selection procedures were performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines, as illustrated in **File S2**[21].

Data collection

From the articles selected through the systematic review, a database was created to extract data of the relative frequencies for each allele with regard to each BRICS country. In one case, the allelic frequency results were provided after contacting the author by e-mail[22]. All allelic frequencies of HLA-DRB1 were independently collected by two investigators (ASM and CBD). From this database, individual files for each allele and country were generated in comma-separated value format for statistical analyses.

Statistical analysis

Statistical analyses were performed using the statistical software program R (version 3.3.3, available at www.R-project.org/) and the package *meta* (available at https://cran.r-project.org/web/packages/meta/index.html). The average frequencies for individual alleles in each country, as well as the 95% confidence intervals (CIs) and the relative weight of each article, were calculated. The first evaluation used to assess publication bias and heterogeneity among the articles was visual inspection of the funnel plots[23], followed by Cochran's Q test and the inconsistency measure (I^2) of the forest plot. Fixed-effect estimates were considered for $I^2 \leq 50\%$ and p-value >0.05, while random-effect estimates were considered for $I^2 > 50\%$ and p-value <0.05[24]. Forest plots were also used to compare among each of the allelic frequencies of the HLA-DRB1 gene according to each country.

RESULTS

Systematic review

The article search and selection results for all the five search terms are presented in **Figure 1**. The search and article selection processes for each search term are illustrated in **Figures S1-S5**.

Overall, during the identification stage, 2,916 articles were analyzed from the five target countries. Of these 2,916 articles, 554 were from Brazil, 188 from Russia, 554 from India, 1,338 from China, and 282 from South Africa. After analyzing the titles and summaries of each article, 394 articles from Brazil, 155 articles from Russia, 336 articles from India, 1,144 articles from China, and

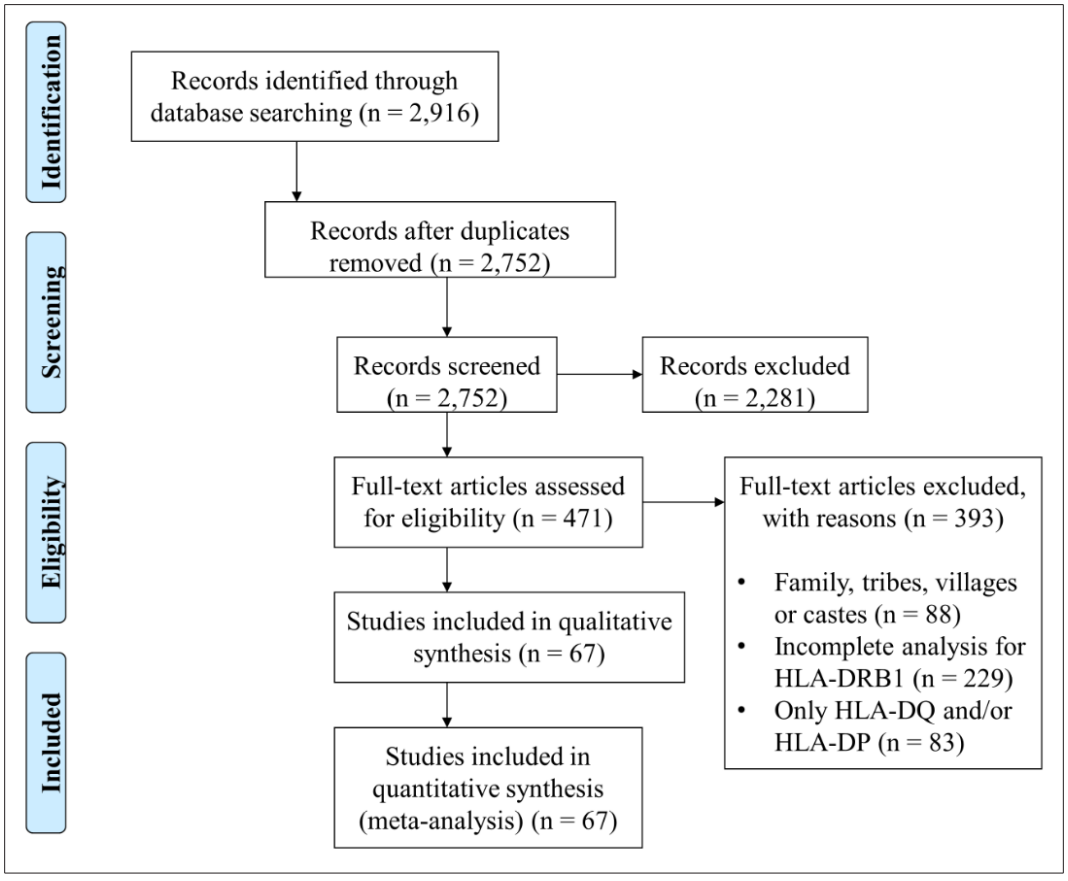


FIGURE 1: Preferred Reporting Items for Systematic Reviews (PRISMA) flow diagram.

252 articles from South Africa were not included. In these cases, the search either retrieved review articles or, in most cases, articles that presented only the allelic frequencies of HLA class I genes.

Following the screening stage, 126 articles from Brazil, 33 articles from Russia, 90 articles from India, 192 articles from China, and 30 articles from South Africa were analyzed for eligibility. In total, 393 of these articles were excluded: 104 from Brazil, 27 from Russia, 76 from India, 168 from China, and 18 from South Africa. These excluded studies were performed in restricted populations or families, were restricted to a specific HLA-DRB1 allele, or had presented insufficient data regarding allelic frequencies.

In the next step, the methodological quality was assessed in the remaining 19 articles from Brazil, six articles from Russia, 14 articles from India, 22 articles from China, and six articles from South Africa, published during 1997-2019. India was the only country in which all the retrieved articles were classified as high quality. Articles from Brazil, Russia, China, and South Africa were classified as of appropriate quality or high quality and were thus considered for meta-analysis. Overall, 39% of the articles included in the meta-analyses were of appropriate quality, while 61% were considered of high quality.

The inclusion and exclusion criteria were clearly defined in 89% of the articles. Less than half of the studies (46%) did not analyze the full sample and did not specify the rationale for this discrepancy. Overall, the number of samples that were not analyzed was less than 5% of the total samples considered. In 21% of the studies, the frequencies of HLA-DRB1 alleles were not fully presented for all samples.

The main method used to determine allelic frequencies was single specific primer-polymerase chain reaction (SSP-PCR), which is considered the gold standard method for HLA genotyping[25]. In addition to SSP-PCR, six articles, one from India and five from China, used other typing methods such as sequence-specific oligonucleotide probe-polymerase chain reaction and sequence-based typing-polymerase chain reaction.

Most studies from Brazil were carried out in the South (6) and Southeast (7) regions, with those from the North (1), Northeast (2), and Midwest (1) being less represented. Articles from India were specific to the West (3), North (3), South (5), and Central West (1) regions of the country. Articles from Russia included individuals from the south (1), west (3), and northwest (2) regions, the latter home to two of the most populous cities in this country: Moscow and St. Petersburg, with approximately 12 m and 5 m inhabitants, respectively.

Among the articles published from China, seven were from the eastern region, five were from the south, and six were from the northeast, with fewer articles from the north (2), southeast (2), and central (1) regions. The articles from South Africa encompassed the Central (1), East (2), and Northeast (1) regions of the country. Some articles from Brazil, China, and South Africa included samples from national bone marrow banks, that is, individuals from the entire country, were considered.

Among all articles, 67 were selected for the meta-analysis, corresponding to 3,207,861 healthy individuals from the BRICS countries, distributed as follows: 3,087,960 individuals from Brazil, in 19 articles; 2,333 individuals from Russia in six articles; 3,111 individuals from India in 14 articles; 110,497 healthy individuals from China in 22 articles and 3,960 individuals from South Africa in six articles. The list of articles selected for meta-analysis, with respective locations, methodological quality scores, and sample size, is presented in **Table S1**.

Meta-analysis

A meta-analysis was performed to estimate the frequencies of the 13 allelic groups of HLA-DRB1 in populations of BRICS countries. The frequencies, heterogeneity, and p-values obtained for each allele are summarized in **Table 1**.

HLA-DRB1 alleles *03, *04, *07, *11, *13, and *15 show combined frequencies varying between 52% and 80% in the BRICS countries. Least variation was observed for HLA-DRB1*04 and *07. Similar frequencies of HLA-DRB1*13 are found in Brazil, Russia, and India, while approximately half of these values are present in China and South Africa. Likewise, HLA-DRB1*11 is present at similar frequencies in Brazil, Russia, and South Africa, but nearly half as much in India and China. The HLA-DRB1*15 allele was found to be present in 23% (95% CI=22-24) of the Indian population, and at least 10% of the populations of the other BRICS countries were considered. The HLA-DRB1*03 allele is most frequent in South Africa, with a frequency of 20% (95% CI=16-25), up to five times higher than that in the other populations studied.

HLA-DRB1 *01, *08, *09, *10, *12, and *14 are relevant in one or two BRICS populations. The HLA-DRB1*01 allele is present at similar frequencies in Brazil, Russia, and South Africa but is present only in approximately 2% of the populations from India and China. The HLA-DRB1*10 allele is present in 9% of the Indian population but is present in less than 2% of other BRICS populations. HLA-DRB1*09 and *12 alleles are present at frequencies of more than 10% in China but less than 5% in the other countries investigated herein. HLA-DRB1*08 and *14 are present in 7% and 6% of the Chinese population, respectively; either of them could be targeted to achieve 80% of minimum coverage in the country.

The heterogeneity among the studies was generally above 50%. China was the only country wherein heterogeneity was considered significant for all 13 alleles. Accordingly, all allelic frequencies considered for China took into account the random effect estimates. Regarding other countries, the frequencies were partly evaluated by fixed-effects analysis, while the remaining part was analyzed by random-effects analysis. The frequencies of the HLA-DRB1*11 and *14 alleles were estimated using random-effects analysis in all the countries. No heterogeneity was observed for the HLADRB1 alleles *10 in Brazil; *07, *08, *15, and *16 in Russia; *12 in India; and *09 in South Africa.

DISCUSSION

The present work reports the most frequent HLA-DRB1 alleles in the populations of Brazil, Russia, India, China, and South Africa, which we propose as targets for the development of new vaccines

TABLE 1: Average frequencies of HLA-DRB1 alleles from BRICS populations.

HLA DRB1	COUNTRIES														
	Brazil			Russia			India			China			South Africa		
	PR (%)	CI 95%	I^2% (p)	PR (%)	CI 95%	I^2% (p)	PR (%)	CI 95%	I^2% (p)	PR (%)	CI 95%	I^2% (p)	PR (%)	CI 95%	I^2% (p)
*01	**9**[a]	9-9	7 (0.38)	**11**[a]	10-13	60 (0.01)	2	1-3	12 (0.3)	2	2-3	97 (0.0001)	7	4-9	89 (0.0001)
*03	**10**[a]	9-10	46 (0.03)	**9**[a]	7-11	75 (0.0001)	**9**[a]	8-10	48 (0.05)	**4**[a]	4-5	87 (0.0001)	**21**[a]	15-27	96 (0.0001)
*04	**11**[a]	10-13	78 (0.0001)	**11**[a]	10-12	1 (0.4)	**7**[a]	5-9	65 (0.04)	**11**[a]	11-12	84 (0.0001)	**9**[a]	6-12	93 (0.0001)
*07	**11**[a]	10-12	70 (0.0001)	**13**[a]	12-14	0 (0.8)	**14**[a]	12-16	67 (0.02)	**10**[a]	9-11	98 (0.0001)	**9**[a]	7-10	70 (0.005)
*08	**6**[a]	6-7	59 (0.002)	4	3-4	0 (0.8)	1	0-2	69 (0.01)	**7**[a]	6-8	91 (0.0001)	2	1-4	91 (0.0001)
*09	1	1-2	81 (0.0001)	1	0-1	58 (0.02)	1	0-1	60 (0.09)	**15**[a]	14-16	96 (0.0001)	1	1-1	0 (0.8)
*10	2	2-2	0 (0.5)	1	0-1	43 (0.09)	8	6-9	53 (0.03)	1	1-2	61 (0.0001)	2	1-2	64 (0.02)
*11	**13**[a]	11-14	87 (0.0001)	**13**[a]	10-16	79 (0.0001)	**7**[a]	6-10	90 (0.0001)	**6**[a]	6-6	83 (0.0001)	**15**[a]	13-18	82 (0.0001)
*12	2	1-2	75 (0.0001)	2	1-2	48 (0.07)	3	2-4	0 (0.5)	**12**[a]	12-13	86 (0.0001)	3	2-5	88 (0.0001)
*13	**14**[a]	13-14	39 (0.06)	**11**[a]	9-13	64 (0.006)	**11**[a]	10-13	40 (0.1)	**6**[a]	5-6	90 (0.0001)	**16**[a]	14-18	65 (0.01)
*14	4	3-5	90 (0.0001)	3	1-4	87 (0.0001)	**9**[a]	7-11	65 (0.004)	6	6-7	92 (0.0001)	2	0-3	92 (0.0001)
*15	**10**[a]	9-10	36 (0.08)	**14**[a]	13-15	0 (0.4)	**23**[a]	21-24	46 (0.06)	**15**[a]	14-16	95 (0.0001)	**10**[a]	9-11	15 (0.3)
*16	4	4-5	55 (0.005)	4	4-5	0 (0.9)	0	0-1	16 (0.3)	2	2-3	96 (0.0001)	0	0-1	52 (0.08)
CFrq	84			82			80			86			80		

PR: Pooled Results. CFrq, combined estimated frequency for the highlighted alleles.

[a]HLA-DRB1 gene alleles with combined estimated frequencies representative of at least 80% of the populations evaluated. The alleles that are present in relevant proportions in most or all of the BRICS countries are highlighted in grey.

against tuberculosis. We performed a systematic review followed by meta-analysis as methodologies of summarizing the estimates of allelic frequencies, using an adapted scale to judge the quality of the studies retrieved (as there are no previously published scales proposed for this goal). It is important to emphasize that the use of systematic reviews and meta-analyses as a means of reaching broad generalizations beyond the estimates of the effect of specific interventions, although not frequent, has a long-recognized value in a broad range of scientific fields[19].

We were able to retrieve not less than six articles per country containing data on all the HLA-DRB1 alleles for inclusion in our meta-analysis. The decision to focus only on HLA-DRB1 was supported by our observation, during the identification stage, that the databases contained only 43 articles measuring the frequencies of HLA-DQ or HLA-DP alleles in any of the BRICS countries. Moreover, all these studies reported isolated, specific allelic frequencies, as compared to the ensemble of alleles for these loci. Likewise, an insufficient number of studies have analyzed HLA class I alleles. Liang et al. reported that a significant overlap can occur between HLA class I and class II alleles regarding their specificity to epitopes of the same antigen[17]. However, it is not clear whether a significant overlap is recognized within the same individual.

Given the frequencies found in the aforementioned populations, HLA-DRB1*03, *04, *07, *11, *13, and *15 should be considered as core alleles in the design of new vaccines providing a high coverage throughout the BRICS countries. In addition to these core alleles, HLA-DRB1*01 and *08 in Brazil, HLA-DRB1*01 in Russia, HLA-DRB1*14 in India, and HLA-DRB1*09 and *12 as well as *08 or *14 in China should also be regarded as important targets to yield at least 80% coverage in these specific populations. By contrast, HLA-DRB1*10 and *16 should not be essential targets for new vaccine candidates, as epitopes with low affinity to these alleles, but with high affinity to the remaining discussed alleles, would nonetheless be capable of triggering responses in at least 80% of the BRICS populations.

Among the most frequently found alleles, HLA-DRB1*15, present in at least 10% of all five populations, is associated with a higher incidence of active pulmonary TB and has been considered a possible marker of disease development[26]. Similarly, HLA-DRB1*09, found at a frequency as much as 15 times higher in Chinese populations than in the other BRICS countries studied has also been associated with susceptibility to TB, especially in East Asian populations. Conversely, HLA-DRB1 alleles *03, *07, *12, and *13 are associated with protection against TB as reported in a meta-analysis that examined studies from 12 countries[27].

Our meta-analysis showed high heterogeneity, which is likely due to the range of study types included[23,28]. All studies included herein were considered to be cross-sectional in nature, as we retrieved only results originating from the control groups; however, the original study designs included prospective cohorts and case-control studies. Meta-analyses of cross-sectional studies tend to show high heterogeneity and frequently employ random-effect modeling[28]. Random-effects analysis tends to produce more conservative results, thereby reducing the risk of bias. However, the inclusion of an extensive number of studies is considered to reduce the impact of discrepant observations[29]. Thus, it was possible to account for the differences in sample sizes while still maintaining CIs similar to those calculated using the fixed-effects analysis.

One limitation of this study was the lack of sufficient articles to explore more specific allelic frequencies within all 13 HLA-DRB1 allelic groups. The affinity of the alleles within each allelic group for a given epitope can vary considerably[17]. However, for most HLA-DRB1 allelic groups, one or a few alleles are responsible for a high proportion of their occurrence[17], and there are tools available to address this issue in vaccine design[30].

In conclusion, we propose that epitope-based candidates for vaccines against TB should have high affinity to the HLA-DRB1 alleles *03, *04, *07, *11, *13, and *15 as core targets, and to *01, *08, *09, *12, and *14 as additional targets, especially with regard to TB control in the BRICS countries.

ACKNOWLEDGMENTS

We thank Artur Queiroz, Pablo Ivan Ramos and Martha Martínez Silveira for expert review of the manuscript; Andris Walter for expert language revision and Iasmim Diniz Orge for assistance as a trainee in this project.

FINANCIAL SUPPORT

This work was supported by National Scientific and Technological Research Council (CNPq) [grant number 158967, 2015] and Bahia's State Foundation for Research Support (FAPESB) [grant number BOL2647, 2013]. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001

AUTHORS' CONTRIBUTION

AS: Data curation; Methodology; Investigation; Formal analysis; Writing - original draft; Writing - review and editing; Visualization; CBD: Data curation; Methodology; Investigation; Formal analysis; Writing - original draft; Visualization; CMCM: Conceptualization; Methodology; Formal analysis; Supervision; TB: Conceptualization; Resources; Investigation; Writing - original draft; Writing - review and editing; Visualization; Supervision; Project administration; Funding acquisition

CONFLICT OF INTEREST

The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. None of the authors has any potential financial conflict of interest related to this manuscript.

ORCID

Alice Sarno: 0000-0001-7440-695X

Cleidiane Borges Daltro: 0000-0001-8395-2768

Carlos Mauricio Cardeal Mendes: 0000-0003-2089-5668

Theolis Barbosa: 0000-0003-1928-0404

REFERENCES

1. WHO. Global tuberculosis report 2020. Geneva, Switzerland: World Health Organization; 2020.
2. Ritz N, Curtis N. Mapping the global use of different BCG vaccine strains. Tuberc Edinb Scotl 2009;89(4):248-51.
3. Peck M, Gacic-Dobo M, Diallo MS, Nedelec Y, Sodha SS, Wallace AS. Global Routine Vaccination Coverage, 2018. MMWR Morb Mortal Wkly Rep. 2019;68:937-42.
4. Roy A, Eisenhut M, Harris RJ, Rodrigues LC, Sridhar S, Habermann S, et al. Effect of BCG vaccination against Mycobacterium tuberculosis infection in children: systematic review and meta-analysis. BMJ. 2014;349(1):1-11.
5. Mangtani P, Abubakar I, Ariti C, Beynon R, Pimpin L, Fine PEM, et al. Protection by BCG Vaccine Against Tuberculosis: A Systematic Review of Randomized Controlled Trials. Clin Infect Dis. 2014;58(4):470-80.
6. McShane H. Insights and challenges in tuberculosis vaccine development. Lancet Respir Med. 2019;7(9):810-9.
7. Schrager LK, Vekemens J, Drager N, Lewinsohn DM, Olesen OF. The status of tuberculosis vaccine development. Lancet Infect Dis. 2020;20(3):e28-37.
8. Smith CM, Proulx MK, Olive AJ, Laddy D, Mishra BB, Moss C, et al. Tuberculosis susceptibility and vaccine protection are independently controlled by host genotype. MBio. 2016;7(5):1-13.
9. Lalor MK, Floyd S, Gorak-Stolinska P, Ben-Smith A, Weir RE, Smith SG, et al. BCG vaccination induces different cytokine profiles following infant BCG vaccination in the UK and Malawi. J Infect Dis. 2011;204(7):1075-85.
10. Abebe F. Is interferon-gamma the right marker for bacille Calmette-Guérin-induced immune protection? The missing link in our understanding of tuberculosis immunology. Clin Exp Immunol. 2012;169(3):213-9.
11. Jouanguy E, Lamhamedi-Cherradi S, Lammas D, Dorman SE, Fondanèche M-C, Dupuis S, et al. A human IFNGR1 small deletion hotspot associated with dominant susceptibility to mycobacterial infection. Nat Genet. 1999;21(4):370-8.
12. Wolday D, Kebede Y, Legesse D, Siraj DS, McBride JA, Kirsch MJ, et al. Role of CD4/CD8 ratio on the incidence of tuberculosis in HIV-infected patients on antiretroviral therapy followed up for more than a decade. PloS One. 2020;15(5):e0233049.
13. Geremew D, Melku M, Endalamaw A, Woldu B, Fasil A, Negash M, et al. Tuberculosis and its association with CD4+ T cell count among adult HIV positive patients in Ethiopian settings: a systematic review and meta-analysis. BMC Infect Dis. 2020;20(1):325.
14. Orme IM, Robinson RT, Cooper AM. The balance between protective and pathogenic immune responses in the TB-infected lung. Nat Immunol. 2014;16(1):57-63.
15. Neefjes J, Jongsma MLM, Paul P, Bakke O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nat Rev Immunol. 2011;11(12):823-36.

16. Xie Y-C, Qu Y, Sun L, Li H-F, Zhang H, Shi H-J, et al. Association between HLA-DRB1 and myasthenia gravis in a northern Han Chinese population. J Clin Neurosci. 2011;18:1524-7.

17. Liang C, Bencurova E, Psota E, Neurgaonkar P, Prelog M, Scheller C, et al. Population-Predicted MHC Class II Epitope Presentation of SARS-CoV-2 Structural Proteins Correlates to the Case Fatality Rates of COVID-19 in Different Countries. Int J Mol Sci. 2021;22(5).

18. Shorten A, Shorten B. What is meta-analysis? Evid Based Nurs. 2013;16(1):3-4.

19. Gurevitch J, Koricheva J, Nakagawa S, Stewart G. Meta-analysis and the science of research synthesis. Nature. 2018;555(7695):175-82.

20. GA Wells, B Shea, D O'Connell, J Peterson, V Welch, M Losos PT. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analyses. Ottawa Hospital Research Institute. 2009.

21. Stroup DF, Berlin JA, Morton SC, Olkin I, Williamson GD, Rennie D, et al. Meta-analysis of observational studies in epidemiology: a proposal for reporting. Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group. JAMA. 2000;283(15):2008-12.

22. Favorova OO, Favorov AV, Boiko AN, Andreewski T V, Sudomoina MA, Alekseenkov AD, et al. Three allele combinations associated with multiple sclerosis. BMC Med Genet. 2006;7(63):1-9.

23. Sterne JAC, Sutton AJ, Ioannidis JPA, Terrin N, Jones DR, Lau J, et al. Recommendations for examining and interpreting funnel plot asymmetry in meta-analyses of randomised controlled trials. BMJ. 2011;343(1):1-8.

24. Higgins JPT, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. BMJ. 2003;327(7414):557-60.

25. Passey, Mike Bunce B. HLA Typing by Sequence-Specific Primers. Methods Mol Biol, vol. 1034. Humana Press, Totowa, NJ; 2013. p. 313-8.

26. Li C-P, Zhou Y, Xiang X, Zhou Y, He M. Relationship of HLA-DRB1 gene polymorphism with susceptibility to pulmonary tuberculosis: updated meta-analysis. Int J Tuberc Lung Dis. 2015;19(7):841-9.

27. Tong X, Chen L, Liu S, Yan Z, Peng S, Zhang Y, et al. Polymorphisms in HLA-DRB1 Gene and the Risk of Tuberculosis: A Meta-analysis of 31 Studies. Lung. 2015;193(2):309-18.

28. Fletcher J. What is heterogeneity and is it important? BMJ. 2007;334(7584):94-6.

29. von Hippel PT. The heterogeneity statistic I(2) can be biased in small meta-analyses. BMC Med Res Methodol. 2015;15(35):1-8.

30. Oyarzun P, Kashyap M, Fica V, Salas-Burgos A, Gonzalez-Galarza FF, McCabe A, et al. A Proteome-Wide Immunoinformatics Tool to Accelerate T-Cell Epitope Discovery and Vaccine Design in the Context of Emerging Infectious Diseases: An Ethnicity-Oriented Approach. Front Immunol. 2021;12:598778.

Vaccine xxx (xxxx) xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Comparing cytokine production and clinical response following vaccination with BCG Moreau and BCG Russia strains in a Brazilian infant population

Julia Bitencourt [a], Alice Sarno [a], Carlos Oliveira [a], Ramon Andrade de Souza [b], Carla Cristina Lima [b], Iukary Takenami [c], Susan M. Pereira [b], Sérgio Arruda [a,d,*]

[a] Laboratório Avançado de Saúde Pública, Instituto Gonçalo Moniz, Fundação Oswaldo Cruz (IGM/Fiocruz), Salvador, Bahia 40296 710, Brazil
[b] Instituto de Saúde Coletiva, Universidade Federal da Bahia (UFBA), Salvador, Bahia 40110-040, Brazil
[c] Colegiado de Medicina, Universidade Federal do Vale do São Francisco (UNIVASF), Paulo Afonso, BA 48607 190, Brazil
[d] Departamento de Ciências da Vida, Universidade Estadual da Bahia (UNEB), Salvador, BA 41150 000, Brazil

ARTICLE INFO

Article history:
Received 20 November 2020
Received in revised form 23 March 2021
Accepted 15 April 2021
Available online xxxx

Keywords:
BCG strains
BCG vaccine
Infants
Cytokines

ABSTRACT

Introduction: BCG is the only licensed vaccine against tuberculosis (TB) and, in Brazil, comprises part of the recommended vaccine schedule within the first month of life. Due to a local manufacturing shortage of BCG Moreau, BCG Russia was introduced in 2017 by the Brazilian Ministry of Health.
Objective: To evaluate differences in immune responses induced by BCG Moreau and BCG Russia in infants, in addition to scar formation.
Methods: The present case series involved 15 healthy infants who were vaccinated within the first seven days of life with one of two strains of BCG, then followed for 12 weeks or longer. Cytokine levels were measured before and after vaccination in whole blood culture supernatants previously stimulated *in vitro* with either BCG strain, heat-killed *M. tuberculosis* H37Rv or in the absence of stimulation. BCG scarring was also documented.
Results: Infants vaccinated with BCG Moreau exhibited increased background IL-2, IL-10 and IL-4 production, yet no differences were found in those vaccinated with BCG Russia. Although both strains induced higher levels of IL-2 and IFN-γ, elevated IL-6, TNF and IL-10 production was also seen in response to BCG Russia. In contrast, no specific responses were observed against heat-killed *M. tuberculosis* H37Rv, with the exception of increased IL-2 following BCG Moreau vaccination. Although documented in both groups, scarring was milder and less frequent following BCG Russia vaccination.
Conclusions: Similar Th1 profiles were found following immunization with either type of BCG vaccine evaluated herein, with more pronounced cytokine production detected in response to the Russia strain. Overall, vaccination was well-tolerated and scarring evolved as expected for both BCG strains.

1. Introduction

The Bacillus Calmette-Guérin (BCG) vaccine was developed by Léon Charles Albert Calmette and Jean-Marie Camille Guérin almost 100 years ago, and today it remains one of the main measures to prevent tuberculosis (TB) disease. Despite its important role in protecting against several forms of TB, a previous study estimated that this vaccine offers limited protection against the pulmonary form, varying between 0 and 80% [1].

Although many low-incidence countries, such as the United States, do not routinely vaccinate with BCG due low protective efficacy following immunization, several countries in Asia, Europe, Africa, Central and South America still consider BCG as a strategy to reduce morbidity and mortality against severe forms of TB [2]. In Brazil, the vaccine is part of the Brazilian Ministry of Health's National Immunization Program (PNI-MS) and is administered intradermally in the first month of life. The effectiveness of BCG against pulmonary TB in school-age children has been estimated at 29% (95% CI: 548%), ranging from 21% (−31 to 53%) to 32%%

* Corresponding author.
E-mail addresses: juliabcp@hotmail.com (J. Bitencourt), sarnoalice@gmail.com (A. Sarno), juniorasoliveira@gmail.com (C. Oliveira), ramon.andrade.souza@gmail.com (R.A.d. Souza), carlaenf78@gmail.com (C.C. Lima), iukary.takenami@univasf.edu.br (I. Takenami), susanmartins@gmail.com (S.M. Pereira), sergio.arruda@fiocruz.br (S. Arruda).

https://doi.org/10.1016/j.vaccine.2021.04.028
0264-410X/

(3–52%) in the capital cities of Manaus and Salvador, respectively [3].

No consensus exists in the literature surrounding the protective mechanism induced by the BCG vaccine [4], primarily because genetic variations in BCG strains may induce different host immune responses. While BCG strains have shown distinct patterns in terms of genetic and immunogenic properties, little is known about how these differences affect the protection induced by vaccination [25]. In addition, other factors may influence vaccine protection, such as environmental mycobacteria presensitization, methodological differences in vaccine preparation, biological variations among the different strains of BCG and, consequently, discordant immunogenic properties among strains [5-8].

Since the 1920s, the BCG Moreau vaccine strain has been produced and administered in Brazil. However, in 2017, the Ataulpho Paiva Foundation (FAP) discontinued the manufacture of BCG Moreau. Thus, the Ministry of Health replaced BCG Moreau with BCG Russia, which is produced by the Serum Institute of India (SII). Although both strains are classified as early strains and are assumed to be very genetically similar, BCG Russia had never been used in Brazil prior to 2018 [5,9].

As the efficacy of the BCG Russia strain in the Brazilian population remains unknown, the present study aimed to evaluate differences between these two BCG strains, focusing on the modulation of cytokine response in infants in an attempt to enhance the understanding of immune response against BCG vaccination in Brazil. We additionally discuss the impacts of our findings with respect to immunogenicity and scarring.

2. Materials and Methods

2.1. Study design

A case series study was carried out to investigate differences in cytokine production patterns in healthy early-vaccinated newborns, comparing the two BCG strains evaluated (BCG Moreau and BCG Russia). Newborns were vaccinated at the José Maria de Magalhães Neto Maternity Hospital in Salvador, Brazil from December 2018 to February 2019, and then followed from March to May 2019. Infants received either BCG Moreau or BCG Russia in their first week of life, depending on the vaccination scheduling at the maternity hospital (alternate days). While the PNI-MS recommends vaccination within the first month of life, neonates are customarily vaccinated before leaving the maternity hospital unit. The present single-blinded study employed no randomization.

2.2. Study population

The study population included newborns aged < 7 days, with a birth weight > 2 kg and no previous BCG vaccination. Exclusion criteria included: evidence of pulmonary or extrapulmonary TB in either mothers or infants; use of any immunomodulatory drug by the mother during pregnancy or while breastfeeding; mother's positive human immunodeficiency virus (HIV) infection status; any apparent or diagnosed congenital disease, or any other acute or chronic disease in both mother and newborn. This study was approved by the Institutional Review Board of the Federal University of Bahia (CEUA-UFBA) (protocol number: 2.850.322), and written informed consent was obtained from all parents or legal guardians. This study was part of a larger research project aimed at evaluating the effectiveness of the first dose of BCG Russia against TB.

After providing written consent, all parents or legal guardians were invited to answer a questionnaire, and newborn were submitted to peripheral blood collection (Pre-BCG). During a three-month follow-up, weekly or semi-weekly home visits were carried out to examine scar formation, and a second blood sample was collected during the last visit (Post-BCG). No more than three attempts to collect blood were performed at each timepoint, and repeated failures led to the interruption of this procedure and consequent infant exclusion from the study. To minimize loss to follow-up, team members kept in direct contact with parents before and after home visits by cellphone.

2.3. Vaccination and scar evaluation

All BCG Moreau (BCG Moreau RDJ, FAP) and BCG Russia (Russian BCG-I, SII) vaccines were provided by the Brazilian Ministry of Health, located 26 km from the state capital, Salvador. All vaccinations were administered via intradermal route in the left upper arm with 0.1 mL containing 2×10^5 CFU/mL of BCG Moreau (lot number: 6209) or 0.05 mL containing 1×10^5 to 4×10^5 CFU/mL of BCG Russia (lot number: 037G6200). BCG scar formation was measured and photographed during home visitations by a single observer. When home visits were not possible, digital photos were taken by parents and sent to the team electronically. Thus, in-person or *online* follow-up was employed to periodically document scar formation and healing until 12 weeks after immunization, or longer if necessary to obtain complete scar tissue formation. All pictures were first assessed by a single team member, and initial observations were then confirmed by an additional collaborator.

2.4. Blood collection and in vitro assays

Whole blood (approximately 2 mL) samples were collected in heparinized tubes. All samples were stored at room temperature for up to 2 h before performing *in vitro* assays and then diluted in culture medium (1:5) as previously described [10]. Total diluted volume was distributed on 96-well U-bottom plates (200 μL/well). Depending on the available blood volume, approximately eight wells were stimulated under each of the following conditions: negative control (media and whole blood only), heat-killed *M. tuberculosis* H37Rv (2×10^3 CFU/mL) and the same BCG strain (Moreau or BCG Russia, (2×10^3 CFU/mL) used for vaccination, resuspended in culture media. It was not possible to investigate cross-stimulation between BCG Moreau and BCG Russia (one strain used in vaccination and another strain used to stimulate *in vitro* cultures) due to limited blood collection volume. Following 72 h of incubation at 37 °C under 5% CO_2, culture supernatants were collected, distributed in two aliquots and stored at −20 °C until further analysis.

2.5. Cytokine quantification

Cytokine levels were measured in whole blood culture supernatants using a BD™ Cytometric Bead Array (CBA) kit (BD Biosciences, San Diego, CA), according to the manufacturer's instructions. A sample volume of 50 μL was used in this assay to quantify IL-2 (lower detection limit: 2.6 pg/mL), IL-4 (lower detection limit: 4.9 pg/mL), IL-6 (lower detection limit: 2.4 pg/mL), IL-10 (lower detection limit: 4.5 pg/mL), IFN-γ (lower detection limit: 3.7 pg/mL), TNF (lower detection limit: 3.8 pg/mL) and IL-17A (lower detection limit: 18.9 pg/mL). The upper detection limit was 5000 pg/mL for all cytokines. By applying the 4- parameter curve fit option for cytokine quantification, it was possible to extrapolate sample intensity values that did not fall within the limits of the standard curve, due to the complexity and kinetics of this multi-analyte assay. All samples were processed simultaneously to avoid inter-experimental variation. Data were acquired using a BD™ LRSFortessa flow cytometer and processed with FlowJo software 10.4.0 (TreeStar, San Carlos, CA, USA).

2.6. Statistical analysis

All data were analyzed using GraphPad Prism v.8.0 software (GraphPad Inc., San Diego, CA). The D'agostino-Pearson test was performed to evaluate sample distribution. The Wilcoxon signed-rank test was used to determine statistical significance in non-parametric paired samples, while categorical variables were assessed using Fisher's exact test. Dunn's multiple comparison test was employed. The level of statistical significance was set at 5%.

3. Results

3.1. Participant characteristics

Between December 2018 and February 2019, 36 newborns were vaccinated with either BCG Moreau or BCG Russia at a maternity hospital. Of these, 13 (36.1%) newborns were excluded: four due to unsuccessful phlebotomy and nine due to an insufficient amount of blood collected, leaving a total of 23 (63.9%) newborns enrolled in this study: seven (19.4%) were vaccinated with BCG Moreau, and 16 (44.4%) with BCG Russia. However, during follow-up, eight (22.2%) infants were excluded from the final analysis: one moved outside the municipality, six were lost to follow-up and one due to difficulties in conducting blood collection procedures. The study flowchart is described in Fig. 1.

Table 1 lists the clinical and demographic characteristics of the 15 infants enrolled in the study. No statistical differences were found with regard to sex, age, weight or height between the groups ($p > 0.05$). The presence or absence of BCG scarring was recorded to determine maternal vaccination status, with scars observed in all six (100%) and eight (88.9%) mothers of infants vaccinated with BCG Moreau and BCG Russia, respectively. In addition, three (50%) mothers of infants vaccinated with BCG Moreau reported having previous contact with patients diagnosed with pulmonary TB ($p = 0.044$).

Table 1
Clinical and demographic characteristics of newborns immunized with BCG Moreau or BCG Russia.

Characteristics	BCG Moreau N = 6	BCG Russia N = 9	*p-value*
Sex, *n* (%)			
Female	4 (66.6)	3 (33.3)	0.314
Male	2 (33.3)	6 (66.6)	
Age at vaccination (days)			0.547
Mean ± SD	3.50 ± 1.04	3.11 ± 0.78	
Median (IQR)	3.5 (2.75–4.25)	3.0 (2.5–4.0)	
Weight (g)			0.437
Mean ± SD	3,080 ± 326.7[#]	3,310 ± 525.9	
Median (IQR)	3,065 (2,760–3,408)[#]	3,255 (2,840–3,790)	
Height (cm)			0.816
Mean ± SD	49.20 ± 1.92[#]	49.13 ± 2.53[#]	
Median (IQR)	49 (47.5–51.0)[#]	49.5 (48.2–50.0)[#]	
Maternal BCG scar, *n* (%)	6 (100)	8 (88.9)	>0.999
Maternal TB exposure, *n* (%)			
Yes	3 (50)	–	**0.044**
No	3 (50)	9 (100)	

[#] Data not available from one newborn; SD = standard deviation; IQR = interquartile range; g = grams; cm = centimeter.

3.2. In vitro cytokine response before and after BCG vaccination

The background immune responses of infants were assessed by detecting cytokine levels from the supernatants of unstimulated whole blood cultures pre- and post-BCG vaccination. After three months, infants vaccinated with BCG Moreau showed an increase in the production of cytokines IL-2, IL-10 and IL-4 ($p = 0.031$), which was not observed in the BCG Russia vaccinated infants. There was no significant difference in the other cytokine levels measured ($p > 0.05$) (Fig. 2 and Table S1).

The infants' whole blood cells before and after vaccination were exposed to the corresponding vaccine strain (BCG Moreau or BCG

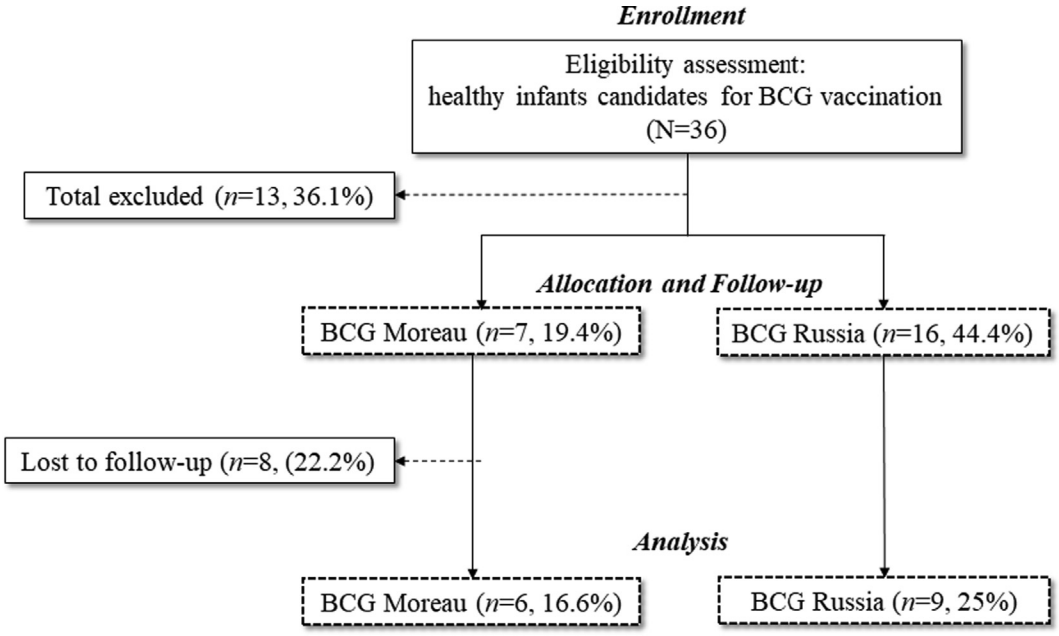


Fig. 1. Representative flowchart detailing study design.

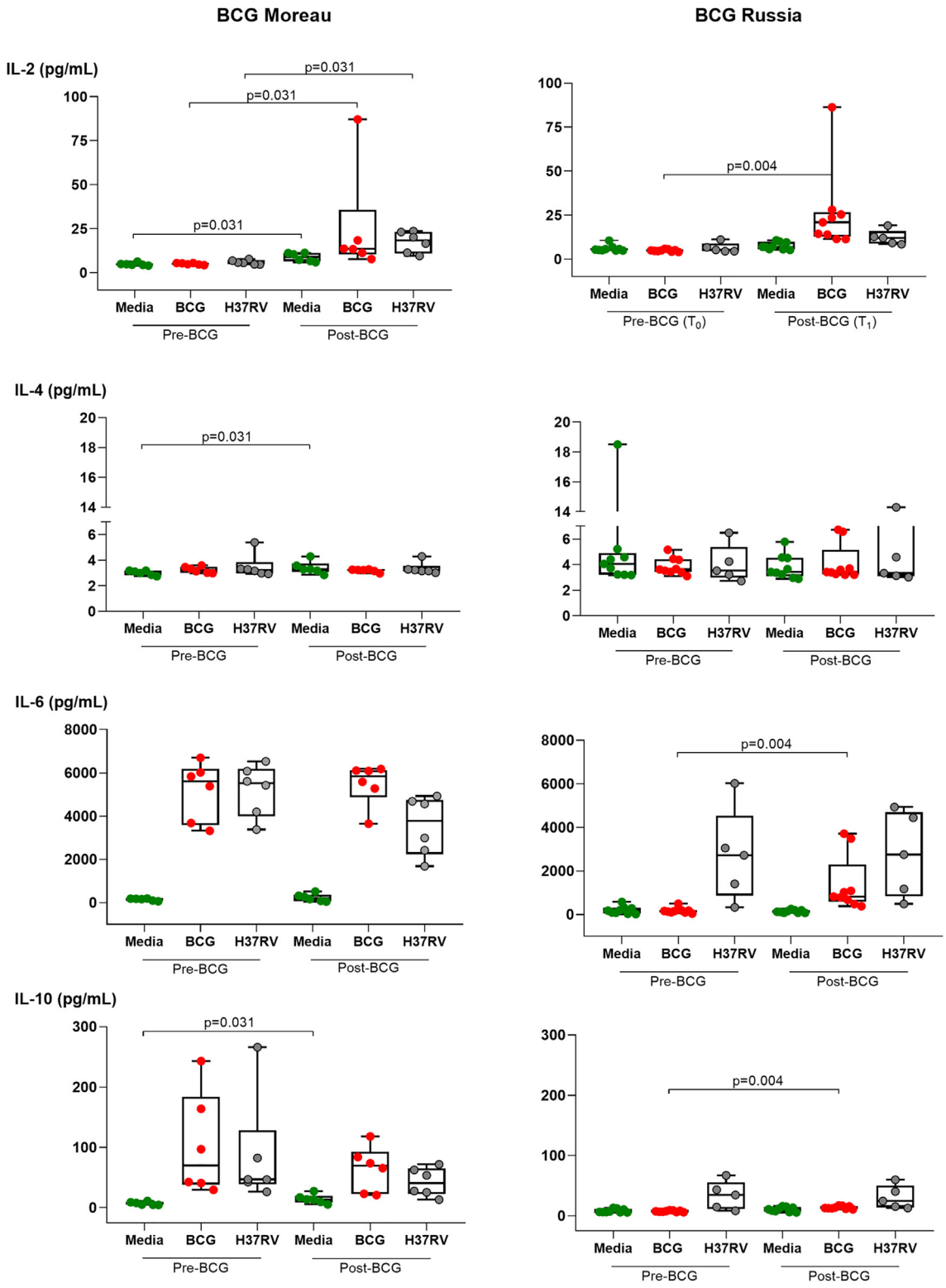


Fig. 2. Cytokine production induced in whole blood cultures under stimulation with BCG strain (red) and H37Rv (light grey) before and after vaccination with BCG Moreau (left) or BCG Russia (right). Medium and whole blood alone were used as negative controls (green). Box plots represent median values and first and third quartiles, while whiskers represent minimum and maximum values. Scatter plots detail individual infants represented by circles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Russia) and to H37Rv. Re-exposure of blood cells from BCG Moreau group to BCG *in vitro* increased the production of IL-2 and IFN-γ (p = 0.031). The same result was observed in BCG Russia group: IL-2 and IFN-γ (p = 0.004). In addition, higher levels of IL-6, TNF and IL-10 were only observed with BCG Russia bacilli *in vitro* stimulus (p = 0.004). Cytokines IL-4 and IL-17a did not change pre and post-vaccination (p = 0.800 and p = 0.796, respectively) (Fig. 2 and Table S2).

The immune response to *M. tuberculosis* was evaluated in whole blood cell cultures in the presence of heat-killed H37Rv stimuli.

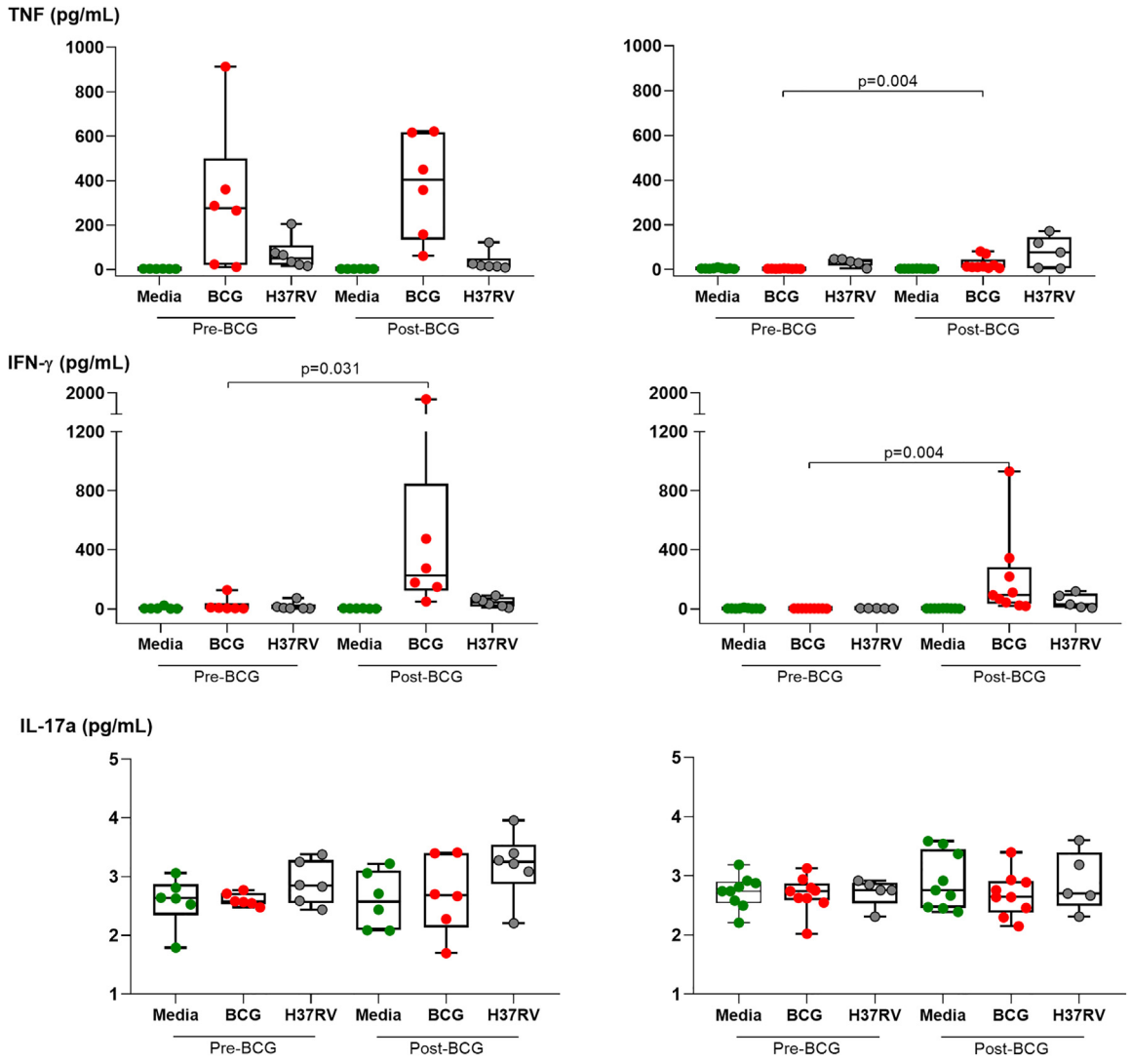


Fig. 2 (*continued*)

With the exception of increased levels of IL-2 following BCG Moreau vaccination (p = 0.031), cytokine quantification remained unchanged over time. Although not statistically significant, higher levels of IL-6 were seen prior to vaccination in the BCG Moreau group (Fig. 2 and Table S3).

3.3. BCG scar evolution

The evolution of scar formation after vaccination with BCG Moreau or BCG Russia is illustrated in Fig. 3. Overall, both vaccines induced initial papules and nodules in the majority of infants, followed by pustules and ulcers, which were later covered by a crust that evolved to a small fibrotic scar. The evolution from smaller papules to scars is characteristic of BCG vaccination, and results from the intradermal administration of the vaccine. In one infant, NB07, the papule observed (Fig. 3A) was less-defined than in the other two infants vaccinated with BCG Moreau. Conversely, NB31 did not present a papule, only a small point of bleeding, after vaccination with BCG Russia (Fig. 3B). In addition, NB17, NB20 and NB31 presented smaller scars, which disappeared after 24 weeks of follow-up. Due to the poor quality of the images obtained, we did not perform in-depth analysis.

4. Discussion

The efficacy of BCG has been a topic of discussion for many years, and several factors are known to influence the degree of protective immune response [7]. One hypothesis posits that genetic differences between vaccine strains, which affect metabolism and immunogenicity, may reduce a given vaccine's capacity to induce protection [5,11]. However, comprehensive testing of this hypothesis and the conduct of comparisons between immune responses induced by BCG strains poses significant challenges, largely due to different methodologies used to assess efficacy, regions and divergences between study populations, variability in levels of exposure to environmental mycobacteria, as well as the use of different BCG strains in vaccination campaigns worldwide.

The background immune response, represented here by non-stimulated cultures, revealed an increase in cytokine production associated with a regulatory effect following BCG Moreau vaccination. The impact that aging, among other factors, could have on cytokine production has been well-described [12,13]. Some authors have argued that prior to receiving any vaccination, all newborns exhibit similar cytokine responses. It is important to consider that age at the time of vaccination may influence immune response[20]. A previous study demonstrated that early vaccina-

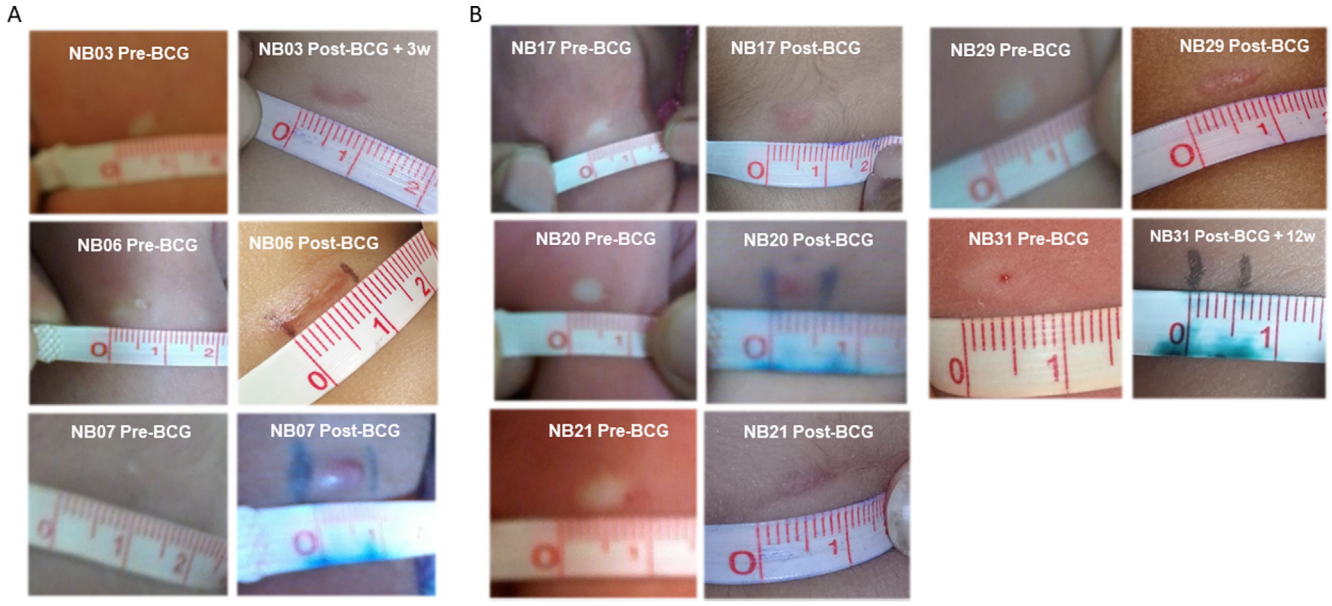


Fig. 3. Images illustrating the evolution of scarring following BCG vaccination. A: newborns vaccinated with BCG Moreau; B: newborns vaccinated with BCG Russia. NB = newborn; W = weeks.

tion induces a broad mycobacterial immune response characterized by a Th1/Th2/IL-17/Treg profile, with significant reductions seen in Th1/Th-17 responses due to the administration of BCG at later times. As all of the infants evaluated herein were vaccinated within the first seven days of life, most therefore exhibited a similar background pattern at baseline [14].

A study published by Raes et. al (2010) found no differences between cytokine quantification at two and six months in cryopreserved serum samples. In contrast, similar to the present results, higher IL-10 levels have been found in infants compared to children with higher environmental exposure early in life [15]. Previous maternal contact with TB might also have influenced the results shown here, as we cannot exclude the possible fetal antigen priming through the placenta – earlier demonstrated in mice [16]. In addition, it is important to consider that making comparisons between study populations do not always yield reliable results, as study design, participant age, differing methodologies and time elapsed between blood collection and *in vitro* culturing procedures tend to vary between studies.

To investigate immune responses induced by BCG Moreau and BCG Russia, cytokine production was quantified, before and after vaccination, in culture supernatants stimulated by each strain. High levels of IL-2 and IFN-γ were found in both groups at similar levels after vaccination, suggesting that both strains shared similarities and that genetic differences between these two strains may not influence the classical Th1 response induced by BCG vaccination [7]. Nonetheless, many studies have noted BCG Russia to be less immunogenic than other strains [11,17]. Although fundamental to the immunopathology of TB, classical IFN-γ production in response to BCG does not reach a sufficient level to offer protection against disease [18,19]. Despite close phylogenic similarities between BCG Moreau and BCG Russia, our results evidenced a more prominent pro-inflammatory cytokine profile associated with BCG Russia, including IL-6 and TNF. These findings are in agreement with the innate immune response maturation shown by Shey et. al (2014).

Most demographic characteristics were similar between groups, except for previous maternal contact with TB in the BCG Moreau group. However, it is important to consider that infants vaccinated with BCG Moreau may have been influenced by the mother's immunity. An association has been reported between latent tuberculosis infection (LTBI) in mothers and a diminished response of IFN-γ-expressing CD4 + T cells in newborns, together with decreased levels of purified protein derived (PPD)-specific antibodies [21]. Further assessment identified a down-regulation in IFN-γ and inflammatory pathways at one week in infants born to LTBI mothers, while an unexpected up-regulation was observed at six weeks when compared to infants from non-exposed mothers [22]. We hypothesize that our finding of increased IL-4 in unstimulated cultures may also affect the Th1 response against BCG in the group vaccinated with strain Moreau, similarly to findings reported by Ritz et al [23].

Despite our efforts to gain insight into differential immune responses induced by two strains of BCG against *M. tuberculosis*, we found no differences over time between the newborns vaccinated with BCG Moreau compared to BCG Russia. Ritz et. al (2012) described similar results under *in vitro* stimulation with *M. tuberculosis,* evidencing no changes in cytokine production by $CD4^+$ or $CD8^+$ T cells. The fact that the mycobacteria used in these vaccines were inactivated by heat may have influenced the quantity of cytokines produced, as the live bacillus is capable of inducing higher cytokine levels compared to heat-killed bacteria [24].

According to Santiago et al. (2003), BCG scarring can be used as a proxy to determine the occurrence of vaccination from the first month of life until three years later [26]. However, the absence of scarring has been related to an increased risk of *M. tuberculosis* infection in children living in endemic regions, who are therefore more likely to become infected [27]. Herein, although no statistical evaluations were performed regarding the size of lesions caused by vaccination with BCG, we did observe less severe scarring in infants vaccinated with the BCG Russia strain.

In a study carried out in Uganda, the proportion of children who developed a scar following vaccination was different among groups vaccinated with different strains of BCG: vaccination with BCG Russia resulted in scar formation in 52.2% of the subjects, while BCG Bulgaria and BCG Denmark were associated with scarring in 64.1% and 92.6%, respectively. Another study revealed that the BCG Russia vaccine also induced a significantly less extensive local reaction (median 2 mm; IQR, 1–4 mm) ($p = 0.001$) than BCG Denmark (median 5 mm; IQR 4–7 mm) or BCG Japan (median 5 mm;

IQR, 3–7 mm) [17]. Although we were unable to confirm these associations between vaccine strains and scar formation, these findings, together with the present results, support the notion that scar formation is influenced by the strain of vaccine administered.

The present study is limited by its small sample size, which impedes the generalization of our findings to the regional population at large. However, while a well-designed larger non-inferiority study comparing BCG Moreau and BCG Russia is undoubtedly the best approach, the present exploratory study does offer preliminary insight into immune response induced by each of these two strains of BCG, and further immunological assessments on antibody production and immunophenotyping would be desirable. Importantly, vaccine effectiveness and TB incidence calculations will be performed in an even larger population from which the present sample was selected (ongoing study).

5. Conclusions

The present study attempted to compare the impact caused by a change in the BCG strain used in Brazil. The immune responses of infants vaccinated with BCG Moreau and BCG Russia were compared under different experimental conditions.

Despite minor genetic variations among early strains, similar cytokine profiles were observed following vaccination with BCG Moreau and BCG Russia. While both strains stimulated a classical Th1 pattern, the *in vitro* induction of inflammatory cytokines was more pronounced in response to BCG Russia. In contrast, BCG scarring was more evident after vaccination with BCG Moreau, as BCG Russia resulted in less severe skin lesions. Thus, we conclude that the substitution of BCG Russia in place of BCG Moreau represents a practical solution for overcoming local manufacturing shortages in the production of BCG Moreau.

CRediT authorship contribution statement

Julia Bitencourt: Writing - original draft, Writing - review & editing, Methodology, Data curation, Formal analysis, Conceptualization. **Alice Sarno:** Methodology, Writing - review & editing. **Carlos Oliveira:** Data curation, Methodology, Writing - review & editing. **Ramon Andrade de Souza:** Project administration, Investigation, Writing - review & editing. **Carla Cristina Lima:** Project administration, Resources. **Iukary Takenami:** Supervision, Visualization, Writing - review & editing. **Susan M. Pereira:** Investigation, Resources, Conceptualization, Writing - review & editing. **Sérgio Arruda:** Investigation, Resources, Conceptualization, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to thank our technical team who provided substantial support to our project, especially Juliane Amorim. We would also like to thank all of the families, and especially the infants who participated. The authors are grateful to Andris K. Walter for English language revision and manuscript copyediting services.

Funding

This study received financial support from the Brazilian Ministry of Health and was also financed in part by the Coordenação de Aperfeiçgoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. The authors would like to acknowledge the support of CNPq and FAPESB for providing student scholarships.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2021.04.028.

References

[1] Mangtani P, Abubakar I, Ariti C, Beynon R, Pimpin L, Fine PEM, et al. Protection by BCG vaccine against tuberculosis: A systematic review of randomized controlled trials. Clin Infect Dis 2014;58:470–80. https://doi.org/10.1093/cid/cit790.

[2] Pereira SM, Dantas OMS, Ximenes R, Barreto ML. BCG vaccine against tuberculosis: Its protective effect and vaccination policies. Rev Saude Publica 2007;41:59–66. https://doi.org/S0034-89102007000800009 [pii].

[3] Pereira SM, Barreto ML, Pilger D, Cruz AA, Sant'Anna C, Hijjar MA, et al. Effectiveness and cost-effectiveness of first BCG vaccination against tuberculosis in school-age children without previous tuberculin test (BCG-REVAC trial): A cluster-randomised trial. Lancet Infect Dis 2012;12:300–6. https://doi.org/10.1016/S1473-3099(11)70285-7.

[4] Kagina BMN, Abel B, Scriba TJ, Hughes EJ, Keyser A, Soares A, et al. Specific T cell frequency and cytokine expression profile do not correlate with protection against tuberculosis after bacillus Calmette-Guérin vaccination of newborns. Am J Respir Crit Care Med 2010;182:1073–9. https://doi.org/10.1164/rccm.201003-0334OC.

[5] Abdallah AM, Hill-Cawthorne GA, Otto TD, Coll F, Guerra-Assunção JA, Gao G, et al. Genomic expression catalogue of a global collection of BCG vaccine strains show evidence for highly diverged metabolic and cell-wall adaptations. Sci Rep 2015;5:1–15. https://doi.org/10.1038/srep15443.

[6] Ponte C, Hacker M, Moraes M, Castello-Branco L, Silva F, Antas P. The patterns of in vitro cell-death and inflammatory cytokines induced by distinct BCG vaccine strains are differentially induced in human mononuclear cells. Hum Vaccines Immunother 2017;14:1–8. https://doi.org/10.1080/21645515.2017.1382788.

[7] Dockrell HM, Smith SG. What have we learnt about BCG vaccination in the last 20 years?. Front Immunol 2017;8.. https://doi.org/10.3389/fimmu.2017.01134.

[8] Angelidou A, Conti MG, Diray-Arce J, Benn CS, Shann F, Netea MG, et al. Licensed Bacille Calmette-Guérin (BCG) formulations differ markedly in bacterial viability, RNA content and innate immune activation. Vaccine 2020;38:2229–40. https://doi.org/10.1016/j.vaccine.2019.11.060.

[9] Ritz N, Curtis N. Mapping the global use of different BCG vaccine strains. Tuberculosis 2009;89:248–51. https://doi.org/10.1016/j.tube.2009.03.002.

[10] Smith SG, Kleinnijenhuis J, Netea MG, Dockrell HM. Whole Blood Profiling of Bacillus Calmette–Guérin-Induced Trained Innate Immunity in Infants Identifies Epidermal Growth Factor, IL-6, Platelet-Derived Growth Factor-AB/BB, and Natural Killer Cell Activation. Front Immunol 2017;8:1–11. https://doi.org/10.3389/fimmu.2017.00644.

[11] Ritz N, Dutta B, Donath S, Casalaz D, Connell TG, Tebruegge M, et al. The influence of bacille Calmette-Guérin vaccine strain on the immune response against tuberculosis: A randomized trial. Am J Respir Crit Care Med 2012;185:213–22. https://doi.org/10.1164/rccm.201104-0714OC.

[12] Decker M-L, Grobusch MP, Ritz N. Influence of Age and Other Factors on Cytokine Expression Profiles in Healthy Children—A Systematic Review. Front Pediatr 2017:5. https://doi.org/10.3389/fped.2017.00255.

[13] Figueiredo CA, Alcântara-Neves NM, Veiga R, Amorim LD, Dattoli V, Mendonça LR, et al. Spontaneous cytokine production in children according to biological characteristics and environmental exposures. Environ Health Perspect 2009;117:845–9. https://doi.org/10.1289/ehp.0800366.

[14] Burl S, Adetifa UJ, Cox M, Touray E, Ota MO, Marchant A, et al. Delaying Bacillus Calmette-Guérin Vaccination from Birth to 4 1/2 Months of Age Reduces Postvaccination Th1 and IL-17 Responses but Leads to Comparable Mycobacterial Responses at 9 Months of Age. J Immunol 2010;185:2620–8. https://doi.org/10.4049/jimmunol.1000552.

[15] Decker ML, Gotta V, Wellmann S, Ritz N. Cytokine profiling in healthy children shows association of age with cytokine concentrations. Sci Rep 2017;7.. https://doi.org/10.1038/s41598-017-17865-2.

[16] Rahman MJ, Dégano IR, Singh M, Fernández C. Influence of maternal gestational treatment with mycobacterial antigens on postnatal immunity in an experimental murine model. PLoS ONE 2010. https://doi.org/10.1371/journal.pone.0009699.

[17] Anderson EJ, Webb EL, Mawa PA, Kizza M, Lyadda N, Nampijja M, et al. The influence of BCG vaccine strain on mycobacteria-specific and non-specific

immune responses in a prospective cohort of infants in Uganda. Vaccine 2012;30:2083–9. https://doi.org/10.1016/j.vaccine.2012.01.053.

[18] Abebe F. Is interferon-gamma the right marker for bacille Calmette-Guérin-induced immune protection? The missing link in our understanding of tuberculosis immunology. Clin Exp Immunol 2012;169:213–9. https://doi.org/10.1111/j.1365-2249.2012.04614.x.

[19] Tameris MD, Hatherill M, Landry BS, Scriba TJ, Snowden MA, Lockhart S, et al. Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: A randomised, placebo-controlled phase 2b trial. Lancet 2013;381:1021–8. https://doi.org/10.1016/S0140-6736(13)60177-4.

[20] Shey MS, Nemes E, Whatney W, de Kock M, Africa H, Barnard C, et al. Maturation of Innate Responses to Mycobacteria over the First Nine Months of Life. J Immunol 2014;192:4833–43. https://doi.org/10.4049/jimmunol.1400062.

[21] Mawa PA, Nkurunungi G, Egesa M, Webb EL, Smith SG, Kizindo R, et al. The impact of maternal infection with Mycobacterium tuberculosis on the infant response to bacille Calmette-Guérin immunization. Philos Trans R Soc B Biol Sci 2015:370. https://doi.org/10.1098/rstb.2014.0137.

[22] Mawa PA, Webb EL, Filali-Mouhim A, Nkurunungi G, Sekaly RP, Lule SA, et al. Maternal BCG scar is associated with increased infant proinflammatory immune responses. Vaccine 2017;35:273–82. https://doi.org/10.1016/j.vaccine.2016.11.079.

[23] Ritz N, Hanekom WA, Robins-Browne R, Britton WJ, Curtis N. Influence of BCG vaccine strain on the immune response and protection against tuberculosis. FEMS Microbiol Rev 2008;32:821–41. https://doi.org/10.1111/j.1574-6976.2008.00118.x.

[24] Ordway DJ, Arroz MJ, Fernandis A, Dockrell HM, Ventura FA. Respostas Th1 e Th2 desencadeadas por Mycobacterium tuberculosis virulento em doentes com tuberculose pulmonar. Rev Port Pneumol 1998;4:393–402. https://doi.org/10.1016/s0873-2159(15)31062-x.

[25] Hayashi D, Takii T, Fujiwara N, Fujita Y, Yano I, Yamamoto S, et al. Comparable studies of immunostimulating activities in vitro among Mycobacterium bovis bacillus Calmette-Guérin (BCG) substrains. FEMS Immunol Med Microbiol 2009;56:116–28. https://doi.org/10.1111/j.1574-695X.2009.00559.x.

[26] Santiago EM, Lawson E, Gillenwater K, Kalangi S, Lescano AG, Du Quella G, et al. A prospective study of bacillus Calmette-Guérin scar formation and tuberculin skin test reactivity in infants in Lima, Peru. Pediatrics 2003;112.. https://doi.org/10.1542/peds.112.4.e298.

[27] Dhanawade S, Kumbhar S, Gore A, Patil V. Scar formation and tuberculin conversion following BCG vaccination in infants: A prospective cohort study. J Fam Med Prim Care 2015;4:384. https://doi.org/10.4103/2249-4863.161327.