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DEPARTAMENTO DE MEDICINA

Alex Ricardo Ferreira

Expressão de Linfócitos T Multifuncionais induzidos por antígenos de *Mycobacterium leprae* em pacientes com hanseníase e controles contactantes

Aracaju
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Trabalho de Conclusão de Curso apresentado ao colegiado do curso de Medicina da Universidade Federal de Sergipe, como requisito parcial para obtenção do título de bacharel em Medicina.
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Autor: Alex Ricardo Ferreira

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LISTA DE ABREVIATURAS E SIGLAS

APC	Célula apresentadora de antígenos
ENH	Eritema Nodoso Hansênico
HD	Hanseníanse Dimorfa
HDD	Hanseníase Dimorfa-dimorfa
HDT	Hanseníase Dimorfa- tuberculóide
HDV	Hanseníase Dimorfa- virchowiana
HI	Hanseníase indeterminada
HT	Hanseníase Tuberculóide
HV	Hanseníase Virchowiana
IB	Índice baciloscópico
IFN	Interferon
IL	Interleucina
M. leprae	*Mycobacterium leprae*
MB	Multibacilar
MS	Ministério da Saúde
NK	Natural-Killer
OMS	Organização Mundial da Saúde
PB	Paucibacilar
PQT	Poliquimioterapia
RH	Reações hansênicas
RIC	Resposta imune celular
RR	Reação Reversa

Th	T helper
TNF	Fator de Necrose Tumoral
T-reg	Linfocito T regulador

SUMÁRIO

RESUMO

FERREIRA, A. R. 2018. **Expressão de Linfócitos T Multifuncionais induzidos por antígenos de *Mycobacterium leprae* em pacientes com hanseníase e controles contactantes**.

A Hanseníase é uma doença infecciosa crônica e de evolução lenta causada pelo *Mycobacterium leprae*. Na população geral, menos de 1% dos indivíduos infectados pelo bacilo evolui com a doença. Diversos autores reportam que o padrão genético e diferenças nos mecanismos da resposta imune do paciente influenciam na susceptibilidade ou resistência à infecção. Os linfócitos T CD4 multifuncionais são células capazes de secretar simultaneamente combinações de IFN-γ, IL-2 e TNF-α. Estudos recentes vêm reportando o papel dessas células na produção de resposta imune efetora e memória contra alguns patógenos. A expressão dessas células é utilizada com parâmetro para avaliar a eficácia de antígenos candidatos as vacinas. Diante disso, este estudo teve como objetivo avaliar o papel de antígenos (bruto e recombinante) de *M. leprae e M. tuberculosis* na expressão de células T multifuncionais, como perspectiva para o desenvolvimento de ferramentas de imunoprofilaxia. Para tanto, avaliamos a resposta imune específica a antígenos brutos e recombinante (MLCS, PPD e ML2028). PBMC de pacientes com hanseníase e de CCS foram estimuladas com os antígenos e o perfil de citocinas e o fenótipo das células T $CD4^+$ e $CD8^+$ multifuncionais (produtoras de IFN-γ, IL-2 ou TNF-α) de memória efetora e central foram analisados. As análises multiparamétricas por citometria de fluxo revelaram maior frequência de células T multifuncionais antígeno-específicas em CCS, do que em pacientes com hanseníase. Nossos dados indicam que controles contactantes, quando estimulados com antígenos recombinantes, produziram mais células T multifuncionais e isto sugere que estas células proporcionam uma resposta imunológica mais eficaz contra a infecção por *M. leprae*, o que previne o desenvolvimento da hanseníase. Estes dados abrem perspectivas para o desenvolvimento futuro de imunoprofilaxia com estes antígenos de *M. leprae*, buscando induzir a expressão de células T multifuncionais e respostas Th1 em indivíduos em risco de adquirir a doença, mesmo que haja uma proteção parcial, pois neste caso haveria uma proteção contra formas MB, reduzindo a transmissão da doença.

Palavras-chaves: Hanseníase; Imunopatogênese; Células T Multifunctionais; Antígenos Recombinantes.

ABSTRACT

FERREIRA, A. R. 2018. **Multifunction T Lymphocytes expression, stimulated by *Mycobacterium leprae* antigens, in leprosy patients and household contacts.**

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*. It is estimated that less than 1% of the individuals infected with *M. leprae* develop the disease. Several authors suggest that the genetic pattern and variations in the mechanisms of the patient's immune response influence the susceptibility or resistance to disease. Multifunctional CD4 T lymphocytes are cells capable of simultaneously secreting combinations of IFN-γ, IL-2 and TNF-α. Recent studies have reported the role of these cells in the production of effector immune response and memory against several pathogens. Expression of these cells is used as a parameter to evaluate the efficacy of candidate vaccine antigens. The objective of this study was to evaluate the role of antigens (crude and recombinant) of *M. leprae* and *M. tuberculosis* in the expression of multifunctional T cells as a perspective for the development of immunoprophylaxis tools. Then, to evaluate the specific immune response to crude and recombinant antigens (MLCS, PPD and ML2028), PBMC from leprosy patients and HHC were stimulated with these antigens, and the cytokine profile and the CD4+ and CD8+ multifunctional T cells (producing IFN-γ, IL-2 or TNF-α) of effector and central memory were analyzed. Multiparameter analyzes by flow cytometry revealed a higher frequency of multifunctional T cells specific for *M. leprae* antigens in HHC than in leprosy patients. Our data indicate that HHC, when stimulated with crude and recombinant antigens, expressed more multifunctional T cells and this suggests that these cells provide a more effective immune response against *M. leprae* infection, which prevents the development of leprosy. These data open perspectives for the future development of immunoprophylaxis with *M. leprae* antigens, seeking to induce multifunction T cells expression and a Th1 immune response in individuals at risk of acquiring the disease, even considering this is a partial protection, because there would be protection against MB forms of leprosy, reducing the disease transmission.

Key words: Leprosy; Immunopathogenesis; Multifunctional T cells; Recombinant Antigens.

REVISÃO DE LITERATURA

A hanseníase (ou doença de Hansen) é uma doença infecciosa crônica e de evolução clínica lenta causada pelo bacilo *Mycobacterium leprae* (*M. leprae*) (Fonseca *et al.*, 2017; Simon *et al.*, 2011). O *M. leprae* é um patógeno intracelular que exibe tropismo tecidual por células da pele e células de Schwann de nervos periféricos e apresenta geralmente período de incubação prolongado (variando média de 2 a 7 anos) (Fonseca *et al.*, 2017; Freitas *et al.*, 2016). Os sinais clínicos da hanseníase podem ser escassos no início da doença, o que pode levar ao diagnóstico tardio ou até mesmo a erros de diagnóstico (Duthie, Gillis e Reed, 2011; Duthie, Saunderson e Reed, 2012). Além disso, a doença pode manifestar-se através de um amplo espectro de sinais e sintomas e o diagnóstico baseia-se primariamente nas características dos sinais clínicos (lesões dérmicas e neurológicas) e no exame histopatológico (Simon *et al.*, 2011). Com base nisso, os pacientes podem ser categorizados segundo a classificação operacional, para fins de tratamento e que baseia-se no número de lesões e no exame baciloscópico, como multibacilares (MB, quando há mais de 5 lesões cutâneas ou baciloscopia positiva) ou paucibacilares (PB, quando há menos de 5 lesões cutâneas ou baciloscopia negativa) (Fonseca *et al.*, 2017; Simon *et al.*, 2011). Além dessa classificação, há ainda um amplo espectro de formas clínicas da hanseníase que foi estabelecido por Ridley e Jopling em 1966 (Ridley DS, 1966). Eles estratificaram a hanseníase em cinco formas clínicas (abrangendo critérios clínicos, histopatológicos, imunológicos e bacteriológicos). Há as formas polares da doença, Hanseníase Tuberculoide (HT) e Hanseníase Virchowiana (HV), e as formas clínicas intermediárias que incluem Dimorfa Tuberculoide (HDT), Dimorfa (HD) e Dimorfa Virchowiana (HDV) (Lockwood, Sarno e Smith, 2007; Ridley

DS, 1966). A forma Indeterminada (HI) foi posteriormente adicionada a esta classificação (Lockwood, Sarno e Smith, 2007).

A forma de HI é considerada a fase inicial da doença. O paciente apresenta manchas hipocrômicas ou eritematosas-hipocrômicas ou áreas circunscritas na pele com algum distúrbio de sensibilidade. Pode ocorrer também perda de pelos e ausência de horripilação. As lesões podem ser únicas ou múltiplas, com locais e tamanhos variados. Nesta forma clínica não há envolvimento de troncos nervosos e, portanto, os pacientes não apresentam grau de incapacidade física e não são contagiantes (Lockwood, Sarno e Smith, 2007; Simon *et al.*, 2011).

A forma HT é caracterizada por máculas hipocrômicas ou eritematosas. As lesões são difusamente infiltradas e com bordas bem delimitadas. Os pacientes podem apresentar de 1 a 5 lesões e geralmente com distribuição assimétrica. Podem ocorrer danos neurais, com alterações sensoriais (hipoestesia e anestesia) e alterações autônomas (hipoidrose e alopecia). O envolvimento dos troncos nervosos geralmente ocorre próximo às lesões cutâneas (Lockwood, Sarno e Smith, 2007; Ridley DS, 1966; Simon *et al.*, 2011).

Na forma HV não ocorre a formação de granuloma e isto leva a uma replicação incontrolável do bacilo com infiltração contínua na pele e nos nervos periféricos. As lesões apresentam limites imprecisos e aspecto ferruginoso. Quando há intesa infiltração bacilar no rosto, com acentuação dos sulcos faciais e conservação do cabelo, configura um quadro clínico denominado de "faces leonina", sinal peculiar na HV. Nesta forma clínica, além do mais, os doentes têm cargas elevadas de bacilo em todo o corpo e é, portanto, a forma mais contagiosa da doença. Contudo, a transmissão é interrompida com

o estabelecimento da poliquimioterapia (PQT) (Lockwood, Sarno e Smith, 2007; Ridley DS, 1966; Simon *et al.*, 2011).

Entre esses dois pólos da doença (HT e HV), existem as formas intermediárias, classificadas como Hanseníase Dimorfa (HD). Pacientes HD têm manchas circulares e hipocrômicas. As lesões costumam ser bem delimitadas e difundidas na periferia. No entanto, algumas lesões não têm limites precisos na periferia (lesões "irregulares", "favo de mel" ou "queijo suíço"). A carga bacilar é elevada sendo, portanto, uma forma contagiosa. Os nervos periféricos são freqüentemente afetados e este envolvimento é intenso e extenso. A neuropatia periférica pode ser perpetuada por vários anos após a cura clínica da infecção. A forma HD também pode apresentar lesões semelhantes às observadas na forma HT e, portanto, é considerada como Dimorfa Tuberculoide (HDT). Quando apresenta lesões semelhantes às da forma HV, é classificada como Dimorfa Virchowiana (HV) (Lockwood, Sarno e Smith, 2007; Ridley DS, 1966; Simon *et al.*, 2011).

A infecção e lesão de nervos periféricos é um sinal clínico relevante na hanseníase, embora os mecanismos subjacentes à lesão do nervo não sejam ainda bem esclarecidos (Duthie, Gillis e Reed, 2011; Oliveira, de *et al.*, 2012; Vital *et al.*, 2013). Nesse cenário, a hanseníase pode evoluir para complicações clínicas limitantes denominadas de reações hansênicas (RH) e grau de incapacidade física (Vital *et al.*, 2013). As RH são um fenômeno de inflamação aguda que pode ocorrer antes, durante ou após o tratamento. Estima-se que 30-50% dos pacientes apresentarão episódios reacionais em algum momento durante o curso da doença (Fonseca *et al.*, 2017; Simon *et al.*, 2011). As RH podem ser classificadas como reação reversa, ou reação do tipo 1 (RR), e eritema nodoso

hansênico (ENH), ou reação de tipo II (Khadge *et al.*, 2015). Pacientes com RH podem apresentar inflamação neural intensa, dolorosa, resultando em perda súbita e até permanente de funções sensoriais, autônomas e até motoras. Na ausência de intervenção precoce, o paciente pode evoluir com sequelas graves que são responsáveis pelo forte estigma social e comprometimento significativo da qualidade de vida (Lockwood, Sarno e Smith, 2007).

Estudos epidemiológicos vem demonstrado que a hanseníase apresenta maior incidência em populações com baixa escolaridade, precariedade nos serviços de saúde e na infra-estrutura domiciliar (Freitas *et al.*, 2016; Santos, dos *et al.*, 2016; Santos *et al.*, 2016). De acordo com dados da Organização Mundial de Saúde (OMS), o número de novos casos de hanseníase relatados em todo o mundo em 2014 foi de 213.899, distribuídos em 121 países, especialmente em áreas da África, Ásia e Américas. Além disso, do total de casos notificados em 2014 pela OMS, 125.785 (59%) ocorreram na Índia, 31.064 (15%) no Brasil e 17.025 (8%) na Indonésia (Vieira *et al.*, 2014). Esses dados indicam, portanto, que apesar do sucesso alcançado com a implementação da PQT, a incidência da hanseníase não apresentou declínio satisfatório (Oliveira, de *et al.*, 2012). Além disso, estima-se que entre 1 a 2 milhões de pessoas no mundo vivem atualmente com alguma deformidade física ou deficiência decorrentes de complicações clínicas da hanseníase e continua a ser uma das principais causas de neuropatia e incapacidade entre as doenças transmissíveis (Freitas *et al.*, 2016).

O Brasil é o país responsável pela endemia da Hanseníase no continente americano, abrangendo 93,8% dos casos relatados (Brito *et al.*, 2016). A taxa de detecção de casos novos em 2014 foi de 15,44 casos por 100.000 habitantes, a taxa de grau de

incapacidade física 2 foi de 0,99 por 100.000 habitantes e a taxa de detecção em menores de 15 anos foi de 5,03 casos por 100.000 habitantes (Freitas *et al.*, 2016). A maior prevalência tem sido relatada nas regiões Norte, Nordeste e Centro-Oeste (Brito *et al.*, 2016). Além disso, as regiões com maior desigualdade social apresentam os maiores coeficientes de detecção e prevalência de hanseníase, o que reforça os argumentos de que indicadores socioeconômicos e ambientais também são importantes preditores da infecção por hanseníase (Santos, Dos *et al.*, 2013).

O tratamento precoce e ininterrupto da doença pode reduzir o risco de transmissão do bacilo e, consequentemente, minimizar o número de novos casos e facilitar a eliminação da hanseníase (Oliveira, de *et al.*, 2012). Apesar do imenso esforço e avanços nas pesquisas e no tratamento nas últimas duas décadas para eliminar a hanseníase, a taxa de incidência da doença tem se mantido constante. Houve uma expressiva redução na prevalência como resultado do uso generalizado da poliquimioterapia (PQT), porém as novas taxas de detecção de casos se estabilizaram na última década e a hanseníase permanece endêmica em várias regiões localizadas em países como Filipinas, Indonésia, China, Índia e Brasil (Duthie *et al.*, 2010; Duthie, Favila, *et al.*, 2016; Duthie e Balagon, 2016; Duthie, Gillis e Reed, 2011; Paula Vaz Cardoso *et al.*, 2013). Diante desse cenário ainda preocupante, são requeridos o desenvolvimento de novas ferramentas de diagnóstico, estratégias de tratamento e uma vacina específica, o que têm sido objetivo de alguns grupos de cientistas.

Estima-se que uma pequena porcentagem (menos de 1%) dos indivíduos que entram em contato com *M. leprae* desenvolve a doença. Diversos autores sugerem que o padrão genético e variações nos mecanismos da resposta imunológica do paciente

influenciam na susceptibilidade ou resistência à doença (Duthie, Gillis e Reed, 2011; Fonseca *et al.*, 2017; Simon *et al.*, 2011). As respostas das células T helper tipo 1 (Th1 - mediadas sobretudo por IFN-γ) estão relacionadas com o controle da replicação e disseminação do *M. leprae* e está presente na forma tuberculóide da hanseníase. Nessa forma clínica, ocorre a formação de granulomas nas lesões epidérmicas (Dupnik *et al.*, 2015; Duthie, Orcullo, *et al.*, 2016; Duthie, Saunderson e Reed, 2012; Saini *et al.*, 2016; Simon *et al.*, 2011). Alternativamente, as respostas das células T helper tipo 2 (Th2) e T reguladoras (T reg – mediada sobretudo pela citocina anti-inflamatória IL-10 e TGF-β), estão associadas às apresentações clínicas multibacilares da hanseníase. Essas formas clínicas são caracterizadas por macrófagos nas lesões epidérmicas que são amplamente infectados e incapazes de controlar a replicação do *M. leprae* (Dupnik *et al.*, 2015; Fonseca *et al.*, 2017; Palermo *et al.*, 2012; Saini *et al.*, 2016; Simon *et al.*, 2011). As células Treg são um dos tipos de células da resposta imune mais potentes que suprimem a função das células T efetoras. Essas células eventualmente apresentam regulação da resposta imunológica provocada pelo hospedeiro durante infecções intracelulares (Palermo *et al.*, 2012; Sadhu *et al.*, 2016; Saini *et al.*, 2016).

Diversos estudos demonstraram papel protetor tanto da IL-1β como da IL-17 (citocinas da resposta Th17) contra patógenos intracelulares (Akdis *et al.*, 2011; Burgler *et al.*, 2009), contudo, relativamente poucos estudos têm investigado a ação dessas citocinas na patogênese da hanseníase (Khadge *et al.*, 2015; Sadhu *et al.*, 2016; Saini *et al.*, 2016; Saini, Ramesh e Nath, 2013; Sampaio *et al.*, 2012). Saini e colaboradores (Saini, Ramesh e Nath, 2013) demonstraram a liberação mais elevada de isoformas de IL-17 em sobrenadante após a estimulação de PBMC de pacientes HT e controles contactantes. Em outro estudo, o mesmo grupo sugeriu que o aumento de citocinas

associadas a células Th17 pode contribuir para a inflamação envolvida no eritema nodoso hansênico (ENH) (Saini *et al.*, 2016). Apesar de pesquisas mostrarem como as respostas Th1, Th2 e Treg podem influenciar na imunopatogênese da hanseníase, o impacto de mecanismos imunológicos sobre a persistência do *M. leprae* e a gravidade da doença ainda carecem de esclarecimentos. Em particular, o papel das células Th17, assim como o papel da resposta imune inata e da geração de células T de memória multifuncionais, relacionadas com à evolução clínica da doença e à ocorrência de episódios reacionais e lesões neurológicas, são objetos de estudo em diveros grupos de pesquisa com hanseníase.

A impossibilidade de cultivo do bacilo *in vitro* limitou por décadas o avanço de pesquisas com a Hanseníase, pela impossibilidade de realizar melhor avaliação dos mecanismos microbicidas de fagócitos e da resposta imune inata e adaptativa específica para antígenos. Contudo, o sequenciamento completo do genoma do *M. leprae* em 2001 permitiu o desenvolvimento de antígenos recombinantes deste bacilo (15). Desde então, progressos consideráveis vêm sendo realizados e um grande número de antígenos da *M. lepra* foram testados *in vitro*. Além desses estudos, os antígenos foram avaliados também em pacientes com hanseníase e em controles contactantes.

Diversos grupos de pesquisa vêm avaliando o papel dos antígenos recombinates *in vitro* e em pacientes com Hanseníase e controles contactantes. Duthie e colaboradores avaliaram a capacidade dos antígenos de *M. leprae* (ML0405 e ML2331) reconhecerem IgG em soro de paciente de Hanseníase (Duthie *et al.*, 2007). Além disso, uma quimera, resultante da fusão dessas duas proteínas de *M. leprae*, designada *Leprosy IDRI Diagnostic* 1 [LID-1] apresentou promissora capacidade de diagnóstico da hanseníase. Testando o LID-1 no soro de indivíduos que desenvolveram hanseníase, essa quimera foi

capaz de diagnosticar a hanseníase com 6 a 8 meses antes da manifestação dos sintomas clínicos (Sampaio *et al.*, 2011). Num estudo subsequente, Duthie e colaboradores (Duthie *et al.*, 2010) ralizaram a fusão quimérica de poliepítopos (ML0405, ML2311, ML2055, ML0049, ML0050 ML0091 e ML0411). Esta quimera foi denominada de *Protein Advanced Diagnostic Leprosy* (PADL). PADL apresentou ação complementar ao LID-1 e permitiu o melhor diagnóstico da hanseníase, sobremaneira em MB em comparação com os controles. Em outro estudo, Spencer e colaboradores avaliaram os antígenos recombinantes de *M. leprae* (ML1877, ML0841, ML2028, ML2038, ML0380 e ML0050) e demonstraram que as respostas dos pacientes com hanseníase (MB e PB) a ML2028 e ML2038 eram geralmente fortes (Spencer *et al.*, 2011). O ML2028 é um antígeno recombinante de *M. leprae*, membro do complexo Ag85B e cuja homologia com o *Mycobacterium tuberculosis* Rv1886c é de 83,3% (Spencer *et al.*, 2011). Diante disso, alguns desses antígenos demonstram ser promissores como potenciais candidatos a uma vacina específica e para o desenvolvimento de testes rápidos para o diagnóstico precoce da doença.

Com o desenvolvimento das técnicas de citometria de fluxo e das análises multiparamétricas, foi possível avaliar as funções efetoras e de memória de células T em nível celular (Seder, Darrah e Roederer, 2008; Talker *et al.*, 2015). As células T multifuncionais, também referidas como células T polifuncionais, são células T $CD4^+$ ou $CD8^+$ que se desenvolvem em células T $IFN\gamma^+TNF^+IL\text{-}2^+$ e apresentam duas ou mais funções (Darrah *et al.*, 2007; Guha *et al.*, 2013; Seder, Darrah e Roederer, 2008; Wimmers *et al.*, 2016). As células T $CD4^+$ normalmente podem permanecer como células T de memória ou diferenciarem-se em efetoras, ou podem morrer por apoptose logo após sua ativação (Seder, Darrah e Roederer, 2008; Wimmers *et al.*, 2016). Além disso, células T $CD4^+$ e $CD8^+$ multifuncionais induzidas por antígenos têm um papel essencial na

proteção contra uma variedade de doenças infecciosas causadas por patógenos intracelulares como já reportado na leishmaniose (Darrah *et al.*, 2007; Guha *et al.*, 2013; Macedo *et al.*, 2012) e na tuberculose (Leung-Theung-Long *et al.*, 2015; Lichtner *et al.*, 2015). A capacidade destas células de permanecerem como células T de memória ou efetoras tem implicações importantes para o desenvolvimento de vacinas para evitar reinfecções. Embora vários estudos tenham investigado o papel dos antígenos recombinantes e sua indução na expressão de células T multifuncionais em algumas doenças infecciosas, não há dados na literatura sobre a expressão dessas células em pacientes com hanseníase.

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Artigo

Differences in multifunctional antigen-specific T cells may account for the diverse outcomes of *Mycobacterium leprae* infection

Márcio B. Santos[1,2,3]; Marise do V. Simon[2]; Micheli L. B. Santos[2]; Aline S. Barreto[2]; Rodrigo A. Cazzaniga[2]; Daniela T. de Oliveira[2]; Mônica R. Barrios[2]; **Alex R. Ferreira**[2,4]; Nanci C. Santos-Bio[2,4]; Steven G. Reed[3]; Malcolm S. Duthie[3]; Roque P. de Almeida[2,4,5]; Cristiane B. Correia[2] and Amélia R. de Jesus[2,4,5]

[1]Departament of Health Education, Universidade Federal de Sergipe (UFS), Brazil.

[2]Laboratory of Molecular Biology (UFS), Universidade Federal de Sergipe, Brazil.

[3]Infectious Diseases Research Institute (IDRI), Seattle, Washington, USA.

[4]Departament of Medicine (UFS), Sergipe Brazil.

[5]Instituto de Investigacão em Imunologia, INCT, CNPq.

Corresponding Author

Amélia Ribeiro de Jesus

Laboratory of Molecular Biology (UFS), Universidade Federal de Sergipe, Brazil. Rua Cláudio Batista, S/N, Hospital Universitário, Bairro Sanatório, Aracaju, Sergipe, CEP 49060-100.

Phone number/FAX:+557921051806 E-mail: ameliaribeirodejesus@gmail.com

Abstract

Leprosy is a slowly evolving chronic manifestation of *M. leprae* infection. Given that multifunctional CD4 T cells capable of simultaneously secreting combinations of interferon-γ (IFN-γ), interleukin (IL)-2 or tumor necrosis factor (TNF) have now been indicated as beneficial for the successful immune outcome of several infections, we hypothesized that they may similarly contribute to the evolution of *M. leprae* infection. To test this hypothesis, we evaluated antigen-specific T cell responses of a total of 87 subjects. Peripheral blood mononuclear cells of control individuals (31 HHC) and leprosy patients (39 PB and 17 MB) were incubated with crude antigen extracts or the recombinant ML2028 antigen. Assessment of the cytokines secreted into the culture supernatants by multiplex assay revealed antigen-specific production of IFN-γ and IL-2 from cells of HHC and PB, confirming the Th1 bias of their responses. We then used multiparameter flow cytometry to determine the source of these cytokines. These analyses revealed a population of multifunctional antigen-specific T cells in HHC that was larger than that observed in PB patients. Our data indicate that HHC generate multifunctional antigen-specific T cells and suggest that these provide a more effective immune response against *M. leprae* infection that prevents the development of leprosy.

Key words: Leprosy; Recombinant antigens; Multifunctional T cells; CD4 T cells; CD8 T cells; Immunopathogenesis.

Introduction

Leprosy, or Hansen's disease, is a chronic infectious disease caused by *Mycobacterium leprae* [1,20]. The widespread use of multidrug therapy (MDT) over the last three decades has caused a large reduction in the prevalence of leprosy [2,40]. Despite these immense efforts, however, new case detection rates have stabilized over the last few years. Leprosy remains as a public health concern in a number of localized regions, including India and Brazil [4,5,9].

There is now strong associative evidence that the genetic background and the immunological response influence both susceptibility to, and outcome of, leprosy [1,2]. T helper 1 (Th1) and T helper 17 (Th17) cells are observed in paucibacillary (PB) patients and are associated with control of *M. leprae* replication [2,20,21,41–43]. In contrast, T helper 2 (Th2) and T regulatory (Treg) cells are associated with the multibacillary (MB) presentations that present with heavily infected macrophages in their epidermal lesions [2,20,21,23,42]. Several attempts have been made to develop a vaccine specifically for leprosy but, at present, BCG (Bacillus Calmette-Guerin) is the only vaccine available [44]. Regardless, systematic meta-analyses indicate that BCG vaccination has a protective efficacy of approximately 50% [5].

Although Th1 cells are associated with protective responses against *M. leprae* infection, assessment of the T cell response by measuring only IFN-γ production may not fully reflect the protective potential of the response [32]. Multiple studies have failed to discriminate healthy, but possibly infected individuals (household contacts - HHC) from

PB patients on the basis of antigen-specific IFN- γ production [1,5]. The development of multiparameter flow cytometry has allowed the analysis of T-cell effector functions at the single cell level [32,33], revealing that $CD4^+$ T cells that secrete only IFN- γ have a limited capacity to develop into memory cells [32,35]. Recently, several studies have evaluated the frequency of multifunctional Th1 cells, characterized by their simultaneous secretion of multiple cytokines (IFN-γ, IL-2 and TNF-α) in various conditions [32–35]. These studies have demonstrated that the proportion of multifunctional Th1 cells positively correlates with protection against cancers and infectious diseases, including leishmaniosis [34,37] and *Mycobacterium tuberculosis* infection [38,39].

The sequencing of *M. leprae* genome, in 2001, have allowed the designing of recombinant antigens [45]. Considerable progress has been made and several antigens have been tried as potential use in clinical and immunological fields. Over the last decade, the Infectious Diseases Research Institute (IDRI) has working on an ambitious research program to develop new tools to aid in leprosy control efforts. The ultimate goal of this program is to provide a vaccine to prevent leprosy, but they also recognize diagnostic screening to permit targeted interventions to assist in the control of the disease [4,5,22,46–48]. The identification of new antigens that are the target of the cellular immune response may be promising for diagnosis and immunoprophylaxis in leprosy [4,19,22,48,49]. Furthermore, how these antigens are recognized is likely the key to protective response. Thus, this research aimed to evaluate the immune response to the recombinant antigen ML2028 and the presence of multifunctional T cells induced in vitro by this antigen.

Material and methods

Ethical considerations

This project adheres to the protocols of the Brazilian Consul for ethics in research (CONEP). The Ethics and Research Committee of the Federal University of Sergipe approved the study (CAAE 0152.0.107.000-07). All subjects signed a free and clarified term of knowledge (IC) and then responded to an investigative questionnaire to collect demographic and clinical data.

Study subjects and procedures

The study volunteers were attended in the dermatology ambulatory of the project (DES)MANCHA-Federal University of Sergipe at the University Hospital (Aracaju city, Brazil). Each subject was thoroughly examined for the presence of leprosy, leprosy reactions and neurological disabilities. Each patient was classified as having one of the clinical forms as characterized by Ridley-Jopling prior to treatment by conventional multidrug therapy (MDT) for leprosy, in accordance with the Brazilian Ministry of Health and International Leprosy Association (ILA) standards. Exclusion criteria were subjects presenting some diseases that affect immune response, as HIV, HTLV-I, diabetes and neurological diseases. A total of 87 subjects were included: 39 PB, 17 MB patients and 31 HHC. As previously mentioned, based on histopathological analysis, patients were further classified as: Indeterminate (IL), Tuberculoid (TT), Borderline (BL) or Lepromatous (LL) Leprosy. HHC were subjects living in close and prolonged contact with the patients and were commonly the patient's spouse.

Antigen stimulation assays

All immunological evaluations were performed before treatment was initiated. Whole blood was collected into tubes with heparin (10 IU/ml). Briefly, the blood was then seeded in 24 well-plates and incubated with crude antigen from *M. tuberculosis* (PPD – Purified Protein Derivative) at 1 µg/ml; crude antigen from *M. leprae* (MLCS) at 10 µg/ml; the recombinant antigen from *M. leprae* (ML2028) at 10 µg/ml; phytohemagglutinin (PHA) at 10 µg/ml or media alone (RPMI 1640: Gibco, Grand Island, New York, USA). Incubations were conducted for 24 hours at 5% CO^2, 37°C before collection of plasma. Concentrations of the cytokines IL-2, IFN-γ, IL-10 and IL-17A were determined by Luminex analyses, according to the manufacturer's instructions (Milliplex kit - Human Th17 Magnetic Bead Panel, Panomics, Affymetrix, Fremont, CA).

Multiparameter flow cytometry

To identify and quantify any multifunctional T cells, peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Hypaque (Ficoll-Paque PLUS™, GE-Healthcare, Menio-Park, NJ). PBMC from subsets of TT (n =5), LL (n =6), HHC (n =6) and control (n =6) were seeded at $1x10^6$ cells/ml in 48 well-plates (Greiner-CELLSTAR®), incubated with PPD (1 µg/ml), MLCS (10 µg/ml) or ML2028 (10 µg/ml) diluted with RPMI 1640 (Gibco, Grand-Island, New York, USA) for 6 hours at 5% CO^2, 37°C before the subsequent addition of GolgiPlug (BD-Biosciences, Franklin Lakes, NJ) for an additional 12 hours. At the end of culture period, cells were washed and incubated with antibodies for cell surface markers CD3 (V500), CD4 (FITC) and CD8 (PE-Cy5) and intracellular cytokines IL-2 (BV421), TNF-α (PE) and IFN-γ (PE-Cy7) (BD Biosciences, San Diego, CA). Cells were resuspended in staining buffer and events were acquired using the BD FACS Canto II. Analysis of the acquired datasets was performed using FlowJo (FlowJo-

LLCv10) software. To evaluate multiple parameters, we performed Boolean gating analysis **(Figure 1)** [15]. Comparison of frequency, median fluorescence intensity (MFI) and integrated MFI (iMFI) was made on a per group basis. iMFI is the value resulting from the multiplication of the frequency of a cytokine-producing subset of cells with the MFI for that cytokine [15]. All frequencies, MFI and iMFI values are reported after background subtraction of the frequency or iMFI of the identically gated population of cells from an unstimulated comparator of the same sample.

Statistical analysis

Cytokine concentrations were compared across the different subgroups (PB, MB and HHC) and according to clinical forms of leprosy. Mean, median and standard deviation of the groups were calculated. D'Agostino and Pearson tests were applied to analyze data that exhibited normal distribution. Statistical differences between the groups were determined by Mann-Whitney U tests. Comparisons of means for a given parameter were made by non-parametric (two-tailed, considering unequal variance of groups) t-tests. Correlation between cytokine levels was performed by Spearman correlation test. All analyses were performed by GraphPad Prism software, version 7. Results were considered statistically different at a p-value < 0.05.

Results

Demographic and clinical characteristics of study groups

No significant differences were observed between the age of patients presenting as PB or MB when compared to HHC, although the proportion of men presenting with MB (58.8%) was higher than that presenting with PB (28.2%; *p*-value =0.02; **Table 1**). As

expected, MB patients had a greater number of lesions (mean ± standard deviation (SD) 10.24 ± 4.69) than the PB patients (2.13 ±2.66; *p*-value < 0.0001). The occurrence of reaction episodes was also significantly higher in MB that PB patients (64.7% vs. 30.77%, *p*-value = 0.01).

Antigen-specific secretion of Th1 cytokines by PB and HHC

To begin to assess the response of patients, we first analyzed the cytokine levels secreted following incubation of whole blood with crude (PPD and MLCS) or recombinant (ML2028) antigens. We detected both IFN-γ and IL-2 in the plasma from PB patients stimulated with PPD and MLCS **(Figure 2A-L).** Similarly, and indicating *M. leprae* exposure or potential lower level infection, blood from HHC also secreted elevated levels of IFN-γ and IL-2. These results were in contrast with those obtained with blood from MB patients, where low or undetectable levels of these cytokines were observed. Differences were not observed between the groups in either IL-10 or IL-17A, both of which were detected at only very low levels. Taken together, these data indicate a Th1-biased anti-mycobacterial response in both PB and HHC.

We also incubated blood with a recombinant version of the ML2028 protein. Relating to antigen-specific Th1 responses, we observed higher concentrations of IFN-γ in PB (31.5 ± 85.1 pg/ml) and HHC (87.6 ± 298.9 pg/ml) than in MB (2.9 ± 4.05 pg/ml; **Figure 2A-L**). Higher concentrations of IL-2 were also observed in samples stimulated with ML2028 in PB (7.59 ± 50.7pg/ml) and HHC (12.8 ± 16.1pg/ml) than in MB (4.6 ± 40.3pg/ml). Interestingly, strong correlations were observed between these cytokines in the responses against each antigen (**Supplementary Figure 1**). As with the crude antigens, differences were not observed in IL-10 or IL-17A levels following incubation with ML2028. These

data indicate that responses to ML2028 are similar to those against the whole *M. leprae* bacteria and can therefore be used as a proxy of the anti-*M. leprae* response.

Recent data indicates that the quality of the Th1 response impacts its ability to protect against intracellular pathogens [32]. The presence of multifunctional cells simultaneously producing Th1 cytokines appears preferable to cells secreting only one of these cytokines. Positive correlations were observed between IL-2 and IFN-γ concentrations in supernatant stimulated with PPD (Spearman r = 0.89, *p*-value < 0.0001), MLCS (Spearman r = 0.79, *p*-value < 0.0001) and ML2028 (Spearman r = 0.61, *p*-value < 0.0001) (**Figure 3A-C**). The correlation in IFN-γ and IL-2 levels suggests that they may be being produced simultaneously by multifunctional T cells.

Multifunctional $CD4^+$ T cells are more abundant in HHC than leprosy patients

To determine the phenotype of the antigen-specific cytokine-producing cells, we first assessed the iMFI (frequency and cytokine-producing capacity) of IFN-γ^+, IL-2^+ and TNF^+ $CD4^+$ T cells. Differences were not observed in percentage of $CD3^+CD4^+$ lymphocytes between the groups assessed (**data not shown**). Following flow cytometry, in support of our ELISA data, our analyses revealed high expression of $CD4^+$IL-2^+ iMFI in samples from HHC (279.6 ± 684.9) and TT patients (329.8 ± 659.6; **Figure 4A**) after incubation with ML2028. We observed also high expression of $CD4^+$TNF-α^+ iMFI in HHC (8.7 ± 17.9; **Figure 4B**). We observed elevated expression of CD4+IFN-γ+ iMFI in HHC (11.68 ± 28.6; **Figure 4C**) than in LL patients (0.0 ± 0.0) samples stimulated with PPD.

As the iMFI is a product of both frequency and quantity, high iMFI values could arise due to a greater frequency of cells that are each producing only small quantities of

cytokine. We therefore used a multi-gating strategy to determine the proportion of antigen-specific cells that were producing the Th1 cytokines, gating for cells that were producing combinations of the cytokines (either all three together, combinations of two or individually). MLCS stimulus presented also higher expression of $CD4^{+}IFN\text{-}\gamma^{-}IL\text{-}2^{+}TNF\text{-}\alpha^{+}$ and $CD4^{+}IFN\text{-}\gamma^{+}IL\text{-}2^{-}TNF\text{-}\alpha^{+}$ in HHC than in TT and LL (**Figure 4D**). We observed high expression of triple positive and $CD4^{+}IFN\text{-}\gamma^{+}IL\text{-}2^{-}TNF\text{-}\alpha^{-}$ in control and HHC (**Figure 4E**) after PPD stimulus. Similarly, the incubation with ML2028 presented higher expression of $CD4^{+}IFN\text{-}\gamma^{+}IL\text{-}2^{+}TNF\text{-}\alpha^{+}$ in control (0.022 ± 0.01) and HHC (0.017 ± 0.01) than in TT (0.003 ± 0.002) and LL (0.001 ± 0.002) patients (**Figure 4F**). ML2028 was also recognized by elevated proportions of $CD4^{+}IFN\text{-}\gamma^{+}IL\text{-}2^{+}TNF\text{-}\alpha^{-}$ (0.026 ± 0.06), $CD4^{+}IFN\text{-}\gamma^{-}IL\text{-}2^{+}TNF\text{-}\alpha^{+}$ (0.094 ± 0.22) and $CD4^{+}IFN\text{-}\gamma^{+}IL\text{-}2^{-}TNF\text{-}\alpha^{+}$ (0.044 ± 0.1) in HHC. ML2028 stimulus presented, however, higher expression of $CD4^{+}IFN\text{-}\gamma^{+}IL\text{-}2^{-}TNF\text{-}\alpha^{-}$ in LL (0.077 ± 0.05) and TT (0.016 ± 0.01), than in control (0.009 ± 0.01) and HHC (0.003 ± 0.00).

We grouped the results of all cells $triple^{+}$, $double^{+}$ and $single^{+}$ (**Figure 4G**) and observed that ML2028 stimulus presented more percentage of $triple^{+}$ (11.29%) and $double^{+}$ (19.96%) in control and $triple^{+}$ (1.22%) and $double^{+}$ (11.7%) in HHC group. PPD stimulus expressed also more percentage of $triple^{+}$ (12.27%) in HHC and $double^{+}$ (22.9%) in control group. ML2028, PPD and MLCS stimulus presented similar percentage of $triple^{+}$, $double^{+}$ and $single^{+}$ cells in TT and LL patients. Taken together, these data suggest that ML2028 recombinant antigen presented multifunctional $CD4^{+}$T cell (IFN- γ, IL-2 and TNF-α triple and double positive) expression, similar to PPD, that have currently been used to vaccinate against leprosy in HHC. It should be mentioned that these evaluations of the contacts subjects were collected before BCG revaccination.

HHC presented elevated expression of multifunctional CD8$^+$T cell

We assessed also the percentage of total CD3$^+$/CD8$^+$ lymphocytes. However, no differences were observed in percentage of these cells stimulated with these antigens (**data not showed**). MLCS stimulus presented elevated CD8$^+$IL-2$^+$ iMFI in controls, but similar values were observed in other groups (**Figure 5A**). Cells stimulated with PPD presented high expression of CD8$^+$IL-2$^+$ and CD8$^+$TNF-α$^+$ iMFI in LL patients (**Figure 5A-B**). Nevertheless, ML2028 stimulus expressed higher CD8$^+$TNF-α$^+$ and CD8$^+$IFN-γ$^+$ iMFI in HHC than TT and LL patients (**Figure 5B-C**). PPD and MLCS expressed also elevated CD4$^+$IFN-γ$^+$IL-2$^+$TNF-α$^+$ (triple$^+$ cells) in control and HHC (**Figure 5D and E**, respectively). We identify also that ML2028 stimulus presented high CD4$^+$IFN-γ$^+$IL-2$^+$TNF-α$^+$ expression in control (0.006 ± 0.008) and HHC (0.005 ± 0.009) and higher percentage of CD4$^+$IFN-γ$^-$IL-2$^+$TNF-α$^-$ in HHC (1.55 ± 1.7) than in TT (0.25 ± 0.43) patients (**Figure 5F**). ML2028 stimulus presented, however, higher expression of CD8$^+$IFN-γ$^+$IL-2$^-$TNF-α$^-$ in LL (0.06 ± 0.07) than in control (0.0 ± 0.0). After grouping all kind of positive cells, we observed that ML2028 stimulus presented high expression of triple$^+$ cells in HHC (5.03%) and control (1.6%; **Figure 5G**). The stimulation with PPD and MLCS, similarly, presented triple$^+$ cells expression in control and HHC. These data indicate that ML2028 stimulus presented either high expression of multifunctional CD8$^+$T cell (triple$^+$), especially in HHC.

Discussion

Recent studies, particularly into vaccine efficacy, have reported the role of multifunctional T cells in chronic infectious diseases as HIV [50], Leishmaniosis [34,37] and Tuberculosis [38,39]. These studies reinforce the role of multifunctional T cells in central and effector memory generation and suggest that some antigens might be

promising for vaccines development. However, this is the first study that analyzed multifunction T cells expression in leprosy patients and controls stimulated with crude and a recombinant antigen from *M. leprae*.

Regarding to leprosy disease, it presents slowly chronic clinical evolution and the multidrug treatment is effective, but current treatments are long term, about half of the patients present reactional episodes and some of them develop physical disabilities [1,2,5,9]. In this study through the evaluation of the immune response of leprosy patients and HHC recognized by recombinant antigens (ML2028, PPD and MLCS), we demonstrate the higher expression of multifunctional T cells in HHC than in leprosy patients. The multifunctional $CD4^+$ T cells usually have three potential features: they can remain as memory or effector T cells; they can further differentiate into less-functional T cells; or they can die following activation [32]. Therefore, these abilities have important clinical implications and our findings suggest that these cells can be involved in the protection against *M. leprae* infection and leprosy development in HHC.

We have found that the recombinant antigen of *M. leprae* (ML2028), as PPD and MLCS, induced higher levels of IL-2 and INF-γ (which regulate the differentiation of Th1 response) in supernatant of HHC and PB compared to MB. Provide that, the Th1 response could lead to more efficient killing of mycobacterium [2] and IL-2 could enhance the expansion of such cells [32], this data reinforce that HHC immune response is more effective in *M. leprae* infection control and hence avoiding the disease development. Our results revealed either that cells from HHC and PB patients secrete higher levels of IL-2 and IFN-γ when stimulated with PPD. Since Bacillus Calmette-Guérin (BCG) has been used as vaccine against leprosy, the high production of these cytokines reinforces the

protective immune response that BCG can generate against *M. leprae*.

As previously mentioned, cellular immune response involving $CD4^+$ and $CD8^+$ T cells is important for controlling infection by intracellular pathogens [32]. Moreover, recent researches have demonstrated also that vaccine-induced generation of multifunctional $CD4^+$ and $CD8^+$ T cells provides better protection against intracellular infections [34]. Our data regarding the secretion of both IL-2 and IFN-γ levels in HHC supernatant, made us to question if these group could present multifunctional T cells expression. Using multiparameter flow cytometry, we observed similarly that ML2028 was recognized by a higher population of antigen-specific multifunctional (double$^+$ $CD4^+$ T cells) in control and HHC than in LL patients. Since that $CD4^+$IL-2^+TNF-a^+ is associated with central memory and $CD4^+$IFN-γ^+IL-2^+ and $CD4^+$IFN-γ^+TNF-a^+ is involved with effector memory generation [32], our data suggest thus that the higher expression of multifunctional T cells in HHC can be involved with bacillus controls and therefore present a rational basis to explain the disease resistant in HHC. In addition, it suggests that ML2028 can provide durable protection by developing memory response. The multifunctional antigen-specific T cells presented also high expression of iMFI $CD4^+$IL-2^+ in HHC and iMFI $CD4^+$IFN-γ^+ in controls. Since iMFI calculation incorporates the magnitude, quality and the potency of the response, it reinforces thus that HHC immune response is more effective against *M. leprae* infection. These data highlight the importance of iMFI to determining a total functional response and show that the iMFI can be useful for correlating with protection [35]. Interestingly, Kim and colleagues [51] suggest that BCG contains many antigens that can augment multifunctional T cell populations. In the current study, our data showed also that PPD antigen was recognized by multifunctional T cells, similar to ML2028. It corroborates our supernatant data and the protective immune response that BCG can generate against *M. leprae* infection.

The role of $CD8^+$T cell during infection for protection has been also recently investigated [32]. Furthermore, $CD8^+$ T cells that produce IFN-γ and TNF-α have enhanced cytolytic activity and the IL-2 secretion promotes cell expansion, it could enhance $CD8^+$ T cell memory function in chronic infectious diseases as HIV [50] and Leishmaniosis [34,35]. We have also found that ML2028 stimulus expressed antigen-specific multifunctional (IFN-γ^+IL-2^+TNF-a^+) $CD8^+$ T cells in control and HHC. Based on these trends, we reported that $CD8^+$ T cells from HHC individuals with better control of infection have an increased frequency of multifunctional cells compared with those patients with leprosy. Altogether, this indicate that multifunctional $CD8^+$T cells can be involved also with *M. leprae* control in HHC group.

We observed also that the antigen-stimulus do not presented high levels of IL-10 in patients and controls. IL-10 is an important cytokine associated with Treg response and some authors have reinforced the role of IL-10 to equilibrium between inflammatory and anti-inflammatory responses, as observed in other infectious diseases, such as mucosal *Leishmaniasis* [52–54]. Therefore, ML2028 stimulus, as PPD and MLCS, were not associated with strong anti-inflammatory responses. Alternatively, we identified IL-17A levels more elevated in HHC than in MB patients. In this regard, IL-17A may attract neutrophils to mediate defenses against different pathogens [25,40,55] and activates other immune mechanisms that help to control intracellular pathogens. Th17 responses can be associated also with a protective effect during *M. leprae* infection. Altogether, these data suggest that the multifunctional antigen-specific T cells do not express higher levels of IL-10 and hence they are not associated with anti-inflammatory responses.

In summary, this is the first study that demonstrated the expression of multifunction T

cells in leprosy patients and HHC, stimulated with crude and a recombinant antigen of *M. leprae*. Taken together, our data reported the higher frequency of triple$^+$ and double$^+$ CD4$^+$ and CD8$^+$ T cells in HHC by antigen-specific. We observed also that ML2028, PPD and MLCS antigens were associated with Th1 response in HHC, but do not presented high levels of IL-10. Therefore, these findings highlight that HHC express high multifunctional antigen-specific T cells and has a more effective immune response against *M. leprae* infection.

Conflicts of interest

The authors declare no conflicts of interest.

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Tables

Table 1. Demographic and clinical characteristics of PB and MB patients and household contacts (HHC).

Variables		PB (n=39)	MB (n=17)	HHC (n=31)	*p* value
Age	Range (years)	11-84	10-77	25-79	
	Mean ± SD	46.87 ± 17.81	40.59 ± 18.76	48.89 ± 10.81	*0.19
Gender (male)	n (%)	11 (28.2%)	10 (58.8%)	11 (35.5%)	**0.02
Lesion number	Range	*0-15	2-20	NA	*<0.0001
	Mean ± SD	2.13 ± 2.66	10.24 ± 4.69	NA	
Leprosy reaction	n (%)	12 (30.77%)	11 (64.7%)	NA	**0.01
Physical disability	Degree 1 n (%)	19 (48.7%)	08 (47.1%)	NA	**0.28
	Degree 2 n (%)	03 (7.7%)	03 (17.6%)	NA	**0.26

SD: Standard Deviation; NA: Not applicable. *Mann-Whitney test; ** Fisher exact test

Figures

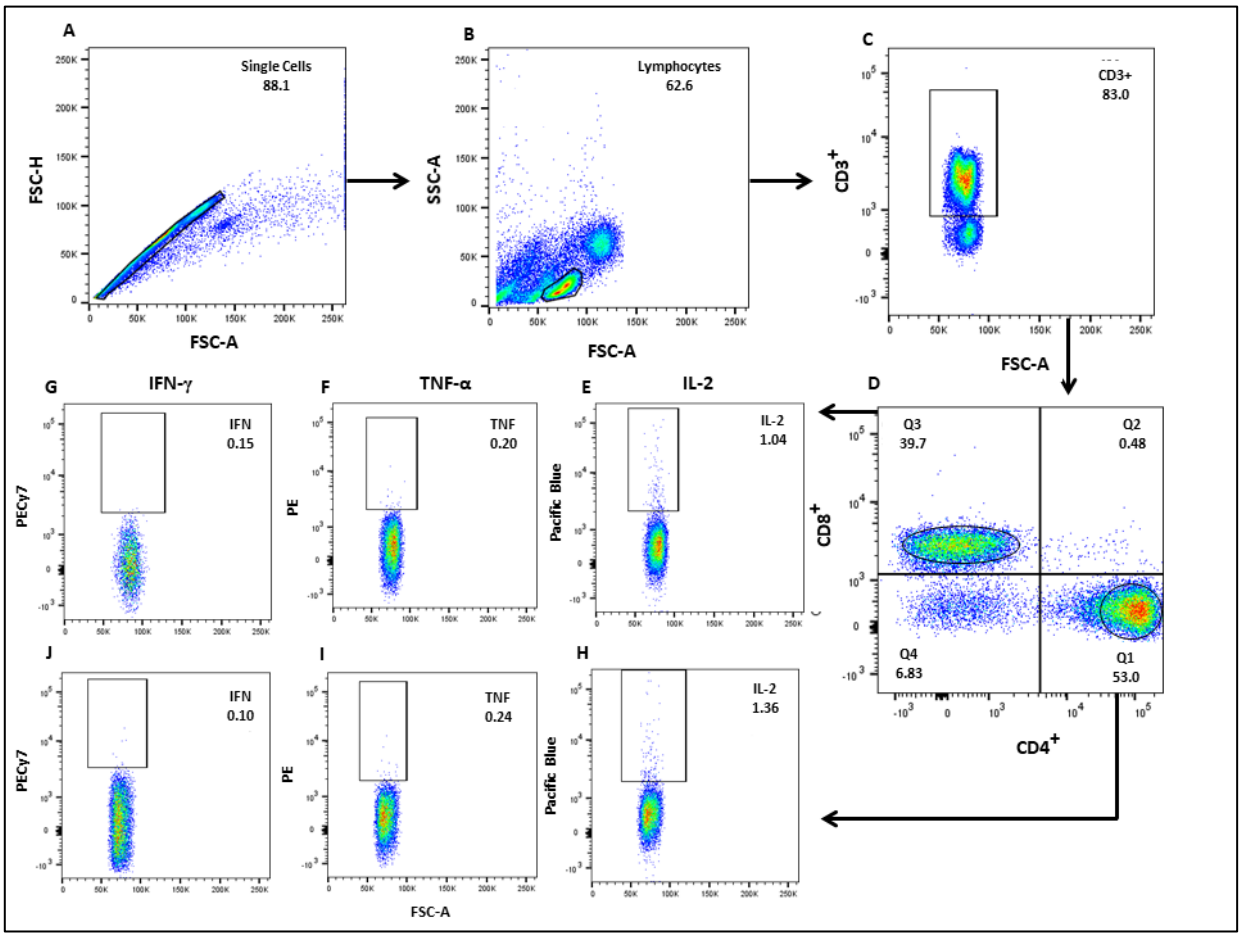

Figure 1. Strategy for the analysis of multifunction T cells response using a seven-color flow cytometry panel to simultaneously analyze multiple cytokines at the single-cell level in PBMC samples stimulated with crude and recombinant antigens. (A) After single cells selection (FSC-A x FSC-H), the lymphocytes were selected according to a (B) FSC-A (size) versus SSC-A (granularity) dot plot, followed by (C) $CD3^+$ gating. (D) $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cell were separated into $CD3^+$ gate. (E-J) $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cell phenotypes were ploted against each cytokine individually: interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). Boolean gating was performed to generate the frequencies of the possible seven combinations of cytokine producing $CD4^+$ and $CD8^+$ cells using FlowJo software.

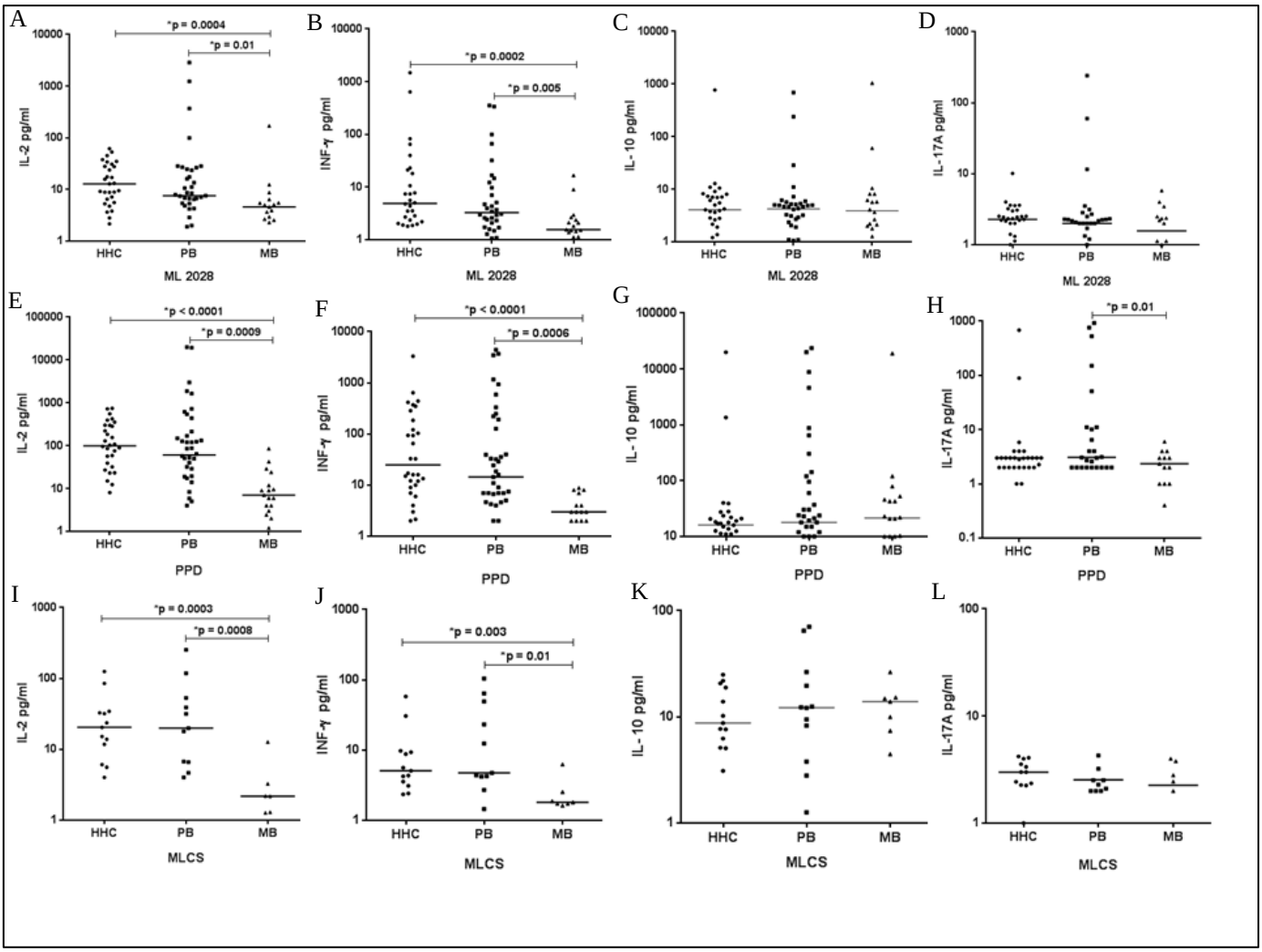

Figure 2. Cytokines released in total blood-stimulated supernatants of Paucibacillary (PB), Multibacillary (MB) and household contacts (HHC). IL-2, INF-γ, IL-10 and IL-17A levels were measures in heparinized total blood supernatants of leprosy patients classified according to Ridley Jopling and HHC, stimulated with recombinant antigen (ML2028) (A-D) and crude antigens of *M. tuberculosis* (PPD) (E-H) and *M. leprae* (MLCS) (I-L). Cytokine concentrations were collected and determined by Luminex.

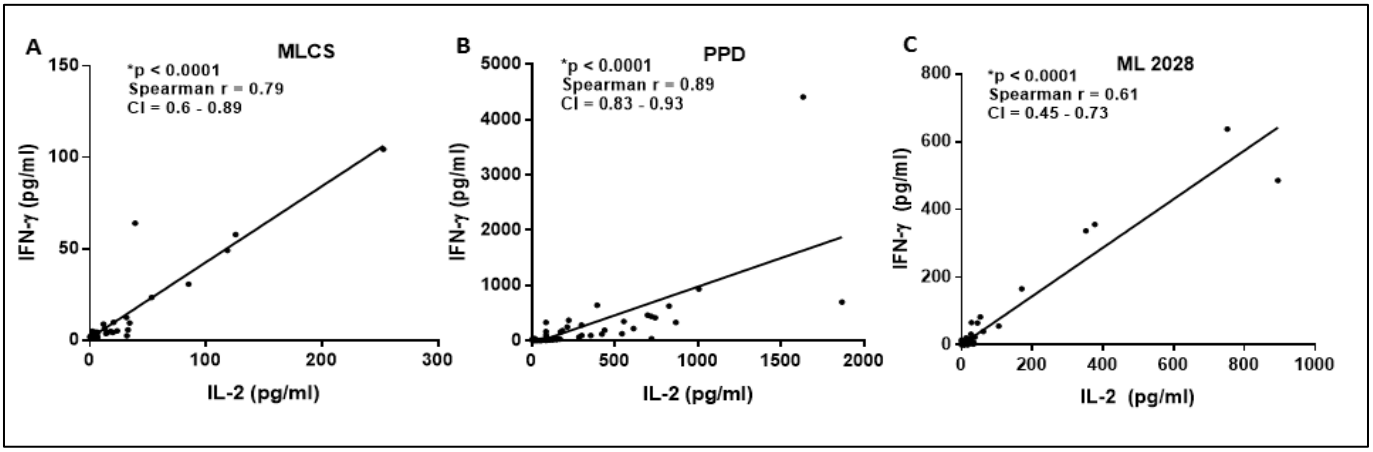

Figure 3. Correlation among supernatant cytokines. Cytokine concentrations from PB (n = 23) and MB n = 28) leprosy patients and HHC (n = 23) samples stimulated with MLCS (A), PPD (B) and ML2028 (C) were analyzed by Luminex Technique and correlation between the cytokines (IFN-γ and IL-2) values were determined by Spearman test. CI = Confidence Interval. *Asterisks indicate statistically significant correlations, at a *p* value < 0.05.

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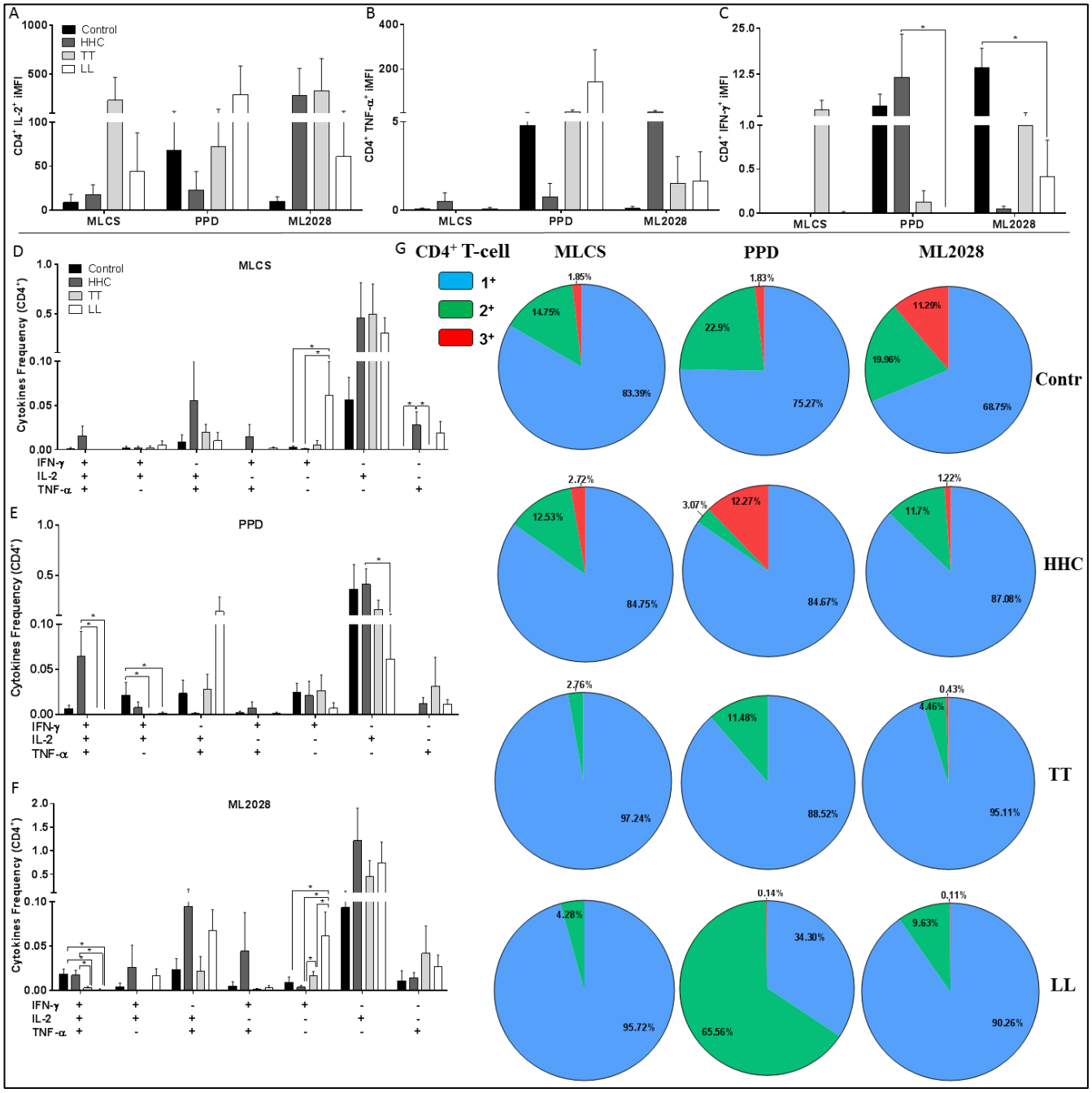


Figure 4. Frequency of multifunction CD4$^+$T cells expression stimulated with crude (MLCS and PPD) and recombinant antigen ML2028 by flow cytometry. The iMFI of CD4$^+$IL-2$^+$ (A), CD4$^+$TNF-α$^+$ (B) and IFN-γ$^+$ (C). The frequency of cells expressing each of the seven possible combinations of IFN-g$^+$, IL-2$^+$ or TNF-α$^+$in PBMC of Contr (control), HHC, TT and LL patients, stimulated with D) MLCS, E) PPD and F) ML2028.

G) The fraction of the total response comprising cells expressing all three cytokines (3^+), any two cytokines (2^+) or any one cytokine (1^+). Statistical analyzes determined by Mann-Whitney and T test. *Asterisks indicate statistically significant differences between groups at a p value < 0.05.

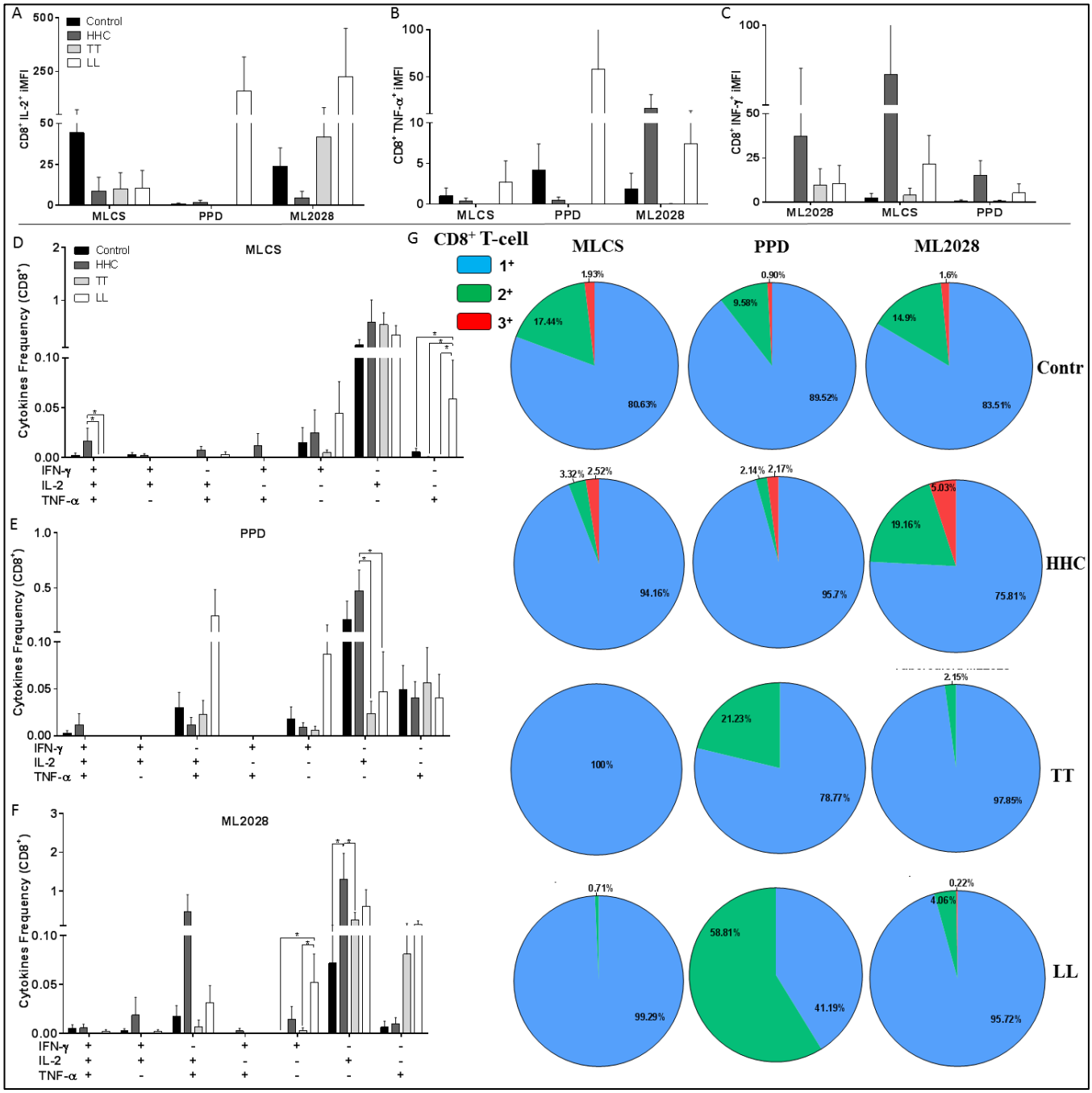
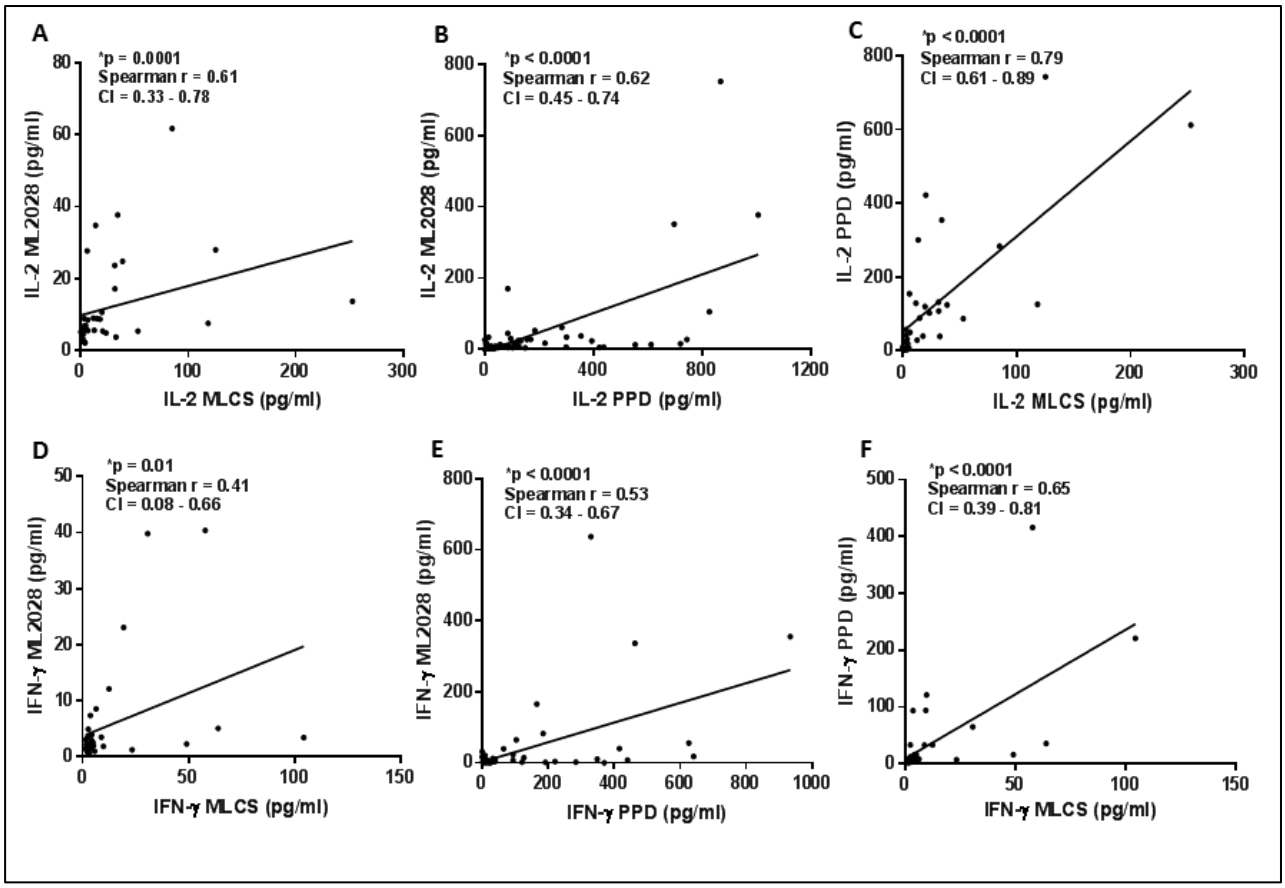


Figure 5. Frequency of multifunction CD8$^+$T cells expression stimulated with crude (MLCS and PPD) and recombinant antigen ML2028 by flow cytometry. The iMFI of CD8$^+$IL-2$^+$ (A), CD8$^+$TNF-α$^+$ (B) and IFN-γ$^+$ (C). The frequency of cells expressing each of the seven possible combinations of IFN-g$^+$, IL-2$^+$ or TNF-α$^+$in PBMC of Contr (control), HHC, TT and LL patients, stimulated with D) MLCS, E) PPD and F) ML2028. G) The fraction of the total response comprising cells expressing all three cytokines (3$^+$),

any two cytokines (2^+) or any one cytokine (1^+). Statistical analyzes determined by Mann-Whitney and T test. *Asterisks indicate statistically significant differences between groups at a p value < 0.05.



Supplementary Figure 1. Correlation among supernatant cytokines. Cytokine concentrations from PB (n = 23) and MB n = 28) leprosy patients and HHC (n = 23) samples stimulated with MLCS, PPD and ML2028 were analyzed by Luminex Technique and correlation between the cytokines (IFN-γ and IL-2) values were determined by Spearman test. CI = Confidence Interval. *Asterisks indicate statistically significant correlations, at a *p* value < 0.05.

ANEXOS

A) Questionário Investigativo

QUESTIONÁRIO	
Estudo Imunológico e Genético na Hanseníase	
1. Nº Estudo: ___ ___ ___ - ___ ___ 3. Classificação: 2. Família de Relação___ ___ ___ CASO □ ÍNDICE (1) □ CONTROLE ÍNDICE (2) □ PARENTE do CASO (3)	
IDENTIFICAÇÃO	
4. Se **PARENTE**: Relação com caso índice:	□ Pai/Mãe (1) □ Irmãos (2) □ Filho(a) (3) □ Avós (4) □ Primos (5) □ Sobrinho(a) (6) □ Tio(a) (7) □ Cônjuge (8) □ Não se aplica (99)
5. Data de Nascimento _____/_____/_____ (dd/mm/aaaa) 8. Raça: □ branca(1) □ negra(2) □ parda(3) □ indígena(4)	6. Idade: _____ anos 7. Sexo: □ F (1) □ M (2)
9. Escolaridade: □ Analfabeto (1) □ Ensino fundamental (completo/incompleto) (2) □ Ensino médio (completo/incompleto) (3) □ Ensino superior (completo/incompleto) (4)	
10. Ocupação:	11. □ Urbana (1) □ Rural (2)
12. Endereço:	13. Telefone:
14. Cidade:	15. Estado:

FATORES AMBIENTAIS	
16. Já trabalhou como caçador ou acompanhou-os?	□ Sim (1) □ Não (2) □ NS (3) □ NA(99)
17. Caso a resposta seja **afirmativa**, por quanto tempo (aproximado)?	____ meses □ NS (3) □ NA(99)
18. Que tipo de animal caçou?	_____ ou □ NA(99) [Se 16 for (2)]
19. Tem luz elétrica na casa?	□ Sim (1) □ Não (2)
20. Tem água encanada?	□ Sim (1) □ Não (2)
21. Tem rede de esgoto?	□ Sim (1) □ Não (2)
22. Etilismo?	□ Sim (1) □ Não (2)
23. Caso a resposta seja afirmativa, tempo total?	____ meses ou □ NA(99) [Se 22 for (2)]
24. . Caso a resposta seja afirmativa, descreva a quantidade (ml) /semana _____, tipo de bebida_____	
25. Ex-Etilista?	□ Sim (1) □ Não (2) ou □ NA(99)
26. Caso a resposta seja afirmativa, há quanto tempo parou?	____ meses ou □ NA(99) [Se 24 for (2)]
27. Há quanto tempo mora na mesma casa que o [caso índice]?	_____ meses ou □ NA(99)
28. Há mais alguém [além do caso índice] com Hanseníase na família?	□ Sim (1) □ Não (2)
29. Caso a resposta seja **afirmativa**, quantos parentes?	_____ parentes ou □ NA(99) [Se 27 for (2)]
30. Caso a resposta seja **afirmativa**, qual o grau de parentesco?	□ Pai/Mãe (1) □ Irmãos (2) □ Filho (a) (3) □ Avô (ó) (4) □ Primo (a) (5) □ Tio (a) (7) □Sobrinho(a) (6) □ Cônjuge (8) □ Outro (10) □ NA (99) [Se 27 for (2)]
31. Esta(as) pessoa(as) **RESIDEM** na mesma casa que você?	□ Sim (1) □ Não (2) □ NA(99) □ Reside na mesma casa, mas não é parente (4)
32. Você já recebeu BCG?	□ Sim (1) □ Não (2) □ NS (3)
33. Você já recebeu a segunda dose da BCG?	□ Sim (1) □ Não (2) □ NS (3)

34. Algum dos seus parentes recebeu a 2ª dose da vacina BCG?	□ Sim (1) □ Não (2) □ NS (3)
35. Caso a resposta acima seja **afirmativa**, quais familiares?	□ Pai/Mãe (1) □ Irmãos (2) □ Filho(a) (3) □ Avós (4) □ Primos (5) □ Sobrinho(a) (6) □ Tio(7) □ Cônjuge(8) □ NA(99) □ Outro(10)

HEREDOGRAMA

HISTÓRIA DA DOENÇA PREGRESSA	
36. Já teve lesão cutânea da Hanseníase anteriormente? Se a resposta for **NÃO**, passe para a **questão 44**.	□ Sim (1) □ Não (2) □ NS (3) □ NA(99)
37. Caso a resposta seja **afirmativa**, qual foi a data de início? _____ / _____ (mm/aaaa)	OU a idade de início? _____ anos □ NA(99)
38. Caso a resposta seja **afirmativa**, apresentou quantas lesões?	_____ lesões cutâneas □ NA(99)
39. Tem até hoje cicatrizes cutâneas características de Hanseníase?	□ Sim (1) □ Não (2) □ NS (3) □ NA(99)
40. Você recebeu tratamento para a Hanseníase?	□ Sim (1) □ Não (2) □ NS (3) □ NA(99)
41. Caso a resposta seja **afirmativa**, você completou o tratamento?	□ Sim (1) □ Não (2) □ NS (3) □ NA(99)
42. Quanto tempo durou o seu tratamento?	_____ meses □ NA(99)
43. Desenvolveu algum tipo de Reação?	□ Sim (1) □ Não (2) □ NS (3) □ NA(99)
44. Caso a resposta seja **afirmativa**, quando desenvolveu (em relação ao tratamento)?	□ Antes(1) □ Durante(2) □ Depois(3) □ NA(99)

HISTÓRIA DA DOENÇA ATUAL	
45. Observa-se lesão cutânea ativa?	□ Sim (1) □ Não (2) □ NS (3) □ NA(99)
46. Caso a resposta seja **afirmativa**, apresenta quantas lesões?	_____ lesões ou □ NS(3*) □ NA(99)
47. Caso a resposta seja **afirmativa**, há quanto tempo apresenta essas lesão(ões)?	_____ (meses) ou □ NS(3*) □ NA(99)
48. Você está tratando a Hanseníase ativa? Caso sim responder a questão 77	□ Sim (1) □ Não (2) □ NA(99)
49. Houve confirmação da Hanseníase por biópsia?	□ Sim (1) □ Não (2) □ NS (3) □ NA(99)
50. Qual o nome do Hospital/UBS que recebe atendimento?	_____ ou □ NS (3) □ NA(99)
51. Qual a Forma Clínica?	□ HI (1) □ HT (2) □ HDT (3) □ HD (4) □ HDV (5) □ HV(6) □ Neural (7) □ NA(99)
52. Qual o esquema terapêutico ou drogas utilizadas?	□ PQT/PB (1) □ PQT/MB (2) □ NS (3) □ NA(99)

53. Tem a marca da vacina BCG?	□ Sim (1) □ Não (2) □ NS (3) □ NA(99)
54. Apresenta sangramento ou formação de crostas nasais?	□ Sim (1) □ Não (2) □ NS (3) □ NA(99)
55. Grau de incapacidade avaliado no exame neurológico:	□ Grau Zero (1) □ Grau 1 (2) □ Grau 2 (3) □ Grau 3 (4) □ NA(99)

INFORMAÇÃO ADICIONAL
_____ _____ _____ _____ _____

DETECÇÃO DE DOENÇAS ALÉRGICAS - <u>ASMA</u>	
56. Alguma vez na vida, você teve sibilos (chiado no peito)? Se a resposta for **<u>NÃO</u>**, passe para a **questão <u>59</u>**.	□ Sim(1) □ Não(2) □ NS(3) □ NA(99)
57. Nos últimos 12 (doze) meses, você teve sibilos (chiado no peito)? Se a resposta for **<u>NÃO</u>**, passe para a **questão <u>59</u>**.	□ Sim(1) □ Não(2) □ NS(3) □ NA(99)
58. Nos últimos 12 (doze) meses, quantas crises de sibilos você teve?	□ Nenhuma(1) □ 1 a 3 crises(2) □ 4 a 12 crises (4) □ + de 12 (5) □ NS(3) □ NA(99)
59. Nos últimos 12 meses, com que freqüência você teve seu sono perturbado por chiado no peito?	□ Nunca acordou com chiado (1) □ Menos de 1 noite por semana (2) □ 1 ou mais noites por semana (4) □ NS(3) □ NA(99)
60. Nos últimos 12 meses seu chiado foi tão forte a ponto de impedir que você conseguisse dizer mais de duas palavras entre cada respiração?	□ Sim(1) □ Não(2) □ NS(3) □ NA(99)
61. Alguma vez na vida você teve asma?	□ Sim(1) □ Não(2) □ NS(3) □ NA(99)
62. Nos últimos 12 meses você teve chiado no peito após exercícios físicos?	□ Sim(1) □ Não(2) □ NS(3) □ NA(99)
63. Nos últimos 12 meses você teve tosse seca à noite, sem estar gripado ou com infecção respiratória?	□ Sim(1) □ Não(2) □ NS(3) □ NA(99)

DETECÇÃO DE DOENÇAS ALÉRGICAS – <u>RINITE</u> (13 A 14 anos) OBS: Todas as perguntas são sobre problemas que ocorreram quando você não estava gripado ou resfriado!	
64. Alguma vez na vida você teve problema com espirros ou coriza (corrimento nasal), quando não estava resfriado ou gripado? Se a resposta for **<u>NÃO</u>**, passe para a **questão <u>67</u>**.	□ Sim(1) □ Não(2) □ NS(3) □ NA(99)
65. Nos últimos 12 meses você teve algum problema com espirros, coriza (corrimento nasal) ou obstrução nasal quando não estava gripado ou resfriado? Se a resposta for **<u>NÃO</u>**, passe para a **questão <u>67.</u>**	□ Sim(1) □ Não(2) □ NS(3) □ NA(99)
66. Nos últimos 12 meses esse problema nasal foi acompanhado de lacrimejamento ou coceira nos olhos?	□ Sim(1) □ Não(2) □ NS(3) □ NA(99)
67. Nos últimos 12 meses, quantas vezes suas atividades diárias foram atrapalhadas por esse problema nasal?	□ Nada(1) □ Um pouco(2) □ Moderado(4) □ Muito (5) □ NS(3) □ NA(99)
68. Alguma vez na vida você teve rinite alérgica?	□ Sim(1) □ Não(2) □ NS(3) □ NA(99)

DETECÇÃO DE DOENÇAS ALÉRGICAS – DERMATITE ATÓPICA	
69. Alguma vez na vida você teve manchas com coceira na pele,que apareciam e desapareciam por pelo menos 6 meses? Se a resposta for **não**, passe para a **questão 73**.	□ Sim(1) □ Não(2) □ NS(3) □ NA(99)
70. Nos últimos 12 meses você teve essas manchas na pele (eczema)? Se a resposta for **não**, passe para a **questão 73**.	□ Sim(1) □ Não(2) □ NS(3) □ NA(99)
71. Alguma vez essas manchas com coceira afetaram algum dos seguintes locais: dobras dos cotovelos, atrás dos joelhos, na frente dos tornozelos, abaixo das nádegas ou em volta do pescoço, orelhas ou olhos?	□ Sim(1) □ Não(2) □ NS(3) □ NA(99)
72. Alguma vez essas manchas com coceira (eczema) desapareceram completamente nos últimos 12 meses?	□ Sim(1) □ Não(2) □ NS(3) □ NA(99)
73. Nos últimos 12 meses, quantas vezes, aproximadamente, você ficou acordado à noite por causa de coceira na pele?	□ Nunca nos últimos 12 meses (1) □ Menos de 1 noite por semana (2) □ 1 ou mais noites por semana (4) □ NS(3) □ NA(99)
74. Alguma vez você teve eczema?	□ Sim(1) □ Não(2) □ NS(3) □ NA(99)

EXAMES COMPLEMENTARES			
75. Baciloscopia	□ Positiva(1) □ Negativa(2) □ NA(99)		
76. Biópsia (descrever)			
77. Parasitológico de Fezes:	1º Exame	2º Exame	3º Exame
78. Há quantos meses trata a Hanseníase ativa?	_____(meses)		
79. É Tabagista? Se a resposta for sim responda a questão seguinte	□ Sim(1) □ Não(2)		
80. Quantos cigarros diários?	_____		

DADOS DA ENTREVISTA	
DATA:	_____ / _____/ _____ (dd/mm/aaa)
ENTREVISTADOR:	

B) CONSENTIMENTO INFORMADO PARA O ESTUDO DA RESPOSTA IMUNE

Nome do Projeto: Estudo Imunológico e Genético na Hanseníase

NOME DO PACIENTE: _____
Registro.HU:_____ Nº: __ __ __ - __ __

Investigador Principal: Amelia Ribeiro de Jesus, médica, Hospital Universitário Rua Cláudio Batista S.N, Bairro Sanatório, Aracajú,-Brazil., Tel: (79)3218-1805.

Convite e Objetivo:

Você é convidado(a) a participar de um estudo que tem como objetivo entender porque as pessoas têm Hanseníase. Este estudo incluirá 90 pessoas com esta doença que apresentam formas diferentes de feridas na pele. Além das informações deste documento você pode perguntar tudo sobre o estudo ao seu médico. Caso decida participar do estudo você será solicitado(a) assinar este formulário de consentimento.

Participação voluntária: A sua participação é voluntária. Você pode decidir não participar do estudo em qualquer momento, sem perder os benefícios dos cuidados médicos prestados e de seu tratamento. Caso, após aceite participar, resolva descontinuar sua participação, isto será feito sem qualquer prejuízo para você. Participando ou não do estudo você receberá o medicamento utilizado para o tratamento da Hanseníase.

Finalidade do estudo: Este estudo vai estudar como o seu corpo se defende quando atacado pela bactéria que causa esta doença. Para isto estudaremos o seu sangue e uma parte do exame de biópsia de sua ferida na pele.

Procedimentos: Caso você concorde em participar do estudo, além de ser examinado por um médico clínico, realizar biópsia da lesão, teste intradérmico e exame de secreção de sua orelha, métodos que são necessários para o diagnóstico da doença, você doará 40ml de sangue (mais ou menos 3 colheres de sopa) para a pesquisa dos mecanismos de defesa do organismo. A retirada do pedaço da pele ou da ferida para diagnóstico da sua doença será feita com anestesia para você não sentir dor e parte deste material poderá ser utilizado para os estudos da defesa do seu corpo contra a bactéria que causa a doença. Caso o

diagnóstico de Hanseníase não seja confirmado, todo o material obtido para pesquisa será destruído.

Duração do estudo: Após a assinatura do termo de consentimento sua participação no estudo é de 5 anos, a contar do primeiro dia de tratamento, caso você tenha Hanseníase. Periodicamente, você será examinado para determinar a cura da doença ou necessidade de utilização de novo tratamento, que também lhe será fornecido gratuitamente.

Confidencialidade: Qualquer informação obtida durante este estudo só será do conhecimento da equipe médica e do órgão que protege o indivíduo em pesquisas (Comitê de ética do Hospital Universitário) Você e qualquer participante desse estudo não será identificado por nome nas publicações dos resultados do estudo. Apenas os representantes do Comitê de Ética em Pesquisa poderão ver sua ficha clínica.

Análises de riscos e benefícios: A retirada de seu sangue e de um pedaço da ferida são feitos se você tiver ferida, ainda antes do tratamento, para confirmar o diagnóstico da doença. Dor leve na retirada de sangue devido à punção com agulha pode ocorrer. Em casos raros a retirada de sangue provoca sangramento ou mancha roxa na pele. Como anestesia local é utilizada, a retirada de um pedaço da ferida não é acompanhada de dor. O tratamento que você receberá é igual ao que todos os pacientes receberão participando ou não do estudo. A participação lhe trará como benefício um acompanhamento clínico mais freqüente. Um médico lhe visitará em sua casa para examinar também sua família. Você deve retornar às consultas médicas regularmente de acordo com marcação de seu cartão do Ambulatório do HU

Retorno de benefícios para o sujeito e para a sociedade: A Hanseníase é relacionada a reação do seu organismo contra a bactéria que causa a doença e o conhecimento destas reações do seu corpo pode contribuir não só para o entendimento da doença como para o aparecimento de novas formas de tratamento ou controle os sintomas e também formas de previnir a doença.

Custos: Você não terá custos com o tratamento. Você não receberá pagamento por sua participação neste estudo.

Esclarecimentos: Caso você precise de atendimento médico durante o estudo, você pode contactar um dos seguintes Médicos pelo telefone (79)3237-7353: Dra. Amélia Ribeiro de Jesus Dr. Emerson Ferreira da Costa ou Dr. Roque Almeida. Caso você queira saber alguma coisa sobre seus direitos e de seu filho, como paciente, você pode procurar o Comitê de Ética do Hospital Universitário, cujo endereço encontra-se no inicio deste consentimento ou pelo telefone (79) 3218-1805.

Consentimento: Se você leu o consentimento informado ou este lhe foi explicado e você concorda em participar do estudo, favor assinar o nome abaixo. A você será entregue uma cópia deste formulário para guardar.

_____	_____	_____
Assinatura do participante	Data	Hora
_____	_____	_____
Assinatura do pesquisador	Data	Hora
_____	_____	_____
Assinatura da testemunha (apenas analfabetos)	Data	Hora

C) FORMULÁRIO DE CONSENTIMENTO PARA MENORES DE IDADE (MENORES DE 18)

ESTUDO DA RESPOSTA IMUNE

Nome do Projeto: Estudo Imunológico e Genético na Hanseníase

NOME DO PACIENTE: _____
Registro.HU:_____ Nº: __ __ __ - __ __

Investigador Principal: Amelia Ribeiro de Jesus, médica, Hospital Universitário Rua Cláudio Batista S.N, Bairro Sanatório, Aracajú,-Brazil., Tel: (79)3218-1805.

Convite e objetivo: Você está sendo convidado a participar de um estudo científico para determinar as razões porque pessoas desenvolvem Hanseníase. Nós perguntaremos a você sobre a sua saúde. Um médico fará exame físico em você, incluindo boca e nariz. Isto não causará dor em você. Então, nós tiraremos um pouco de sangue (cerca de duas colheres de sopa) de seu braço usando uma seringa e agulha descartáveis para realizar alguns exames que ajudarão a explicar a doença. Nós também iremos fazer um teste na pele, onde nós injetaremos uma pequena quantidade de líquido (duas gotas) no seu braço usando uma agulha fina. Nós também vamos precisar remover um pequeno pedaço da pele ou do nariz para confirmar se você tem a doença. Isso será feito por um médico no hospital, com anestesia local para evitar dor. Nós esperamos através deste estudo esclarecer mais sobre a doença, entendê-la e assim poderemos preveni-la no futuro.
Você pode não participar deste estudo. Se você quer nos ajudar, por favor, assine ou coloque sua impressão digital abaixo.

_____ _____ _____
Assinatura ou impressão do paciente Data Hora

_____ _____ _____
Assinatura ou impressão do responsável Data Hora

_____ _____ _____
Testemunha Data Hora

_____ _____ _____
Pesquisador Data Hora