UNIVERSIDADE FEDERAL DE UBERLÂNDIA

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PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

PERFIL IMUNOLÓGICO DE PACIENTES COM COCCIDIOIDOMICOSE CRÔNICA E PÓS-CURA E NOVOS REAGENTES IMUNOBIOLÓGICOS APLICADOS AO DIAGNÓSTICO DA DOENÇA

FAUSTO EMILLIO CAPPARELLI

UBERLÂNDIA – MG

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Luiz Ricardo Goulart Filho

Dedico este trabalho

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AIDS	Síndrome da Imunodeficiência Adquirida)
Ag2/PRA	(Antigeno coccidioidal rico em prolina)
APC	(Células Apresentadoras de Antígeno)
Cy7	(Corante de cianina 7)
BAD-1	Adesina e fator de virulência-1
BALB/c	Albino Inbred (Espécie de camundongo)
BFA	Brefeldina- A
BLG2	Gene da β-glucosidase
cDNA	DNA complementar
CD3	Cluster de Diferenciação 3
CD11c	Cluster de Diferenciação 11c
CD14	Cluster de Diferenciação 14
CD19	Cluster de Diferenciação
CD206	Cluster de Diferenciação 206 (Receptor de Mannose)
CD209	Cluster de Diferenciação 209 (DC-SIGN)
CR3	Receptor Complementar 3
C57BL/6	C57 black 6.
DBA/2	Diluted Brown Non-Agouti Mouse (Espécie de camundongo)
DC-SIGN	Dendritic Cell-Specific intercellular adhesion molecule-3-Grabbing non-integrin
FBS	Soro Fetal Bovino
FITC	Fluoresceína Isotiocianato
HIV	Vírus da Imunodeficiência Humana
HLA-DR	Antígeno Leucocitário Humano
IL-2	Interleucina 2
IL-4	Interleucina 4
IL-10	Interleucina 10
IL-12	Interleucina 12
IFN-γ	Interferon gama
IGG	Imunoglobulina G
IGM	Imunoglobulina M
ISH	Hibridização in-situ
kDa	Kilo-dalton
MCP-1	Proteína Quimiotática de Monócito -1
MEP-1	Metaloproteinase 1
MHC II	Complexo Principal de Histocompatibilidade II
MyD88	Gene da resposta primária de diferenciação mielóide 88
OD	Densidade Otica
OPD	*o*-Phenilenodiamina dihidroclorido
pAb	Anticorpo policlonal
PAMP	Padrão Molecular Associado à Patógeno
PBMC	Células Mononucleares de Sangue Periférico

PCR	Reação em cadeia da Polimerase
PE/Cy5	Corante de Ficoeritrina cianina 5
PerCP/Cy5.5	Complexo proteico Peridinina-chlorofil /cianina 5.5
PFA	Paraformaldeído
PRR	Receptor de Reconhecimento Padrão
pIII	Proteína 3
pVI	Proteína 6
pVII	Proteína 7
pVIII	Proteína 8
pIX	Proteína 9
Qdot 605	Quantum dot 605
Qdot 655	Quantum dot 655
RNA	Acido Ribonucleico
RPMI	Meio de cultura Roswell Park Memorial Institute
RT	Temperatura ambiente
scFv	Fragmento variável de cadeia simples
SOWgp	Glicoproteína da parede externa da esférula
TEMED	Tetrametiletilenodiamina
TH1	T helper 1
TH2	T helper 2
TLR2	Receptor do tipo Toll 2
TLR3	Receptor do tipo Toll 3
TLR4	Receptor do tipo Toll 4
TLR5	Receptor do tipo Toll 5
TLR7	Receptor do tipo Toll 7
TLR8	Receptor do tipo Toll 8
TLR9	Receptor do tipo Toll 9
TNF-α	Fator de Necrose Tumoral alfa
VH	Cadeia pesada
Vκ	Cadeia leve κ
Vλ	Cadeia leve λ

Apresentação

Coccidioidomicose, conhecida também como Febre do Vale, é uma doença causada pelos fungos geofílicos e dimórfico térmicos das espécies *Coccidioides immitis* e *Coccidioides posadasii*. Estudos mostram que o fator climático possui um papel importante para o desenvolvimento da Coccidioidomicose, já que os maiores índices de infecção ocorrem em regiões áridas e semi-áridas dos Estados Unidos, México e América do Sul.

Em relação a taxa de incidência, dados epidemiológicos revelam que a doença tem aumentado drasticamente nas últimas décadas nos estados do Arizona e Califórnia. No entanto, no Brasil, somente a poucas décadas a região semi-árida do nordeste foi incluída como área endêmica. O pouco conhecimento da coccidioidomicose e prováveis diagnósticos errôneos como pneumonias inespecíficas, tuberculoses ou mesmo pneumoconiose e silicose podem ter dificultado o diagnóstico da doença.

A infecção ocorre pela inalação de artroconídios infectantes presentes no solo que acabam por se instalar no pulmão, o qual é considerado fonte primária de infecção. As manifestações clínicas da micose ocorrem sob três formas clínicas principais: forma pulmonar primária, forma pulmonar progressiva ou forma disseminada, sendo que, na forma primária 60% das pessoas infectadas com o fungo permanecem assintomáticos ou com a presença de leves sintomas respiratórios. Além disso, a semelhança dos sintomas clínicos da coccidioidomicose com outras doenças respiratórias dificulta o reconhecimento da doença, necessitando, portanto, de novas metodologias de reconhecimento.

Uma das formas de identificar a infecção é através do teste cutâneo contra *Coccidioides*. A reação de hipersensibilidade tardia após o teste com o antígeno coccidioidal tem sido associado à uma resposta celular, e a forte resposta imune é considerada fator crítico para o controle da infecção. Estudos clínicos mostraram que há uma estreita relação entre a resposta imune coccidioidal específica e a severidade da infecção clínica. Pacientes com HIV ou

sob terapia por longo período a base de corticóides, por exemplo, possuem maiores chances de desenvolver sintomas severos da coccidioidomicose. Além disso, estudos *in vitro* relatam que a liberação da citocina interferon-gama pelas células aumenta o poder fungicida/efetor dos monócitos, enquanto que a liberação de Il-10 está associada com a maior chance de adquirir e disseminar o fungo.

A disseminação da doença ou o sucesso do *Coccidioides* como patógeno, contudo, principalmente em imunocomprometidos, está relacionado com a capacidade do fungo em alterar a composição de sua parede celular possibilitando assim, um escape do sistema imune. Uma via pela qual isso ocorre é promovendo o desequilíbrio do balanço Th1/Th2. Estudos em modelo de camundongos têm mostrado que o direcionamento da resposta para a via Th2 aumenta o sucesso de infecção pelo fungo. Dessa forma, a alteração de algumas proteínas e carboidratos da parede celular do fungo, considerados como padrões moleculares associados aos patógenos (PAMPs), promovem a virulência e o escape imune. Assim, estudar a imunologia da doença é um aspecto importante para conhecer a patologia e desenvolver as melhores formas terapêuticas.

Phage display tem sido considerado uma ferramenta poderosa para a seleção de peptídeos miméticos ou ligantes para epítopos de superfície celular, bem como de fragmentos de anticorpos, como moléculas de fragmentos variável de cadeia simples (scFv) com potencial aplicação clínica. Por apresentar sequências randômicas de peptídeos na superfície dos fagos, essa metodologia tem sido utilizada para gerar mimetopos para diagnóstico, vacina ou com ação bloqueadora a diversos patógenos como vírus, bactérias e fungos.

Assim, o objetivo deste trabalho foi postular que exista um possivel escape imune por parte do fungo *Coccidioides*, analisando por meio do perfil imunológico dos diferentes grupos de pacientes (saudáveis, curados e crônicos) estímulados com antígenos coccidioidais (T27K, endosporo e esférula). Além disso, produzimos por *Phage display* prováveis biomoléculas que poderiam vir a

estimular uma resposta imune (mimetopos e fragmentos de anticorpos-scFv) como uma alternativa de terapêutica para combater a infecção. A inovação desse estudo se fundamenta em analisar três tipos diferentes de estímulo sobre PBMCs em três diferentes grupos de pacientes ao mesmo tempo e ainda por ser o primeiro grupo a buscar uma nova alternativa (Phage Display) de desenvolvimento de novas biomoleculas a serem utilizadas como terapêutica ou detecção da coccidioidomicose.

Fundamentação teórica:

1. Coccidiodomicose

1.1 Aspectos gerais e Epidemiologia:

Coccidioidomicose é uma doença que acomete humanos e animais causada por *Coccidioides immitis/posadasii,* um fungo presente no solo [1] . Esta doença foi primeiramente reconhecida e relatada por um estudante de medicina na Argentina, Alejandro Posadas, em 1892 [2]. Posteriormente, Thorne and Rixford, em 1894, apresentaram dois casos clinícos semelhantes no estado da California, EUA [1]. C.immitis e C. posadasii causam coccidioidomicose tanto em humanos como em animais, o que, para constar, sao doenças aparentemente indistintas quando se considera manifestações clínicas e resposta imune de hospedeiro. As espécies são classificadas em agentes de nivel 3 de biosegurança por manual a biobliografia [3].

A recente história da Coccidioidomicose é relatada por Smith e colaboradores [4]. Eles desenvolveram um importante diagnóstico, o teste cutâneo de coccidiodina e o teste sorológico, que permanece útil até os dias de hoje. Em um dos seus estudos com os soldados no campo do Vale de São Joaquim, Smith foi capaz de demonstrar que 60% dos novos casos de infecção coccidioidal eram completamente assintomáticos. Além disso, eles demostraram que as pessoas que perderam ou nunca demostraram reatividade com o teste cutâneo eram mais propensas a desenvolver coccidioidomicose disseminada pela região toráxica [4].

Coccidioides immitis aparentemente possui a menor distribuição biogeografica quando comparado com *C. posadasii*, ficando assim centralizada no Vale de São Joaquim, Califórnia. Nesta região *C. immitis* parece ser a única espécie, no entanto dois isolados clínicos de *C. posadasii*, Silveira e K-727, foram recuperadas destas áreas. Silveira foi isolada de um paciente em Bakersfield em 1951 e K-727 de uma paciente em 1992 [5]. Estima-se, que todo

ano nos Estados Unidos, 150 mil pessoas tornam-se infectadas com *Coccidioides ssp*. e 50 mil desenvolvem sintomas. No caso dos estados do Arizona e California, estudos têm mostrado que a coccidioidomicose aumentou drasticamente, com taxa de incidência de 97,8% e 91,1% dos anos de 2001-2006, nos dois estados, respectivamente [6].

Mais recentemente, o Brasil foi incluido como uma área endêmica, incluindo os estados de Ceará, Piauí, Bahia e sul do Maranhão [7]. No Brasil, os primeiros casos foram registrados no final da década de 70, mas o país só foi incluído como região endêmica para coccidioidomicose em 1994 após surtos da doença no Piauí e Ceará [8]. Em 1991 em Oeiras (PI) foi registrada a primeira microepidemia brasileira [7]. No entanto, a real incidência no Brasil ainda é desconhecida, provavelmente por três razões: por não ser uma doença altamente notificada, por ser erroneamente diagnosticada (não mais nos dias atuais), assim como a tuberculose, a qual é endêmica no Brasil e por apresentar altas porcentagens de pacientes assintomáticos [8].

1.2.Ciclo de vida e Transmissão

Coccidioides é um fungo presente no solo, em que o homem é eventualmente o hospedeiro final, apesar de que outroas animais como cavalo, tatu, gato e cães também desenvolverem coccidioidomicose. No solo, o fungo existe como filamentos de hifas septadas (Fig. 1), os quais posteriormente são degenerados e liberados na forma de artroconídios. Esses artroconídios apresentam-se em forma de barris e possuem um tamanho de 2 X 5μm, o que facilita a dispersão e aumenta a probabilidade de atingir os brônquios após a inalação por um hospedeiro sensível [8].

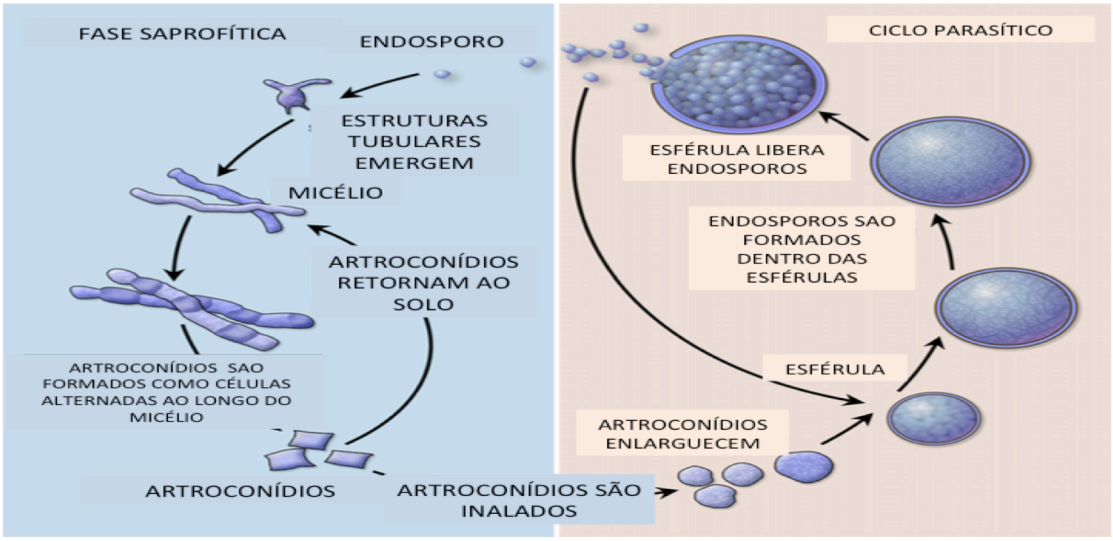

Fig. 1 Ciclo de vida *Coccidioides immitis/posadasii.* Filamentos de micelas crescem no solo e formam artroconidios. Após ser disperso pelo ar os artroconidios podem ser re-implantados no solo ou ser inalado pelo hospedeiro, os quais se transformam em esferula no pulmão. A esferula libera endosporo, o qual se desenvolve em esferula novamente dentro do tecido do hopedeiro. Extraído de DiCaudo, 2006.

Uma vez dentro do hospedeiro, o fungo passa por uma profunda alteração morfológica em que a parede externa fratura-se e o interior da parede espessa-se. O aumento da temperatura e da concentração de CO2 [9], o decréscimo do pH, e uma interação com os fagócitos [10] tendem a facilitar essa metamorfose. Este processo leva à formação da esférula que pode crescer até 120 μm, e apresenta em seu interior com múltiplos segmentos, chamados de endosporos, os quais são liberados após o rompimento da esférula, desenvolvendo-se em uma nova esférula no interior do hospedeiro, reiniciando seu ciclo [11].

1.3.Fatores de risco para desenvolver Coccidioidomicose severa e disseminada

Existem muitos fatores de risco que predispõem o hospedeiro a desenvolver coccidioidomicose. A susceptibilidade determinada geneticamente é um desses fatores. Estudos epidemiológicos tem mostrado que filipinos, afro-americanos, e latino-americanos possuem maior susceptibilidade a desenvolver

coccidioidomicose disseminada [12]. Verificaram que filipinos possuem 176 vezes mais chances de desenvolver a doença disseminada quando comparados com caucasianos; afro-americanos e mexicanos nascidos nos Estados Unidos possuem 14 e 3 vezes mais chances, respectivamente, do que caucasianos [12].

Embora a base genética para este aumento da susceptibilidade permaneça indefinida, os pesquisadores têm realizado estudos para avaliar polimorfismos genéticos que possam controlar ou estarem associados à predisposição de determinadas populações étnicas para Coccidoides. Louie et al. [13] examinaram a influencia genética do hospedeiro na severidade da doença entre os grupos de loci HLA de classe II e no tipo sanguíneo (ABO). Neste estudo verificou que entre os hispânicos a predisposição para os sintomas, bem como, para a severidade da doença é associada ao tipo sanguíneo A e B, respectivamente. Já os alelos HLA class II DRB1*1301 promovem a predisposiçao da severidade da doença, enquanto que os genes DRB1*0301-DQB1*0201 e DRB1*1501-DQB1*0602, diminuem o risco de obter coccidioidomicose severa em caucasianos e hispânicos como nos afro-americanos.

Outros fatores seriam o gênero, idade, gravidez e imunossupressão. Para o gênero, foi evidenciado que homens apresentam uma predisposição de desenvolver a doença severa de 3.5 a 5 vezes mais quando comparado às mulheres [14] e, que crianças com menos de 5 anos de idade e pessoas acima de 50 anos possuem maior susceptibilidade à doença em uma população indígena americana [15]. Já em relação a gravidez, estudos mostraram que mulheres em gestação possuem grandes chances de adquirir coccidioidomicose, principalmente no terceiro mês da gravidez [16]. Drutz et al [17] mostrou que a progesterona e o 17 β-estradiol estimulam o crescimento da esférula e endosporo *in vitro*. Além disso, a imunossupressão atribuida a mulheres grávidas poderia ser uma das razões do desenvolvimento da doença, assim como em pacientes imunossuprimidos, seja por medicamento ou outra

enfermidade como na Sindrome de Imunodeficiência Adquirida (AIDS)[12, 17, 18].

1.4.Manifestação Clínica

C. immitis é transmitido por uma rota respiratória. Na infecção pulmonar primária, cerca de 60% dos indivíduos infectados evoluem para a cura espontânea sem apresentar manifestações clínicas [19]. Os demais (40%) geralmente apresentam manifestações de doença respiratória aguda, simulando gripe, com febre, sudorese noturna, tosse e/ou dor torácica tipo pleurítica que pode evoluir para pneumonia. A intensidade dos sintomas depende diretamente da carga infectante, variando desde um estado gripal até o quadro de uma grave infecção respiratória inespecífica [20]. Nesta forma da doença apresentam-se com múltiplos infiltrados difusos, os maiores podendo apresentar cavidades que cursam com manifestações respiratórias graves, que podem levar a insuficiência respiratória, e são mais comumente observados em pacientes imunocomprometidos [20]. Além da doença pulmonar, a coccidioidomicose pode disseminar e causar doença miliar óssea e infecção conjunta, doença de pele, abcessos dos tecidos moles, e meningite. Estas complicações extrapulmonares são pouco frequentes (<5% das infecções) [21].

1.5. Diagnóstico e Tratamento

Apesar dos avanços proporcionados por técnicas moleculares e imunológicas recentemente, o isolamento e crescimento de *Coccidioides* ainda é considerado o padrão ouro em laboratórios de diagnóstico para coccidioidomicose [22]. Ensaios imunoenzimáticos, aglutinação de partículas de látex e imunodifusão podem ser usados como técnicas qualitativas, que produzem resultados positivos no início do curso da infecção. Ensaios imunoenzimáticos fornecem uma avaliação qualitativa rápida de IgM e IgG para a coccidioidomicose [23]. No entanto, reações positivas requerem outra confirmação por testes mais específicos.

Técnicas moleculares, como a hibridização in situ (ISH) e reação em cadeia da polimerase (PCR), podem auxiliar na identificação de *C. immitis* e *C. posadasii* em tecidos parafinados [24]. Nos casos em que os organismos não são morfologicamente distintos, ISH pode fornecer a confirmação específica em cortes histológicos. [25]. Foi desenvolvido ainda um teste para detecção direta de DNA de C. Posadassi em amostras de escarro por amplificação do gene altamente específico Ag2/PRA. Os resultados obtidos sugerem que a detecção direta da sequência de Ag2/PRA em amostras de escarro é um excelente método de diagnóstico rápido e específico de coccidioidomicose [24].

Testes cutâneos de hipersensibilidade tardia para antígeno coccidióidico são úteis em estudos populacionais, que avaliam as tendências globais na incidência da micose. No entanto, o teste cutâneo é de utilidade limitada no diagnóstico de uma infecção aguda. Um teste cutâneo positivo não distingue infecção atual de infecção prévia. Além disso, anergia pode se desenvolver durante a infecção ativa em alguns casos. Indivíduos com testes cutâneos positivos frequentemente mostram declínio na reatividade após cerca de 12 a 15 anos [26].

Para o tratamento da coccidioidomicose, drogas estão disponíveis. A anfotericina B tem sido usada para tratar a coccidioidomicose severa [27]. No entanto, a limitação da administração via intravenosa na meningite coccidioidal, tem requerido tratamentos que ultrapassem a barreira hemato-encefálica tal como terapia intratecal. Devido a esses problemas e à toxicidade da anfotericina B, antifungicos tais como azois tem sido utilizados. Vericonazol, bem como Fluconazol e intraconazol foram relatados como eficazes terapias primárias para a meningite coccidióidica [28].

Outras classes de antifúngicos também sao utilizadas. Caspofungina, um inibidor da sintase de 1,3-β-D-glucano, uma equinocandina, foi considerado eficaz no tratamento da coccidioidomicose em murinos [29]. Nicomicina Z, um inibidor da quitina sintase, também se apresentou eficaz no tratamento da coccidioidomicose [29].

2. Resposta immune

A resposta imune tem papel fundamental no combate a agentes infecciosos e constitui o principal impedimento para a ocorrência de infecções disseminadas. No caso da Coccidioidomicose, a imunidade celular é considerada o mecanismo chave para o controle da proliferação do fungo. Além disso, recentes estudos [30, 31] têm ajudado a elucidar o mecanismo pelo qual se promove a defesa celular [1].

A resposta imune inata é considerada a primeira linha de defesa após a inalação de artroconídios infectantes. Pesquisas têm mostrado que o influxo celular ocorre em resposta aos extratos solúveis derivados do fungo, o qual ativa o sistema complemento que atua com atividade quimiotática [10]. Células mononucleares, compostas principalmente por monócitos e macrófagos, realizam a fagocitose dos artroconídios [32], endosporo [33] e esférulas recém formadas [34], além de promoverem a apresentação de antígeno para os linfócitos T CD4+, o qual inicia a resposta adaptativa, conforme mostrado na Fig. 2 [30].

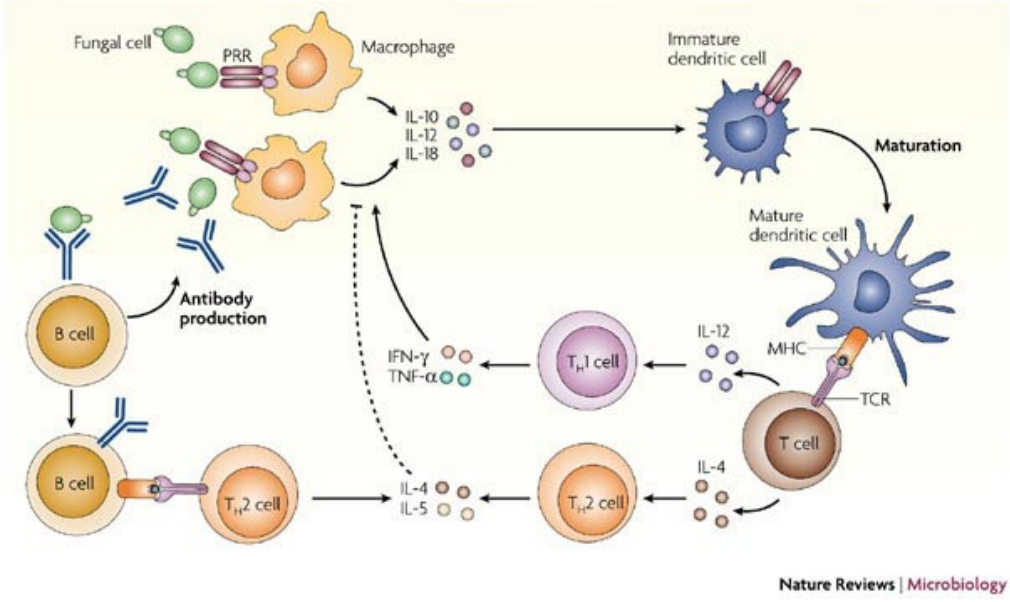

Fig. 2 Ilustração resposta celular contra fungos. Reconhecimento de moleculas conservadas nos patógenos (PAMPs) pelos receptores Toll-Like e apresentação antigênica pelas células apresentadoras de antigeno (APCs) para a ativação da resposta imune adaptativa. Figura extraída de Cutler *et al.* [37]

O reconhecimento do agente infeccioso pelas células do sistema imune inato ocorre via receptores de reconhecimento padrão (PRRs), tal como receptor Toll-like [35] e Dectina-1 [36]. Estes PRRs reconhecem os padrões moleculares associados ao patógeno e permitem a eliminação do mesmo [37].

Receptores Toll-like têm sido identificados como a maior classe de PRRs. Pesquisas mostram que os TLRs exibem um reconhecimento restrito. TLR-2, por exemplo, reconhece peptidoglicano, lipoproteinas bacterianas, zimosan, ao passo que TLR-3 reconhece dupla fita de RNA; TLR-4, lipopolissacarideos e proteinas de choque térmico (Heat-shock proteins); TLR-5, flagelina; TLR7/8 reconhece RNA de fita simples e, por fim, as sequencias CpG de DNA não metilado bacteriano são identificados por TLR-9 [35]. No entanto, a função dos TLRs na infecção por fungos ainda não foi totalmente esclarecida. Ampel [38], nos estudos da complexidade imunológica da coccidioidomicose humana mostrou, contudo, nos ensaios com células mononucleares do sangue periférico (PBMC) e monócitos aderentes, que TLR-2 e TLR-4 parecem mediar uma resposta imune celular em resposta ao estímulo com T27K, um antigeno presente em *Coccidioides*.

Dectina-1 é um receptor lectina do tipo C que reconhece β-glucano, um carboidrato presente na parede celular do fungo. Esse receptor reconhece diversos fungos tal como *Saccharomyces*, *Candida* e outros [31]. Na coccidioidomicose, há relatos de que β-glucano, carboidrato que corresponde a 60% do peso-seco da esferula, estimula dectina-1 de maneira dependente para a produção de citocinas pró-inflamatórias [30].

Já para resposta adaptativa, a estimulação de linfócitos T CD4+ na ativação de TH1 ao invés de TH2 parece ser crucial para a proteção contra *C. immitis.* Magge, 1995, mostrou que camundongos resistentes (DBA/2) à infecção por *Coccidioides* produz muito mais IFN-γ quando comparado com a linhagem susceptível (BALB/c), a qual libera mais IL-4. Além disso, ao tratar a linhagem susceptível com IFN-γ recombinante, este apresentou um efeito protetor à

infecção [39]. Da mesma forma, a estimulação de PBMCs de pacientes imune saudáveis, com resultado positivo para o teste cutâneo de hipersensibilidade, produz uma aumento significativo de IFN-γ, IL-2, IL-12 e TNF-α, porém baixa produção de IL-4 e IL-10, quando comparado a indivíduos saudáveis não-imunes e pacientes com coccidioidomicose disseminada [40, 41]. Interferon gama é uma das citocinas pró-inflamatórias produzidas por TH1 que aumenta a ativação dos macrófagos além de regular a expressão de moléculas de classe II do complexo principal de histocompatibilidade (MHC II) [42]. A alta susceptibilidade dos indivíduos com HIV e imunossuprimidos em desenvolver coccidioidomicose severa, também é um outro indicativo de que a defesa é feita por meio de linfócitos T CD4+, já que esses pacientes têm depleção neste tipo de linfócito T [18, 43, 44].

Em experimentos com camundongos, foi analisada a importância de IL-10 na resistência dos mesmos à coccidioidomicose. Existe uma relação direta entre níveis de IL-10 e a susceptibilidade à peritonite por *Coccidioides immitis* em camundongos C57BL/6 (B6), DBA/2 e BXD [45]. Esta mesma citocina tem um efeito significativo na habilidade de camundongos C57BL em resistir à infecção por *C. immitis*, ou seja, influencia na susceptibilidade desta espécie de camundongos, bem como de outras, à coccidioidomicose [46].

Em relação a resposta humoral, pouca atenção tem sido dada ao papel do anticorpo na proteção à coccidioidomicose, já que Kong et al. [47] relataram que a transferência passiva de soro de camundongos vacinados com o antigeno coccidioidal não protege o receptor da vacina. Beaman et al. [48] complementou essa afirmação ao mostrar que a pré-incubação do soro com artroconídios de camundongos imunizados falha na neutralização da infectividade do artroconídios. Dessa forma, embora os anticorpos nao pareçam estar associados com a proteção, maiores estudos são necessarios para confirmar esses dados.

3. Mecanismo de virulência e imuno evasão dos fungos

Os fungos apresentam diversas estratégias para evadir a imunidade inata e adaptativa. Um dos mecanismos que *Coccidioides* parece utilizar para tal é a transformação de artroconídias em esférulas no interior das células mononucleares, o qual este último pode lisar a célula devido ao seu grande tamanho (60-80μm) quando comparado com células (12μm). O outro mecanismo seria pelo qual artroconídias e endosporos conseguem inibir a fusão fagossomo-lisossomo [49].

Existem proteínas de *C. Immitis* com grande importância em relação à infectividade deste fungo. Uma glicoproteína de parede externa de esférula (SWOgp) aparentemente funciona como adesina e contribui para virulência do *C. Immitis*. [50] e ainda sua urease recombinante pode ser usada como candidata à uma vacina para coccidioidomicose. [51].

Um outro exemplo é a produção de uma proteína específica, a adesina e fator de virulência-1 (BAD-1) liberada por *Blastomyces dermatitidis*. BAD-1 promove a diminuição da produção de TNF-α, pela interação com os receptores (CR3 e CD14) presentes em macrófagos e neutrófilos, e a estimulação e liberação de TGF-β, a qual atua de maneira autocrina ou parácrina, na inibição de TNF-α. Além disso, BAD-1 solúvel liga-se a CR3 e promove a endocitose mediada pelo receptor, alterando, também, a sinalização intracelular para a inibição de TNF-α. A capacidade de alterar a composição da parede celular dos fungos durante suas diferentes fases e consequentemente a alteração da resposta imune pode ser uma explicação pelo qual permite que o patógeno evada o sistema imune [52].

4. Phage Display

Diversas metodologias de apresentação de moléculas na superfície de vírus e células têm sido descritas, incluindo as técnicas de *Phage-Display* [53], *Ribossome Display* [54, 55] e C*ell-Surface Display* [56], através das quais

anticorpos ou fragmentos de anticorpos podem ser selecionados pela reatividade a antígenos de interesse.

As bibliotecas de peptídeos recombinantes apresentados em fagos são uma ferramenta importante para identificar os sítios ligantes de moléculas biológicas de interesse e no desenvolvimento de novas vacinas. *Phage Display* é um método de seleção no qual uma biblioteca de peptídeos, anticorpos ou proteínas são expressos na superfície de uma partícula viral (fago). Assim, é possível a correlação entre cada seqüência de proteína variante e sua respectiva seqüência de DNA [57].

Smith [58], em 1985, foi o pioneiro em conseguir a expressão da enzima de restrição *Eco* RI em fusão com proteína oito (pVIII) do capsídeo do fago. É comum, utilizar o fago M13 [59] para a infecção, um bacteriófago filamentoso que infecta bactérias gram-negativas, que por sua vez apresentam pilus F. O vírus utiliza a maquinaria de replicação, transcrição e tradução da bactéria para se reproduzir. Este bacteriófago não provoca lise na célula hospedeira, mas induz um estado no qual a célula infectada origina e libera partículas virais, causando uma queda na taxa de reprodução bacteriana [60]. Vetores virais, como o fago lambda [61], bacteriófagos T4 e P4 [62, 63], além dos vírus de eucariotos, tais como baculovírus, também podem ser utilizados neste processo[64].

A apresentação de peptídeos em fagos apresenta a vantagem sobre outras tecnologias pela facilidade de mapear grandes números de clones simultaneamente. Bibliotecas de cDNA, tal como as expressas em fagos lambda, são limitadas pelo número de colônias com o qual podem ser detectadas por hibridização, tipicamente na ordem de 10^4 colônias. Tecnologias baseadas em síntese química (química combinatorial) apresentam um limite máximo de peptídeos randômicos que podem ser mapeados com 10^3 a 10^4 seqüências diferentes [65].

O sucesso de seleção por *Phage Display* depende da complexidade da biblioteca: a maior diversidade de clones dentro da biblioteca aumenta a probabilidade de conter sequências que ligarão a um determinado alvo com maior afinidade [65].

Bibliotecas de peptídeos randômicos compreendem um vasto número de peptídeos (10^7-10^9) de um dado tamanho, onde suas seqüências são geradas aleatoriamente por uma variedade de resíduos de aminoácidos em cada posição. Os peptídeos expressos, em forma linear ou conformacional, são capazes de mimetizar estruturas conformacionais e epítopos contínuos ou descontínuos. A construção dessas bibliotecas é feita principalmente pela inserção de oligonucleotídeos degenerados, sintetizados quimicamente, no gene que codifica a proteína do capsídeo [60, 66, 67].

O vetor M13KE, derivado do vetor de fagos m13mp19, possui uma rápida propagação e não necessita de seleção por antibiótico ou superinfecção por fago helper. Além disto, o gene *lac*Z presente neste vetor facilita a distinção entre colônias bacterianas, infectadas com fagos de bibliotecas, das colônias não- infectadas ou infectadas com fagos selvagens [68, 69]. O vetor M13KE permite a construção e propagação de bibliotecas de *Phage Display* pelo uso de técnicas padronizadas para fagos M13. Para pequenos insertos, a biblioteca pode ser amplificada repetidamente com pouca perda de seqüências e diversidade [70].

A partícula de fago é formada por uma fita simples de DNA envolta por uma capa protéica constituída por cinco proteínas (pIII, pVI, pVII, pVIII e pIX) (Figura 3). Destas cinco proteínas, existem aproximadamente 2800 cópias da pVIII e cinco cópias da pIII. Neste sistema, o gene codificador do peptídeo ou proteína de interesse é geralmente fusionado a um dos genes destas duas proteínas da capa protéica do fago [71].

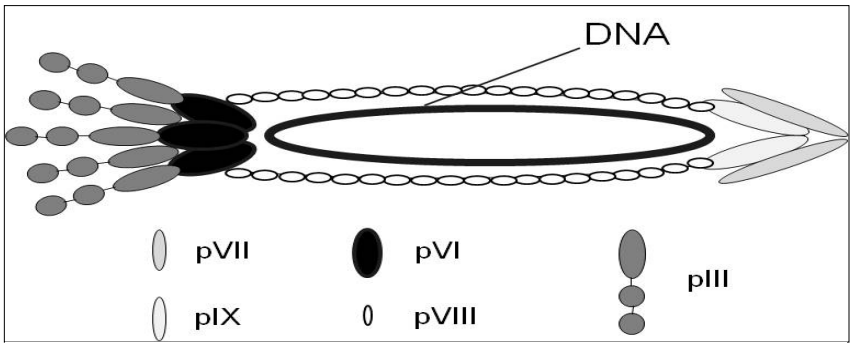

Figura 3. Constituição do Bacteriofago. Capa proteica (pIII, pVI, pVII, pVIII e pIX) e DNA.

A descoberta de que sítios funcionais de anticorpos, como os scFvs, podem ser apresentados na superfície de bacteriófagos permitiu a seleção de anticorpos contra antígenos de interesse sem a necessidade de utilização da tecnologia do hibridoma [53]. Bibliotecas de scFv apresentadas em fagos consistem de diversos domínios de cadeias leves e pesadas fusionados à proteína pIII do fago e apresentados externamente como um scFv [72]. Os fragmentos de anticorpos podem ser expressos em bactérias rapidamente, em grandes quantidades e a baixos custos, se comparados à expressão de anticorpos completos em cultura de células animais. O DNA codificante para uma biblioteca de scFv pode ser clonado no genoma de um fago ou em um vetor fagomídeo para produzir uma fusão scFv-pIII. Em vetores, o DNA codificante da biblioteca pode ser clonado no genoma de fagos filamentosos [73] ou pode ser inserido como um cassete gênico que codifique a seqüência completa da fusão scFv-pIII dentro do genoma do fago. Ambos os tipos de vetores possuem seqüências codificantes para todas as proteínas necessárias para a replicação e montagem do fago [74].

O método mais comum de Ph.D. para bibliotecas de anticorpos é realizado com vetores fagomídeos, uma vez que esse sistema utiliza uma estratégia de clonagem mais fácil e apresenta uma estabilidade genética maior quando comparados aos demais vetores, além de permitir a ligação mais eficiente do scFv ao alvo. Fagomídeos têm sido desenvolvidos para aumentar a

eficiência da expressão [75] e fagos auxiliares têm sido remodelados para reduzir sua influência negativa (‘*bald phage*’ *background*) nos processos de seleção dos fagos ligantes [76].

A seleção é realizada pela incubação da biblioteca de peptídeos ou anticorpos apresentados em fagos contra o alvo, a molécula alvo é imobilizada, geralmente, em uma placa de microtitulação (utiliza-se, também, “beads”, resinas ou membranas); então uma população de fagos, em solução, é incubada com a molécula alvo. Os fagos, contendo peptídeos recombinantes com afinidades pelo alvo, são capturados e permanecem ligados; já os fagos não ligados (não específicos) são eliminados por sucessivas lavagens. O *pool* de fagos, ligados ao alvo é eluído e amplificado em *Escherichia coli*. Os fagos resultantes deste processo são titulados e submetidos a um novo ciclo (ligação ao alvo, eluição e amplificação) visando selecionar mais seqüências específicas para o alvo. Após três ou quatro repetições deste processo, clones individuais são submetidos a ensaios imunológicos e suas seqüências de DNA podem ser obtidas por seqüenciamento [70].

O processo de eluição dos fagos ligados a alvos protéicos imobilizados compreende um ponto chave nos protocolos de seleção. Algumas alterações realizadas neste processo, durante o primeiro passo de seleção por afinidade, podem levar ao isolamento de clones com maior afinidade para a molécula alvo. D’Mello e Howard (2001) [77], por exemplo, testaram tampões de eluição com pHs decrescentes, enquanto Gaskin *et al*. (2001) [78] usaram a solução de proteína alvo como tampão de eluição. Como resultado, todos obtiveram aumento na seleção de clones fortemente ligantes às respectivas proteínas.

Anticorpos protetores contra doenças infecciosas reconhecem peptídeos extraídos de bibliotecas recombinantes como seus epítopos. estes peptídeos são capazes de induzir a formação de anticorpos efetivos contra a doença [79] [80]. Peptídeos recombinantes dispersos em fagos podem se ligar a receptores hormonais [81], proteínas [82] e componentes inorgânicos [83] e, assim, elucidar suas moléculas alvo.

A tecnologia de *Phage Display* pode levar a produção de uma enorme gama de ligantes, incluindo anticorpos recombinantes e peptídeos, com especificidades predefinidas. Além disso, tecnologias emergentes baseadas em *Phage Display* podem beneficiar testes diagnósticos através da produção de moléculas que seria impossível de serem obtidas por métodos tradicionais. Foram expressos na superfície viral anticorpos [84-86], peptídeos [65], enzimas [87], receptores de superfície celular [88], entre outras estruturas.

Phage display já foi utilizado para selecionar diferentes alvos, tais como, agonistas e antagonistas de receptores [89], inibidores de angiogênese tumor-específico [90], desenvolvimento de vacinas [91] e biomarcadores [92, 93],sendo muitos deles direcionados para doenças infecciosas, causadas por protozoários (*Plasmodium spp*.) como malária [94, 95], bactérias (*Staphylococcus aureus* e *Staphylococcus epidermidis*) [96] e fungos (*Paracoccidioides brasiliensis* [93], *Saccharomyces sp.*, *Candida sp. [97]* e *Phytophthora sp. [98]*.

Kioshima e colaboradores [89] encontraram um marcador de virulência para *Paracoccidioides brasiliensis*, utilizando uma biblioteca de fagos CX7C (C, cisteína, X, qualquer resíduo de aminoácido). Ensaios *in vitro* do peptídeo sintético selecionado demonstraram que este era capaz de matar apenas os fungos mais virulentos, pois o peptídeo era internalizado apenas pela linhagem mais virulenta. Ensaios *in vivo* também foram realizados demonstrando que a infecção pulmonar era significativamente inibida quando antes de ser injetado no camundongo o fungo mais virulento era pré-incubado com o peptídeo selecionado [93]. Esses trabalhos utilizando a tecnologia de Phage Display nos mostra a importância desta técnica como uma nova alternativa a ser utilizada no desenvolvimento e aprimoramento de técnicas de detecção e tratamento da coccidioidomicose.

Neste trabalho buscou-se uma nova estratégia na busca por novas moléculas no tratamento e detecção prévia da coccidioidomicose, selecionando anticorpos e peptídeos, respectivamente. Tal abordagem apresenta inúmeros resultados de impacto em outras doenças relacionadas nao somente à fungos,

mas infecciosas de uma forma geral, mas inédita no estudo de coccidioidomicose. Nas análises de citometria, apesar de alguns trabalhos recentes trazendo informações de importância no manejo da doença, procuramos especificar com maior acurácia os tipos celulares que realmente apresentam influência no entendimento desta doença, bem como o uso dos três estágios do C. Immitis afim de identificar qual destes realmente seria a mais crítica durante a infecção.

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Capítulo 1

EVALUATION OF NEW BIOMARKERS WITH POTENTIAL USE IN THERAPEUTICS AND DIAGNOSTIC OF COCCIDIOIDOMYCOSIS BY PHAGE DISPLAY

EVALUATION OF NEW BIOMARKERS WITH POTENTIAL USE IN THERAPEUTICS AND DIAGNOSTIC OF COCCIDIOIDOMYCOSIS BY PHAGE DISPLAY

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Resumo

Coccidioidomicose, conhecida com Febre do Vale, é uma doença causada pelo fungo C. immitis/ *C. posadasii*. Esta doença é endêmica no sudoeste dos Estados Unidos da América. Devido à complexidade da doença, múltiplos testes, tanto sorológicos, microbiológicos ou microscópicos, devem ser realizados na identificação desta micose. Por causa disto, nós propusemos uma nova abordagem no desenvolvimento de novos biomarcadores, baseados em uma estratégia proteômica subtrativa conhecida por Phage Display, para identificar ligantes que mimetizam mimotopos antigênicos. Interessantemente, a

mesma estratégia foi utilizada para selecionar fregmentos de anticorpos, tais como moléculas scFv (single chain fragment variable), as quais podem ser utilizadas para bloquear ou neutralizer um microorganismo, reforçando seu potencial como moléculas terapêuticas. Nós mostramos que um de nossos scFvs selecionados, clone B8, apresentou alta especificidade e sensibilidade com diferença significativa entre pacientes crônicos e saudáveis. Diversos clones do *biopanning* de peptídeos foram capazes de distinguir significativamente IgGs de pacientes cronicos e saudáveis. Esta investigação nos trouxe importantes perspectivas no diagnostic e terapêutica desta doença.

Abstract

Coccidioidomycosis, known as “Valley Fever”, is a disease caused by the fungus *C. immitis*/ C. posadasii. This disease is endemic to the Southwestern United States. Due to the complexity of the disease, multiple tests, such as serological, microbiological and microscopical, should be performed to identify this mycosis. Because of that we have proposed a new approach for the development of novel biomarkers, which is based on the subtractive proteomic strategy known as Phage Display to identify ligands that mimics antigenic mimotopes. Interestingly, the same strategy was used to select antibody fragments, such as scFv (single chain fragment variable) molecules, which may be used to block or neutralize a microorganism, reinforcing its potential use as therapeutic molecules. We showed that one of our selected scFv, clone B8, had high specificity and sensitivity with significant difference between chronic and healthy non-immune patients. Several clones from the peptide biopanning were able to significantly distinguish between purified IgG from chronic patients and healthy non-immune individuals. This investigation brought important perspectives for the diagnosis and therapeutics of the disease.

1.BACKGROUND

Coccidioidomycosis, known as “Valley Fever”, is a disease caused by the pathogenic dimorphic soil-dwelling fungus of the genus *Coccidioides* [1]. This

disease is endemic to the Southwestern United States with estimated 150,000 cases every year. Epidemiological data have shown that incidence rates of disease have risen in Arizona and California with a 97.8% and 91.1% increase from 2001 to 2006 in the two states, respectively [2]. In other endemic countries, however, is probably underreported. In Brazil, for example, coccidioidomycosis is not a notifiable disease, so there is not enough information about its epidemiology. A study performed in Ceará State, Brazil, has reported 19 cases from May 1999 to September 2007, from which 14 patients presented non-specific respiratory symptoms, and 5 cases were found in other reports. So, improvements in diagnosis should be performed in order to understand the disease epidemiology, and to bring information for proper coccidioidomycosis treatment [3, 4] in the Northeast Brazil.

There is great difficulty to stablish a precise and fast diagnostic for coccidioidomycosis, due to the complexity of the disease, and multiple tests, such as serological and microscopical, should be performed to identify this mycosis. Even skilled clinicians experience problems in its diagnosis and in the choice of proper treatment for fungal infections. Thus, early clinical, radiological and biological confirmation of the diagnosis is essential in order to avoid the possible complications of pulmonary mycosis [5].

Currently, antigen detection and fungi serology are useful in the diagnosis of endemic mycoses as causes of pneumonia [6]. Enzyme immunoassay (EIA) can also be performed quickly and locally, but has the potential of false-positive results in patients manifesting a positive EIA for immunoglobulin M (IgM) antibodies and a negative EIA for IgG [7].

Due to the variable efficacy in the coccidioidomycosis diagnosis, we have proposed a new approach for the description of novel biomarkers, which is based on the subtractive proteomic strategy known as Phage display. This technology has been widely used to identify ligands that bind specifically to biological molecules through cycles of selections. The most exciting potential of phage

displayed libraries is to obtain short peptide molecules that mimic antigens, defined as mimotopes, which may be useful as diagnostic tools or therapeutics [8, 9]. Interestingly, the same strategy can be used to select antibody fragments, such as scFv (single chain fragment variable) molecules, which may also be used to block or neutralize a microorganism, reinforcing its potential use as therapeutic molecules [10, 11] or antigen detection.

In this investigation, we aimed to develop novel biomarkers for antigen and antibody detection to the coccidioidomycosis diagnosis using the T27K protein complex as a target.

2.RESULTS

2.1. *ScFv selection against T27K*

The biopanning performance of Phage Display libraries was analyzed, after 2 rounds of selection against the antigen T27K fraction. We have produced soluble scFv antibodies, which were tested by enzyme-linked immunosorbent assay (ELISA). Although we selected 48 clones in the biopanning, only three clones showed reactivity to the immobilized target (Figure 1). Furthermore, antigen-specific binding affinity was done with human serum samples (healthy subjects and chronic patients) pre-coated on a plate. In this assay, we showed that clone B8 had high specificity and sensitivity (Figure 2), with significant difference between chronic and healthy non-immune patients ($p<0.05$), therefore confirming the success of the selection.

2.2. Peptide selection against a polyclonal antibody (pAb) anti-T27K

Sixteen clones were selected against the pAb anti-T27K after three rounds of selection using the 7-mer peptide library. Several peptides presented significant immunoreactivity in ELISA tests, and were able to significantly distinguish between purified IgG from chronic patients and healthy non-immune individuals ($p < 0.0001$) (Figure 3), and the peptides proved to be potential mimotopes of coccidioidal antigens of the T27K complex.

2.3. Epitope mapping of Coccidioides proteins

We have observed that peptides that mimic different exposed sites of known proteins SOWgP and MEP-1, both predicted. We obtained a great diversity of motifs when comparing with the linear sequence of the SOWgp, which are GXXXAXY (peptide1), SSSVXXX (peptide2), SXXKXXY,SVXXSXX (peptide3) and AXXGNXX (peptide4). All of them are aminoacids exposed on the surface and therefore accessible to solvent so that, increasing the possibility of high antigenicity of the mapped regions. For MEP-1, only peptides 2 and 3 presented alignments higher than 3 hits (SXSXXRX and SXTXSXY, respectively) (Figure 4).

2.4. Protein-G magnetic beads immunoprecipitation/ Western-blot.

After immunoprecipitation, bands were detected in both sera (healthy non-immune individuals and chronic patients) by Western-blotting, using the anti-T27K pAb as the primary antibody (Figure 5B). The two between 25 and 37 kda bands were cut, and sequenced by mass spectrometry, which revealed an Ig gamma 3 chain C region from *Homo sapiens* (Figure 6).

2.5. Histoplasmosis cross-reaction

After blotting the T27K protein fraction, spherule and endospore on the membrane, we have observed that two mAb produced in mouse for histoplasmosis diagnosis cross-reacted with antigens from the two developmental stages of *Coccidioides immitis* and also with T27K (Figure 7)

3.DISCUSSION

3.1 Novel biomarkers for Coccidioidomycosis diagnostics

We have successfully used the phage display technology for the discovery of new antigens and antibodies targeting the T27K heterogeneous antigen in order to map the epitopes that could contribute to both diagnosis and protective immunity. We have also provided new insights on how to develop novel molecules and targets for treatment, which might help us to better understand the

pathology and improve diagnostics and therapeutics.

We have used two strategies: a combinatorial fragment antibody phage display and a random peptide phage-display. On the first one our goal was to develop monoclonal antibodies that could be used to detect circulating *Coccidioides* antigens, and the later was used to obtain peptides expressed on the phage's surface that were immunoreactive against polyclonal antibody anti-T27K. Successful examples of Phage Display selections have been presented elsewhere showing ligands that bind to the surface of microorganisms [12] and that also act as antimicrobial peptides [13].

A specific antibody selected against T27K was able to discriminate chronic patients from controls; however, it also cross-reacted with samples from healthy Brazilian individuals. The immunoprecipitation and sequencing of the ligand found in the serum of patients that was bound to the scFv indicated that a putative idiotope, defined serologically by the reaction of anti-idiotopic antibodies with antibodies bearing the idiotopes [14], was identified as the Immunoglobulin gamma 3 chain C. We postulate that the pAb anti-T27K might be recognizing an idiotope present on antibodies against Coccidioidomycosis in healthy immune patients. Also idiotopes from antibodies against other types of fungus from healthy non-immune patients are apparently being recognized by the pAb. This idea was reinforced by cross-reaction of mAbs developed for histoplasmosis with the *Coccidioides* antigens (T27K, endospores and spherules).

Regarding the selected peptides against the polyclonal antibody anti-T27K, mimotopes were aligned in conformational structures that matched the metalloproteinase-1 (MEP-1) and regions of the surface of SOWgp (Spherule outerwall glycoprotein). This is the first description of such attempt to map functional antigens and epitopes in the heterogeneous protein preparation from *Coccidioides immitis*.

Despite the peptides had presented no common motifs between them, we can propose that the selection was still efficient, due to the hits presented were

higher than 3 aminoacids (Figure 4) on each region aligned with the peptides and of course because the selection was against a polyclonal antibody, which would increase anyway the variability of selected clones. The same was observed when we analyzed the peptides over the MEP-1 predicted structure. Certainly, the more clones we sequence and analyze, the more regions of mapping over the 3D structures of these two proteins and others of interest, we will obtain, consequently increasing the chances of these peptides being used as target biomolecules against coccidioidomycosis.

4.CONCLUSION

Our findings support the idea of different literatures over the demanding studies about the APCs response to *Coccidioides*. Fungal infections are normally of high complexity to understand, and for that, various technologies should be applied at the same time to assess which would be most effective way to detect and control the disease. We presented a broad view of what we obtained so far, but gene expression, higher amounts of clones sequencing, increasing numbers of patients and testing the scfvs and the peptides as new stimuli will give us great perspectives of driving this disease to a greater management and/or prevention.

5.MATERIALS AND METHODS

5.1 Antigen preparation

T27K was purified by water-soluble coccidioidal preparation isolated from disrupture of mature spherules, which were killed with thimerosal and after centrifugation at 27 000 g. Spherule-endospore (SE) cultures were prepared as previously described using the *C. posadasii* Silveira strain [15]. Spherules and endospores were harvested from SE phase cultures and placed in thimerosal (1:10,000) for 72 hours. An aliquot was then cultured to confirm no viable organisms remained prior to other experiments.

5.2 scFv library

The scFv library used for antibody selection against the T27K antigen complex was constructed in the Laboratory of Nanobiotechnology, Federal University of Uberlândia, MG, Brazil by Ana Paula Carneiro dos Santos and kindly donated to this work. It was constructed according following protocol using total RNA of leukocytes. First strand cDNA was generated from 5 µg of total RNA using SuperScript III kit (Invitrogen). The genes for variable regions of heavy chain, κ light chain, and λ light chain (VH, Vκ, and Vλ) were amplified separately in 55 independent reactions to generate variable domains of the heavy and light chains, and then were precipitated, purified and equal amount of PCR products were pooled into collections of VH, Vκ, and Vλ gene repertoire. That gene repertoire were used for two overlap PCR reactions (VH+Vκ) and (VH+ Vλ), the samples were purified by a Promega Kit for the cloning (digested with SfiI), ligated and electroporated into *E. coli* XL-1 Blue. The library stock was grown to log phase and rescued with VCM13 helper phage.

5.3 scFv selection over T27K

T27K antigen (10-100 µg/ml) in 0.1 mM NaHCO3 pH 9.4 were coated on Immuno 96 plate MicroWell Plates (Nunc, Denmark) overnight at 4°C. Following blocking with 2% (w/v) BSA/PBS, a scFv library containing between 10^{10} and 10^{11} phage particles were added and the plate was incubated for 2 hours at room temperature. For elimination of non-specific binding phages, wells were washed 10 times with PBS-Tween 0.1%, and then incubated with 100 µL of 0.2M glycine-HCl, pH 2.2 for specific phages detach, neutralized with 15 µl of 1M Tris-HCl, pH 9.1. Eluted phages were used to infect exponentially growing *E.coli* XL-1 Blue incubated for 15 minutes at 37°C.

5.4 Peptide selection over pAb anti-T27K

NuncPlates were coated with policlonal anti-T27K (kindly provided by Dr. Pappagianis' laboratory UC Davis) overnight at 4°C, after blocking (2% skimmed milk powder in PBS) we incubated for 1 hour with $2x10^{10}$ phage (Ph.D.C7C,

Phage Display Peptide Library, New England Biolabs, Inc.). Non-bound phages were eliminated by washing 10 times with PBS containing 0.1% Tween 20 (PBS-T). The bound phages were eluted by incubation with 100 µL of 0.2Mglycine-HCl buffer, pH 2.2 (immediately neutralized with 1M Tris-HCl pH 9.1) then, after three rounds, eluted phages were amplified in *Escherichia coli* (ER2738 host strain, New England Biolabs Inc.), titrated and sequenced.

5.5 Sequencing of selected phage clones

Peptide phage clones single-strand DNA was isolated by the iodide buffer extraction procedure (Instruction Manual Ph.D.-C7C Phage Display Peptide Library Kit). The nucleotide sequence of the gene III insert was sequenced with −96 gIII sequencing primer 5′-HO CCC TCA TAG TTA GCG TAA CG-3′ by automated dye terminator cycle sequencing. The amino acid sequence of the insert was deduced from the nucleotide sequence in program DNA2PRO (http://www.relic.bio.anl.gov).

5.6 Soluble scFv production

Bacteria *E. coli* TOP10 (carbenicilin resistant) carrying the phagemid encoding the scFv antibody were grown in 1 ml of SB medium containing carbenicilin (50 µg/ mL) and glucose (2% w/v). After 4 hours, cells were harvested and grown at 30°C in glucose free SB medium containing 50 µg/mL carbenicilin and 2.5 mM isopropyl-µ-d-thiogalactopyranoside (IPTG). The cell culture supernatant containing scFv was collected after induction for 18 hours.

5.7 Phage/scFv ELISA (Selection confirmation)

Antigen (T27K) or antibody (pAb anti-T27K) were added to the plate (1.0 µg/well) in 0,1M $NaHCO_3$ pH 9.4 overnight at 4°C. That was subsequently washed three times with PBST 0,1%, blocked with skym milk 3% for 1 hour at 37°C. The wells were washed three times and were incubated with 100 µL of scFv anti-T27K supernatant (plate coated with T27K) or phage supernatants (plate coated with pAb anti-T27K) for one hour at 37°C. After washed five times,

the bound scFvs and the phage particles were detected with peroxidase conjugated anti-HA (Roche Applied Science) and anti-M13 monoclonal antibody (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands), respectively diluted 1:5000 in the blocking buffer. The peroxidase staining reaction was developed in the presence of SIGMA*FAST*™ OPD (*o*-Phenylenediamine dihydrochloride) tablets (Sigma–Aldrich). Then incubation at room temperature for five minutes and stopped with diluted acid solutions, the OD 492nm values were measured on a microplate reader (TP-READER - Thermo plate).

5.8 Phage/scFv ELISA (Specificity)

Two plates were coated for determine specificity of selected phage clones and scFv B8. One with serum from 4 chronic and healthy non-immune donors (individually in duplicate) and other plate with a pool of magnetic bead-purified IgG from the serum of the same patients (diluted to 1µg/well in 0,1M $NaHCO_3$ pH 9.4 overnight at 4°C). The plates were washed three times with PBST 0,1%, blocked with skym milk 3% for 1 hour at 37°C. The wells were washed three times and were incubated with 100 µL of scFv (clone B8) anti-T27K supernatant (plate coated with serum) or phage supernatants (plate coated with magnetic bead-purified IgG) for 1 hour at 37°C. After washed five times, the bound scFvs and the phage particles were detected with peroxidase conjugated anti-HA (Roche Applied Science) and anti-M13 monoclonal antibody (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands), respectively diluted 1:5000 in the blocking buffer. The peroxidase staining reaction was developed in the presence of SIGMA*FAST*™ OPD (*o*-Phenylenediamine dihydrochloride) tablets (Sigma–Aldrich). Then incubation at room temperature for five minutes and stopped with diluted acid solutions, the OD 492nm values were measured on a microplate reader (TP-READER - Thermo plate).

5.9 Immunoprecipitation/Magnetic Protein-G beads

The Dynabeads® Protein G (Invitrogen) were resuspended by pipetting or rotating on a roller (5 min). Only 50 µl were transferred to a tube, placed on magnet and the supernatant removed. The tubes were removed from the magnet and 10 µg of the pAb anti-T27K were added to resuspend the beads and a 10 minutes incubation with rotation at room temperature was performed. The supernatants were removed by placing the tubes on the magnet and the beads were washed with PBS-Tween 0.05% twice (200 µl). The tubes were placed on the magnet to remove the supernatant and the samples -100 µl of pool of chronic patients in one tube and healthy donors in the other tube- were added and incubation was proceeded for 30 minutes at room temperature. We removed the supernatant as previous steps and washed the beads 3 times with PBS-Tween 0.05%. The elution step occurred by adding 100 µl of 2M glycine-HCl, pH 2.2 for target detach during 10 minutes agitating at room temperature and finally neutralized with 15 µl of 1M Tris-HCl, pH 9.1.

5.10 SDS-PAGE

To run the samples from the immunoprecipitation with the pAb anti-T27K we used a 15% SDS PAGE gel - lower Buffer (for the separating gel) with 1.5M Tris pH to 8.8, 0.4% SDS; Stacking (Upper) buffer (for the stacking gel) with Tris 0.5M pH 6.8, 0.4% SDS; 30% acrylamide for both, TEMED and 10% ammonium persulfate. The samples were boiled for 5 minutes with sample buffer and 10% total volume with beta-mercaptoethanol (PIERCE-THERMO SCIENTIFIC). After that, the gel was loaded for running at 100V.

5.11 Western Blot

For the Western blot, we used the iBlot™ Dry Blotting System (Invitrogen) to transfer the gel to the membrane, which was blocked for 1 hour at 37oC with 3% skym milk in 1X PBS pH7.4 (Thermo Scientific). After three washing steps with PBS +Tween 20 (0.05%) the anti-T27K rabbit pAb was added (1:100 diluted in Blocking buffer- 3% skym milk in 1X PBS pH7.4) and incubated at 37oC for 1

hour. Three more washing steps were performed with PBS Tween 20 (0.05%) and we added the secondary antibody, monoclonal anti-rabbit peroxidase conjugated in a 1:5000 dilution with blocking buffer (3% skym milk in 1X PBS pH7.4), for 1hr at 37oC. After that we developed the reaction with SIGMA*FAST*™ OPD (*o*-Phenylenediamine dihydrochloride) tablets (Sigma–Aldrich) and the reaction was stopped with 2M Sulfuric Acid.

5.12 Dot Blot (Histoplasmosis mAbs)

We got a nitrocellulose membrane ready and using a narrow-mouth pipet tip, we spotted 2 µl of samples of T27K, spherules and endospores onto two nitrocellulose membrane strips and left the membrane to dry. The non-specific sites were blocked by soaking in 5% BSA in TBS-T (0.5-1 hr, RT) using 10cm Petri Dish for reaction chamber. The membranes were than incubated with primary antibody (1.0 µg/ml for mAb 1 and mAb2 anti-histoplasmosis), dissolved in BSA/TBS-T, for 30 min at RT, washed three times with TBS-T (3 x 5 min) for later incubation with secondary antibody conjugated with HRP (anti-rabbit 1:5000) for 30 min at RT. After washing three times with TBS-T, DAB (Diaminobenzidine) was used to check the reaction.

5.13 Bioinformatics analysis

After testing peptide clones reactivity, they were sequenced and their translated peptides were aligned with known proteins and structures in the GeneBank. We had to predict the SOWgp and also MEP-1 structures using I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER) so that the alignment of the clones selected by peptide Phage Display could be highlighted over the predicted structures. The mimetic epitope mapping was performed using the pepitope server (http://pepitope.tau.ac.il) using the pepsurf algorithm and later was visualized with Pymol (http://www.pymol.org).

5.14 Statistical analysis

Statistical analyses were performed by comparisons among healthy

immune, healthy non-immune and chronic patients groups using the Mann – Whitney test or Student's t test, both with two-tailed P values. The level of significance was set at 0.05.

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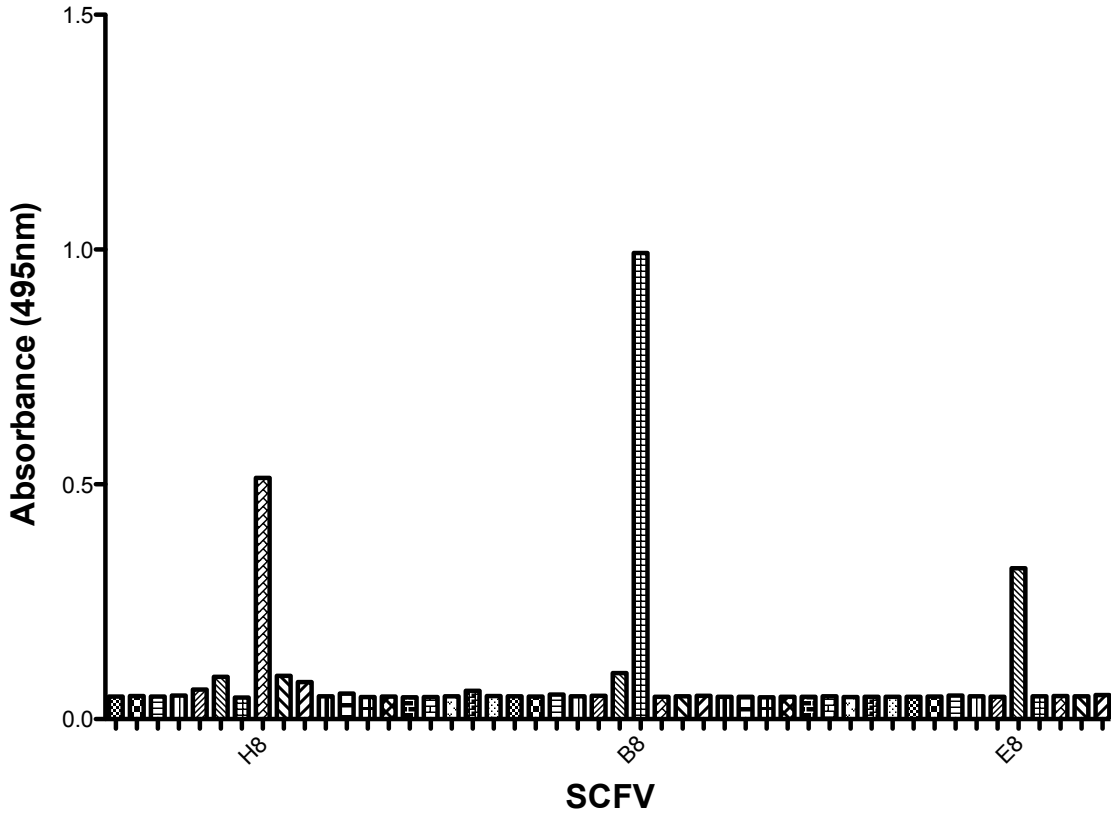

Figure 1. ***ELISA for scFvs selected against T27k*****.** A total of 48 clones were analyzed with T27K antigen. Only three had reactivity with T27K (E8, B8, H8).

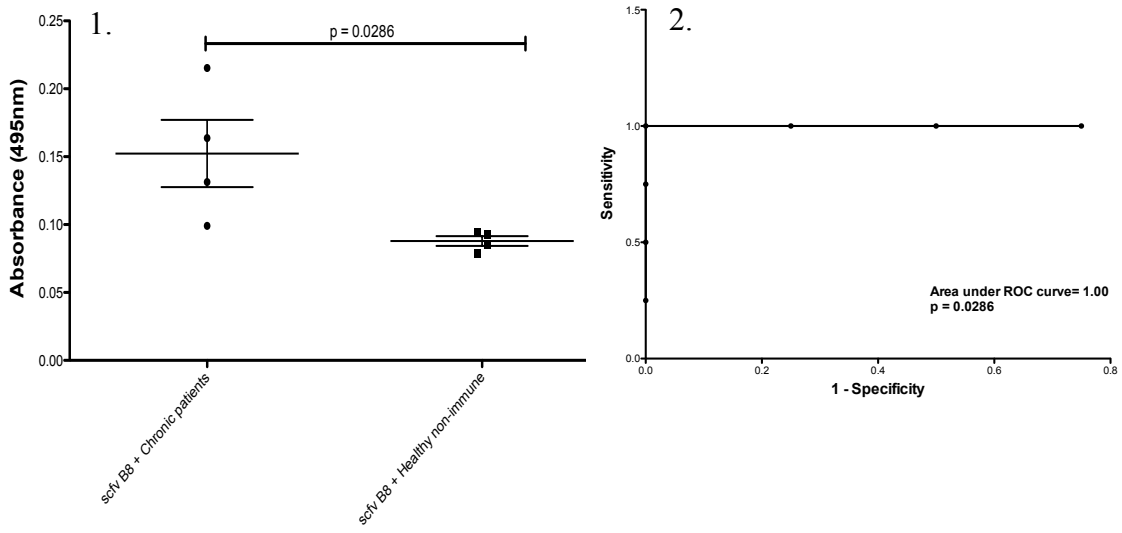

Figure 2. ***ScFvs over Chronic and healthy non-immune patients*****. A.** Detection and distinction of healthy non-immune (scfv B8+Patients) and chronic patients serum (scfv B8+Patients) using one of the most reactive selected scfv (B8 / p=0.0286) and **B.** its respective ROC-curve.

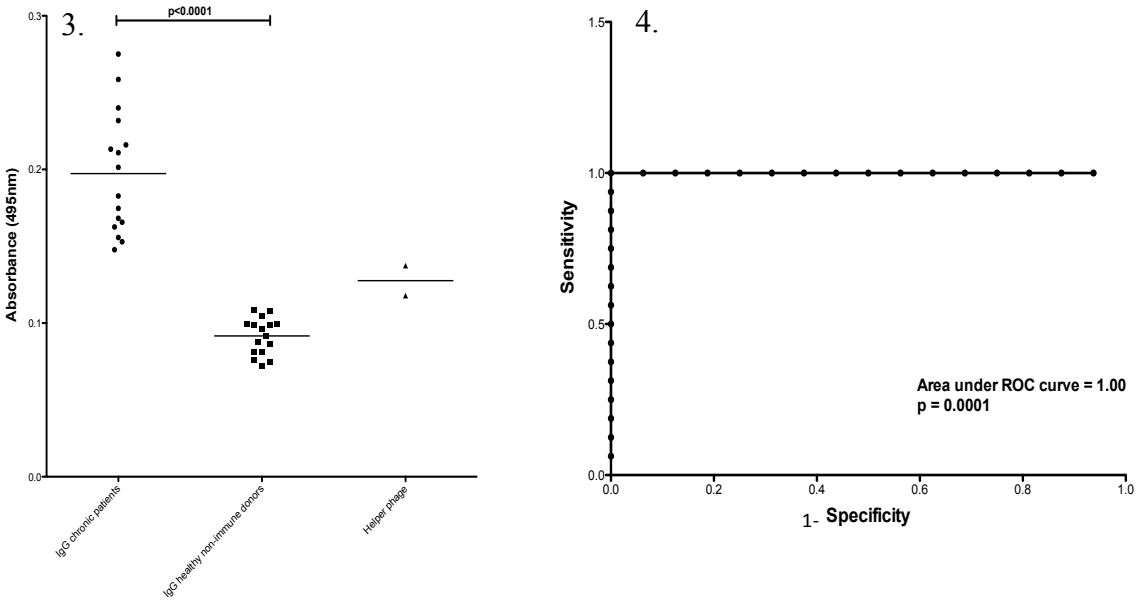

Figure 3. ***ELISA with clones selected against anti-T27k*****. A.** Peptides from clones selected by Phage Display reacting with magnetic bead-purified IgG from healthy non-immune donors and chronic patients. There was a great significance between IgG from Chronic and Healthy non-immune patients ($p<0.0001$ – t-student) and **B.** the specificity and sensitivity of the reaction is presented at its ROC curve (AUC =1.0 and $p = 0.0001$).

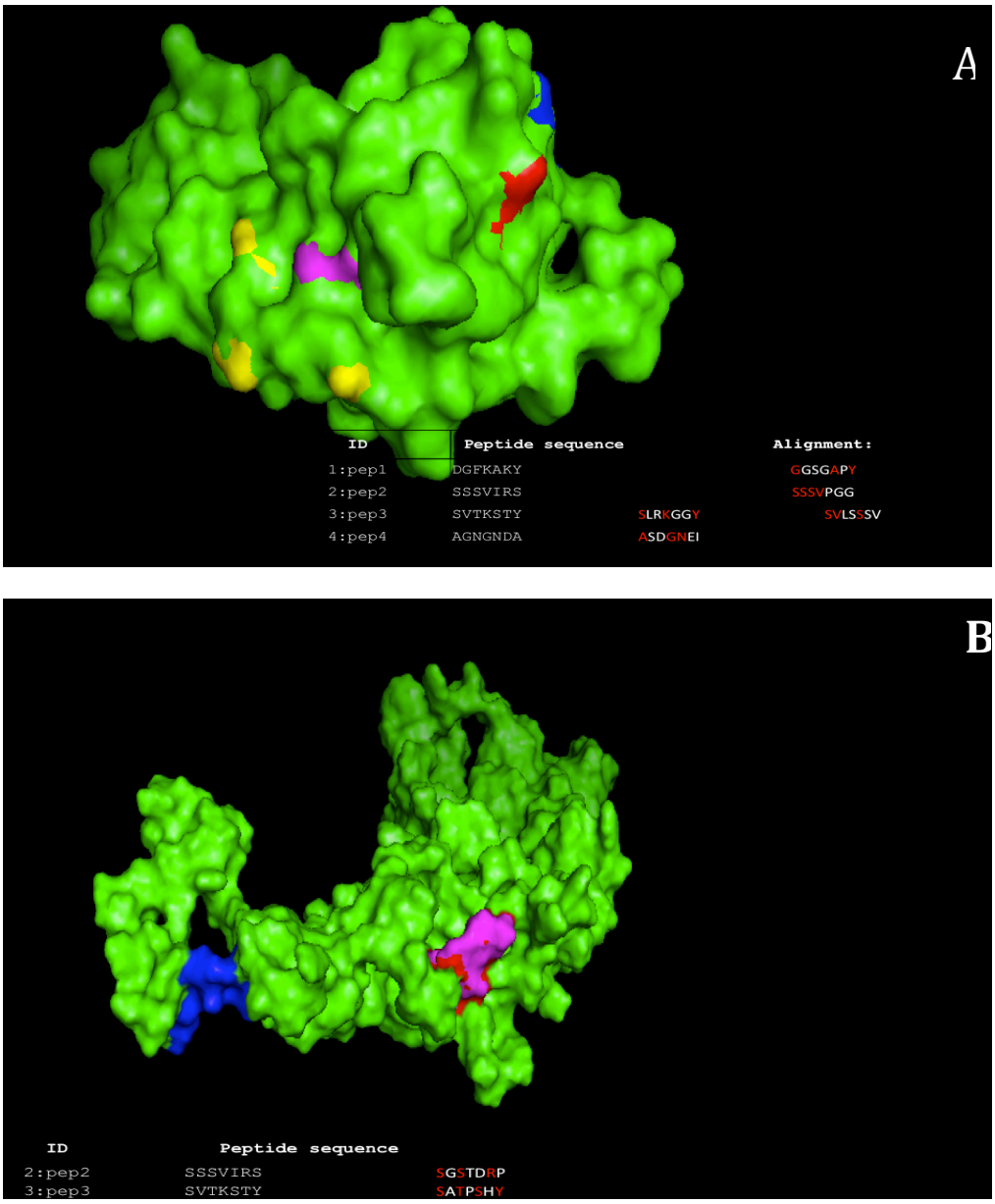

Figure 4. ***3D predicted structure analysis.*** **A.** The clones mimetic to some regions of the surface of MEP-1 (Metalloproteinase-1) are highlighted in different colors peptide 1 (yellow), peptide 2 (purple), peptide 3 (blue) and peptide 4 (red). The table shows (in red) the aminoacids identical to the protein sequence. **B.** The clones mimetic to some regions of the surface of SOWgp (Spherule outerwall glycoprotein) are highlighted in different colors peptide 2 (purple) and peptide 3 (blue). The table shows (in red) the aminoacids identical to the protein sequence.

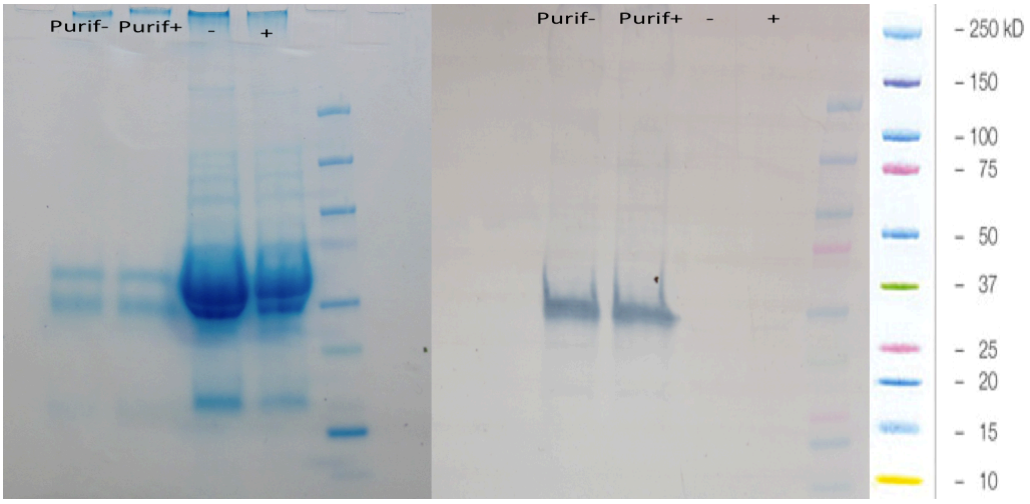

Figure 5. ***Western blot/immunoprecipitation with pAb anti-T27K*****. A.** The serum from chronic and healthy non-immune patients for coccidioidomycosis (+ and -, respectively) before and after immunoprecipitation (Purif+ - chronic patients and Purif-, Healthy non-immune patients)are shown on an SDS 15% Gel and **B.** the western blot presented two bands of approximately 41kda.

Probability Legend:
over 95%
80% to 94%
50% to 79%
20% to 49%
0% to 19%

Bio View:
Identified Proteins (8)
Including 0 Decoys

#	Visible?	Starred?		Accession Number	Molecular Weight	Protein Grouping Ambiguity	Human_DIR
1	☑	☆	Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2	sp|P0276...	69 kDa	★	12
2	☑	☆	Ig gamma-3 chain C region OS=Homo sapiens GN=IGHG3 PE=1 SV=2	sp|P0186...	41 kDa	★	7
3	☑	☆	Fibrinogen beta chain OS=Homo sapiens GN=FGB PE=1 SV=2	sp|P0267...	56 kDa		4
4	☑	☆	Fibrinogen gamma chain OS=Homo sapiens GN=FGG PE=1 SV=3	sp|P0267...	52 kDa		3
5	☑	☆	Ig gamma-1 chain C region OS=Homo sapiens GN=IGHG1 PE=1 SV=1	sp|P0185...	36 kDa	★	2
6	☑	☆	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3	sp|P3552...	62 kDa		1
7	☑	☆	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6	sp|P0426...	66 kDa		2
8	☑	☆	39S ribosomal protein L36, mitochondrial OS=Homo sapiens GN=MRPL36 PE=2 S...	sp|Q9P0J...	12 kDa		1

Figure 6. ***Scaffold 3 analysis*****.** The pos-sequencing analysis of the two bands extracted from the SDS 15% gel, where there is a 95% of similarity with Ig gama-3 chain C.

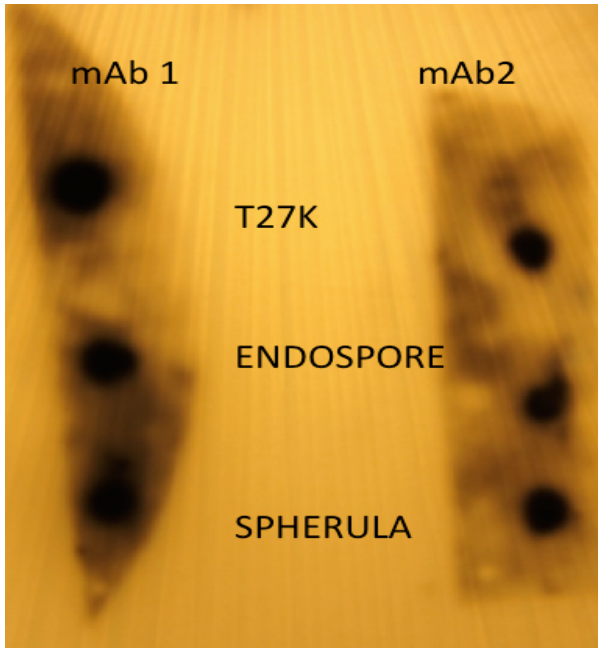

Figure 7. *Dot-blot analysis*. Cross-reaction of two histoplasmosis monoclonal antibodies against the T27K protein complex, endospores and spherules from *Coccidioides immitis*. The figure shows the reaction of two mAbs (mAb1 and mAb 2) from histoplasmosis, against T27K, endospore and spherule.

Capítulo 2

IMMUNE RESPONSE PROFILE OF CHRONIC AND POST-DISCHARGED (HEALTHY IMMUNE) PATIENTS WITH COCCIDIOIDOMYCOSIS

IMMUNE RESPONSE PROFILE OF CHRONIC AND POST-DISCHARGED (HEALTHY IMMUNE) PATIENTS WITH COCCIDIOIDOMYCOSIS

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Resumo

Coccidioidomicose é causada por um fungo dimórfico presente no solo, pertencente ao gênero *Coccidioides.* A falta de um entendimento profundo de sua resposta imune nos guia à investigar o perfil de células apresentadoras de antígenos e, tivemos sucesso ao demonstrar que linhagesn específicas (CD3-CD19-CD14+/-CD11c+/-) apresentaram expressão diferenciada de receptores e citocinas. Após estímulo com endosporos e esférulas, pacientes curados apresentaram maiores níveis de IL-10, e foram similares aos de pacientes crônicos. Nós ainda observamos uma redução significativa na expressão de PRRs na superfície de células CD14+CD11c-, tais como TLR2, CD206 (Receptor de Manose) e Dectin-1. A expressão de TLR2 por células HLA-DR+CD14-CD11c+, HLA-DR+CD14+ e CD14+CD11c+ de pacientes crônicos foi reduzida significativamente após os mesmos estímulos, o que pode estar

relacionado com o processo de internalização de receptores após a fagocitose do fungo.

Abstract

Coccidioidomycosis is caused by the pathogenic dimorphic soil-dwelling fungus of the genus *Coccidioides*. The lack of a thorough understanding of its immune response guided us to investigate the antigen presenting cells profile, and we have successfully demonstrated that specific cell lineages (CD3-CD19-CD14+/-CD11c+/-) present differential expression of receptors and cytokine production. Under stimulation of endospores and spherules, healthy-immune patients presented higher levels of IL-10, and were similar to the chronic patients. We have also observed a significant reduction of PRRs surface expression in CD14+CD11c- cells, such as TLR2, CD206 (Mannose Receptor) and Dectin-1. TLR2 expression from HLA-DR+CD14-CD11c+, HLA-DR+CD14+ and CD14+CD11c+ cells of chronic patients were significantly decreased under same stimuli, which maybe is related with internalization of the receptor after phagocytosis of the fungus.

1.BACKGROUND

The systemic fungal disease coccidioidomycosis is caused by the etiologic agent *Coccidioides immitis* and *Coccidioides posadasii*, a dimorphic fungus which lives as an arthroconidium-forming saprophyte in the earth and on the ground [1].

Infection in humans usually occurs through the inhalation of coccidioidal arthroconidia [2] that are carried in dust and install in the terminal alveoli of the lung, and differentiate into large endosporulating spherules once they are in the host [3, 4]. The disease presents a diverse clinical spectrum that includes unapparent infection, primary respiratory disease (usually with uncomplicated resolution), stabilized or progressive chronic pulmonary disease, and extrapulmonary dissemination, which can be acute, chronic, or progressive [4]

The severity of coccidioidomycosis is variable, and it is influenced by several factors, such as the concentration of inhaled arthroconidia, the genetic predisposition of the host, and especially the human immune response. Immunocompromised and immunocompetent individuals have been clearly identified as at increased risk for developing severe and disseminated coccidioidomycosis [5-7]. Therefore, the identification of host genetic variants that predispose persons to severe disseminated coccidioidomycosis would be of great value in vaccine development [4].

Delayed-type hypersensitivity tests and *in vitro* studies have shown that immune responses against infection are achieved by cell-mediated immune responses [8, 9]. In the innate immune response the cell receptor binds to microbial molecules [10] and activate the T-helper type 1 response rather than Th2 [11] by releasing cytokines, such as interleukin-2 (IL-2) and gamma interferon (IFN-γ) [8]. Among the innate immune receptors, the Toll-like receptors (TLRs) represent a major class of Pattern Recognition Receptors (PRRs), which mediate the production of cytokines that are necessary for the development of effective immunity. The TLRs consist of 10 members (TLR1-TLR10) and MyD88 is an essential signaling component for the activation of innate immunity [12]. The TLR2 recognizes peptidoglycan and lipoteichoic acid (LTA) of gram-positive bacteria and lipoproteins of both gram-negative and gram-positive bacteria [13]. Also it may be an important receptor for fungi recognition, once macrophages of TLR-2-deficient mice infected with *Paracoccidioides brasiliensis* displayed low phagocytic activity, resulting in the secretion of low concentrations of NO and MCP-1 (Monocyte Chemotactic Protein-1) [14].

Another pattern recognition receptor involved in the innate immune response against fungal pathogens is Dectin-1, which is a C-type lectin that recognizes β-glucans present on cell walls of fungi. Studies have shown that Dectin-1-deficient mice presented significant impaired responses to fungi [15, 16]. Furthermore, mouse cytokine responses to *C. posadasii* spherules in mouse peritoneal macrophages are dependent on TLR2, MyD88, and Dectin-1,

highlighting the importance of these receptors to control this infection of diseases [17].

However, subversion of protective immunological mechanisms of the host by the fungus may lead to a successful infection. Results published elsewhere have suggested that the susceptible host with a disseminated disease mounts an early and sustained Th2 immune response to coccidioidal infection [18]. It has been reported that the dominant spherule outer wall glycoprotein (SOWgp) is capable of modulating host immune responses and may result in compromised cell-mediated immunity to coccidioidal infection by driving Th2 response. Moreover, mouse models (BALB/c or C57BL/6) that are susceptible to *Coccidioides immitis* manifested a predominant IL-4/IL-10 response respectively, whereas the resistant strain (DBA/2) showed increased levels of IFN-γ [11, 19].

For this study our objective was to partially characterize the antigen presenting cells profile under stimulation of the T27K protein mixture, endospores and spherules of non-immune healthy individuals, post-discharged immune patients, and chronic patients with Coccidioidomycosis.

2.RESULTS

2.1. Flow citometry

To analyze the immunological profile of subjects, we have performed isolation and stimulation of PBMCs of healthy non-immune individuals, healthy-immune and chronic patients. We observed a significant increase on IL-10 production of CD19+ cells on healthy non-immune patients when stimulated with endospores and spherules from Coccidioides (Figure 1). Discharged immune patients presented higher levels of IL-10 in all stimulations, and were similar to the chronic patients.

We have also observed a significant reduction of PRRs surface expression in CD14+CD11c- cells, such as TLR2, CD206 (Mannose Receptor) and Dectin-1 under stimulation of endospores and spherules. Interestingly, both

CD206 and Dectin-1 presented a similar pattern of reduction in all patients and controls (Figures 2 and 3A). However, contrary to the previous data, the CD14+CD11c+ cells presented higher expression of Dectin-1 only in discharged immune patients, maintaining a significant difference between control media and the endospores and spherules stimulations in chronic patients (Figure 3B).

TLR2 expression from HLA-DR+CD14-CD11c+, HLA-DR+CD14+ and CD14+CD11c+ cells of chronic patients (Figure 4A, B and C, respectively) were significantly decreased under stimulation with T27K, endospores and spherules and a similar pattern was observed for healthy non-immune individuals (Figure 5).

TNF-alpha production was also significantly increased in HLADR+CD14-CD11c+ cells of chronic patients stimulated with T27K and spherules. A similar pattern could be observed on healthy non-immune individuals, but was not significant (Figure 6A). Also CD14+CD11c- cells from healthy non-immune patients had significant TNF-alpha production when stimulated with endospore and spherule. The chronic patient's cells were also significantly stimulated by spherules for TNF-alpha production (Figure 6B). Interestingly, the CD14-CD11c+ (Figure 6C) CD14+CD11c+ (Figure 6D) and CD14-CD11c- (Figure 6E) cells of healthy-immune patients also presented much higher expression of TNF-alpha than chronic patients or controls, despite it was not significant.

3.DISCUSSION

Coccidioidomycosis is a very difficult disease to work with due to its great complexity, no vaccine is available, diagnostics is difficult to achieve and disease may progress to a chronic stage even under treatment. The lack of a thorough understanding of its immune response guided us to further investigate the antigen presenting cell profile, and we have successfully demonstrated that specific cell lineages (CD3-CD19-CD14+/-CD11c+/-) present differential expression of receptors and cytokine production, indicating possible defects in the pro-inflammatory cytokine expression of these cells in chronic patients. We

have also developed markers for detection of antigens and antibodies associated with this disease, which may become potential markers for both diagnosis and therapeutics. This investigation provides new information about the immune response of coccidioidomycosis, and brings important perspectives for the diagnosis and therapeutics of the disease.

3.1 Antigen Presenting Cells profile under different stimuli

To investigate the APCs response in chronic and healthy-immune patients, we have stimulated PBMCs with endospores and spherules of the fungus stages, and with the protein fraction T27K, consisted of whole killed spherules mechanically disrupted and centrifuged at 27,000 *g* to collect the supernatant, which was designated T27K. This protein fraction is known to generate a protective response in mice [20]. However, the T27K is antigenically heterogeneous, and the protective component has yet to be identified [21]. This explains why we have incorporated this antigen in our cell stimulations and later on in our search for epitopes and antibody fragments.

The interaction between the pathogen and the host involves the simultaneous recognition of several microbial products, each one with specific agonist activities. A deeper understanding of such a complex process can only be achieved by first characterizing each recognition event separately. Once the response to each microbial agonist is defined, one can start to analyze the synergies and antagonisms caused by the simultaneous recognition of different microbial products [22]. Different techniques can help us on better understanding how a disease progress occurs.

Flow cytometry panels were designed to identify specific cell subsets representing antigen presenting cells under three different stimuli. Patients and healthy control profiles under stimulation presented similar behavior across patients and control groups in CD14(+/-)CD11c(+/-) cells, but the most striking difference was observed for the T27K and media control when compared to endospores and spherules' stimuli with opposite expressions of dectin-1 and

mannose receptor (CD206) in CD14+CD11c- cells, and for TLR2 in CD14+CD11c+ cells. CD14+CD11c- cells also presented higher TNF-alpha production when stimulated with endospores and spherules, but not with T27K. However, two cell phenotypes CD14+CD11c+/Dectin-1 and CD14-CD11c-/TNF-alpha could differentiate chronic from discharged immune patients, with the highest expression of dectin-1 and TNF-alpha in the immune patients' group in both cell types, respectively, and again the T27K response was also much higher than the other fungus stages. The contrasting response between T27K and the *Coccidioides* stages suggests that this cell lineage may be critical for the fungus survival or for the disease establishment, and that the T27K may also present a protective action against the pathogen, inducing higher TNF-alpha production in all cells, except in CD14+CD11c-.

It has been shown that conidial and hyphal forms of *A. fumigatus* are able to differentially modulate host response and generate distinct cytokine responses [23]. The same can be observed in coccidioidomycosis, where three spherule-specific genes, BLG2, SOWgp, and MEP1, contribute to virulence of *Coccidioides* spp. BLG2 encodes a 120-kDa **β** -glucosidase that cleaves **β** -1,3-glucan, enabling the cell wall to expand during spherule growth [24].

A. fumigatus modulates the host TLR responses by directly decreasing the capacity of the host cells to respond to TLR2 and TLR4 ligation, a mechanism that can be interpreted as a means to evade detection by host immune system or to interfere with the resultant signaling pathways [25]. Our study corroborates those findings by demonstrating that whole cell constituents of *Coccidioides* endospores and spherules may modulate host innate immune responses through the TLR2 pathway, which is evidenced by its decreased expression in CD14+CD11c+ cells. Another study also supports this result through a confocal microscopy analysis after incubation of *Aspergillus* conidia with monocytes, demonstrating internalization of the TLR2 into the phagosome together with the *A. fumigatus* conidia [23]. We suggest that this mechanism could be an explanation for the low expression of TLR2 onto the surface of monocytes.

Inhibition of TLR2-mediated responses by *Aspergillus* conidia was due to induction of TLR2 engagement and internalization, rather than by mediation via other PRRs like mannose receptor, TLR4 or Dectin-1. In the case of *A. fumigatus* conidia, phagocytosis may conversely result in surface receptor depletion and consequently decreased receptor mediated signaling [23]. The evidence that phagocytosis modulates TLR activation [26] can partially explain our results showing that chronic and immune patients had increased IL-10 production by B cells in comparison to healthy non-immune controls, which also showed increased IL-10 production when stimulated with endospores and spherules, suggesting that the *Coccidioides* surface constituents are triggering this immunosuppressive response, an event that is counteracted by TNF-alpha production by CD3-CD19-CD14- cells. For example, spherule outer-wall glycoprotein encoded by SOWgp is an adhesin that binds to host extracellular matrix. Although the cellular and humoral immune systems recognize SOWgp, it promotes a Th2 cytokine response that favors pathogen survival. During spherule maturation, the developing endospores become coated with SOWgp, which MEP1 (Metalloproteinase-1) degrades, retarding immune recognition [24].

Another interesting mechanisms of escape by fungi is the **α**-(1,3)-glucan, which effectively shields **β**-(1,3)-glucan from dectin-1 on myeloid cells. This may explain how **α**-(1,3)-glucan promotes virulence in dimorphic fungi such as *H.capsulatum*. *C. albicans* displays **β**-(1,3)-glucan only on the bud scars of dividing yeast, and not on the more pathogenic hyphae. The large capsule of *C. neoformans* also shields **β**-(1,3)-glucan [24].

Apparently each fungus species has its unique way of escaping the immune response, and this is mainly related to the Th2 pathway. Supporting our results, capsular polysaccharides of the pathogenic fungus *Cryptococcus neoformans* (GXM, GalXM and MPs) display various immunomodulatory effects on the host response, therefore enhancing the production of anti-inflammatory IL-10 [27].

Another explanation for the Th2 (IL-10 B-cell production) response

probably related to the Dectin-1 internalization is demonstrated by the decreased expression of Dectin-1 on the surface of CD14+CD11C-cells triggered by each *Coccidioides* developmental stages. It was strongly suggested that internalization is the first step to attenuate Dectin-1-mediated pro-inflammatory responses in DC [28]. Furthermore macrophage internalization of fungal ß-Glucans is not necessary for initiation of related inflammatory responses [29].

The Th1 response due to fungus recognition is mediated by TLR2 and Dectin-1, and both may work synergistically. For example, TLR2 signals pro-inflammatory cytokines that control fungal growth during *C. albicans* keratitis [30]. Dectin-1 collaborates with TLRs in promoting responses associated with Th1 immunity including production of IL-12 and activation of antimicrobial killing (ROS) [31]. Dectin-1 is likely to be centrally involved in the innate response to fungal pathogens. It has also been shown that pathogen-specific receptors, in addition to the TLR receptors, are required to generate proinflammatory responses to pathogens [32].

Proinflammatory cytokine responses in mouse peritoneal macrophages to *C. posadasii* spherules are dependent on TLR2, MyD88, and Dectin-1 [17]. Our results with human samples support such evidences observed in mouse models, but apparently there are other pathways that are convoluted in the Coccidioides immune response. The increased TNF-alpha production by CD14-CD11c- and CD14-CD11c+ cells in the immune patients group of human coccidioidomycosis is the first evidence that shows a subset of cells associated with the cure of the disease, but because due to the large variation in expression, it is quite probable that other factors may also be involved in combination with this specific immunophenotype. The first data showing the production of TNF-alpha in PBMCs from both immune and non-immune subjects incubated with arthroconidia or spherules derived from the dimorphic fungus *Coccidioides immitis* were performed elsewhere [33, 34], but were unable to differentiate the response from those that progressed to a chronic disease. However, the production of these acute inflammatory cytokines was associated with the

immunopathogenesis of active coccidioidomycosis [41].

By our evidences we suggest that might have a Th2 polarization in the PBMC response to *Coccidioides*, mainly due to endospores and spherules stimuli. Furthermore, our results also suggest that TNF-alpha production may be necessary in certain APC subtypes to counteract the immunosuppressive effect of IL-10 produced by B cells, which is especially observed in the discharged immune patients. Apparently, the TNF-α production may be induced by CD14+CD11c+ mediated by dectin-1, which is highly expressed only in immune patients, but we need to perform more tests, like blocking Dectin-1, before we confirm that.

Despite of so many evidences of Dectin-1 acting in different fungi infections, our results reinforce a notion that other pathways in different cell types may also be acting towards the progression of Coccidioidomycosis, which is also dependent on the type of stimulus.

4.CONCLUSION

This is the first study that uses three different stimuli from *Coccidioides* with two patients' groups and a healthy control. Our hypothesis was that the stimulus would allow us to identify specific subsets of cells that could differentiate immune patients from chronic ones. But most important, we could prove that both fungus stages (forms) behaved with an immunosuppressive effect in chronic patients, while the T27K antigen complex generated an opposite profile, suggesting that the protective effect observed in mouse may also be induced in humans, reinforcing the vaccine approach in the near future.

5.MATERIALS AND METHODS

5.1 Antigen preparation

T27K was purified by water-soluble coccidioidal preparation isolated from disrupture of mature spherules, which was killed with thimerosal and after

centrifuged at 27 000 g. Spherule-endospore (SE) cultures were prepared as previously described using the *C*. *posadasii* Silveira strain [35]. Posterior, spherules and endospores were harvested from SE phase cultures and placed in thimerosal (1:10,000) for 72 hours. An aliquot was then cultured to confirm no viable organisms remained prior to other experiments.

5.2 Human subjects

Blood samples of thirteen patients were obtained by a licensed phlebotomist at the University of California Davis Medical Center (Sacramento, CA) and samples placed on ice until testing begun. Donors known to be infected with human immunodeficiency virus or known to have undergone allogeneic transplantation were excluded from the study. The clinical score of severity of coccidioidomycosis was determined by serologic testing for the presence of antigens reactive with IgM and IgG antibodies by immunodiffusion (ID). It was performed as previously described using heated antigen to detect IgM antibody reactivity, and unheated antigen to detect IgG reactive with the antigen (chitinase *Cts1*) [36]. The study protocol was approved by the Internal Research Board of the University of California, Davis. The patients are described as shown on **Table 1.**

Chronic	Sex	Ethnicity	Age	Healthy-Immune	Sex	Ethnicity	Age	Healthy non-immune	Sex	Ethnicity	Age
PATIENT 1	MALE	Caucasian	61	PATIENT 9	MALE	Caucasian	83	PATIENT 13	MALE	Caucasian	30
PATIENT 2	MALE	Asian	39	PATIENT 10	MALE	Hispanic	40	PATIENT 14	MALE	Caucasian	22
PATIENT3	MALE	African American	54	PATIENT 11	FEMALE	Caucasian	22	PATIENT 15	FEMALE	Filipino	40
PATIENT 4	FEMALE	Caucasian	70	PATIENT 12	MALE	Caucasian	37	PATIENT 16	MALE	Caucasian	34
PATIENT 5	MALE	African American	59								
PATIENT 6	FEMALE	Caucasian	71								
PATIENT 7	MALE	Caucasian	75								
PATIENT 8	MALE	African American	33								

Table 1: Description of all the patients that volunteered for this experiment.

5.3 Isolation and stimulation of PBMC

Approximately 30 ml of peripheral blood was drawn by venipuncture from volunteer healthy donors, immune donors and chronic patients and collected into sodium heparin. Whole blood was spin and collected 2ml plasma. Blood was transferred to 50ml falcon tube added with 20ml of PBS. Isolation was performed layering over ficoll gradient (Ficoll-Hypaque; Amersham Pharmacia, GE Healthcare, Piscataway, NJ). PBMC were collected from the interface layer, and these were ressuspended in culture media (RPMI-1640 supplemented with 10 % FBS, 100 U ml $^{-1}$ penicillin / streptomycin, and 1 % glutamine (Gibco). Then, 3 million cells (1ml) were added to Falcon Round-bottom for stimulation with T27K (20ug/ml), endospore and spherules (10^4 cells/ml each) overnight at 37 ° C with 5 % CO2. After 4 hours of incubation we added 2uL of BFA (Brefeldin- A) 10ug/ml (Biolegend) for intracellular cytokine analysis.

5.4 Flow Cytometric analysis of PBMC

For surface receptors and intracellular cytokine analysis, nonadherent cells (three million cells per tube) were incubated first with Aqua Live / Dead Dye (Invitrogen,Carlsbad, CA), for 18 min at 37°C with 5 % CO2 and then we proceeded with surface staining by adding fluorochrome-conjugated mouse anti-

human monoclonal antibodies specific for the surface antigens CD3 Pacific Blue conjugated, CD3 APC-Cy7 conjugated (Biolegend, San Diego, CA), CD4 Qdot 605 conjugated, CD14 Qdot 655 conjugated (Invitrogen), CD8 AF700 conjugated, CD11c PE/Cy5 conjugated, CD19 Pacific Blue conjugated, CD19 PE/Cy5 conjugated, HLA-DR APC/Cy7 (allophycocyanin [APC]-Cy7 conjugated), TLR2 FITC conjugated, CD206 (Mannose receptor) PE/Cy5 conjugated (Biolegend, San Diego, CA), DECTIN-1 FITC conjugated (Ab SEROTEC) and then fixed the cells using the 2%PFA (Caltag, Burlingame, CA). After incubation for 30 min at RT, cells were washed and the intracellular staining, IL-10 PE conjugated and TNF alpha PerCP/Cy5.5 (Biolegend, San Diego, CA) were added with Perm&Fix buffer (Invitrogen), incubated for 30 min at RT and then fixed with 2%PFA (Caltag, Burlingame, CA). Flow cytometry was performed using LSR II flow cytometer (Beckton Dickinson at the UC Davis core facility) with a minimum acquisition of 1.000.000 events collected. The data was analyzed by Flow Jo (Tree Star, San Carlos, CA). For one panel cells were gated based on forward and side-scatter characteristics first and then CD3/CD19 (PE conjugated) gate was performed so that the monocytes and DC gate for cells expressing DECTIN-1 could be possible. For the lymphocyte panel, CD3, CD8, CD4 and CD19 were gated separately. At a last panel the gate was for the TLR2 expressing cells. The TNF alpha was analyzed at all panels and the IL-10 only at the lymphocyte panel.

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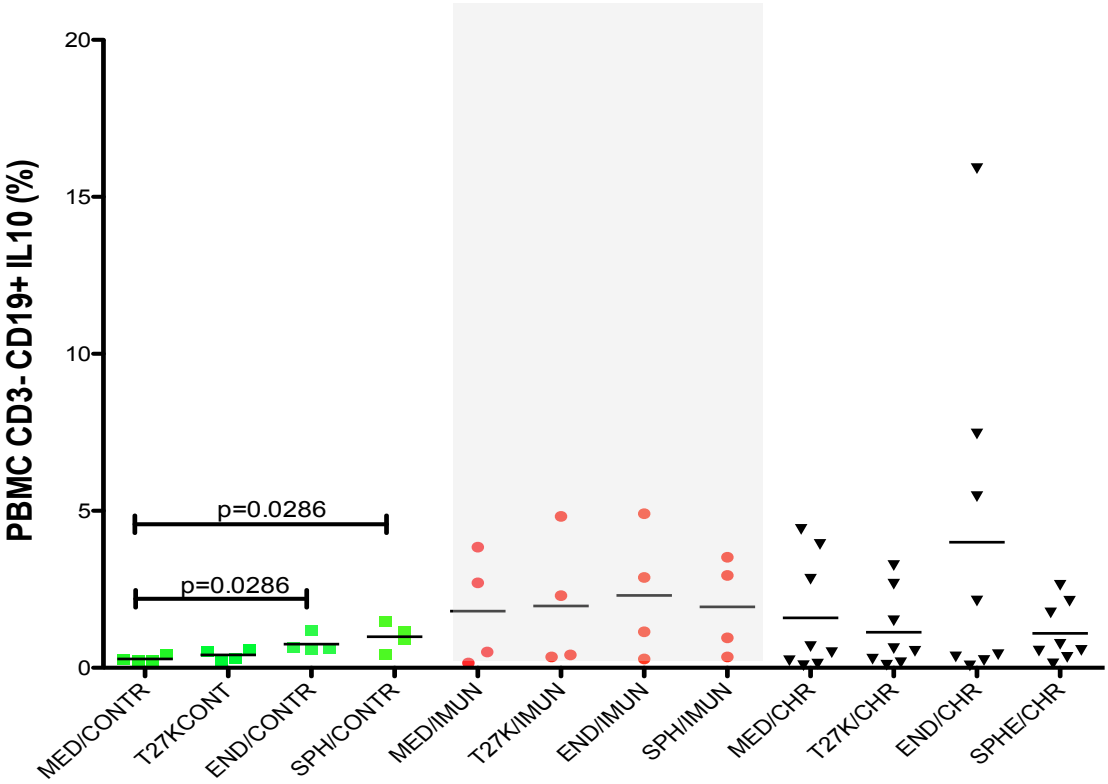

Figure 1. IL-10 production by CD19+ cells after stimulation with T27K, endospore and spherules within 3 distinct groups of patients: The p values are showing the significance after stimulation with endospore and spherules, where p=0.0286 and p=0.0286 for both stimulations (using two tailed T-student test). Stimuli : MED=Media (without stimuli), T27K=T27K antigen, END=endospore, SPH=spherules. Patients : CONT=healthy non immune patients, IMUN= healthy immune patients, CHR= chronic patients.

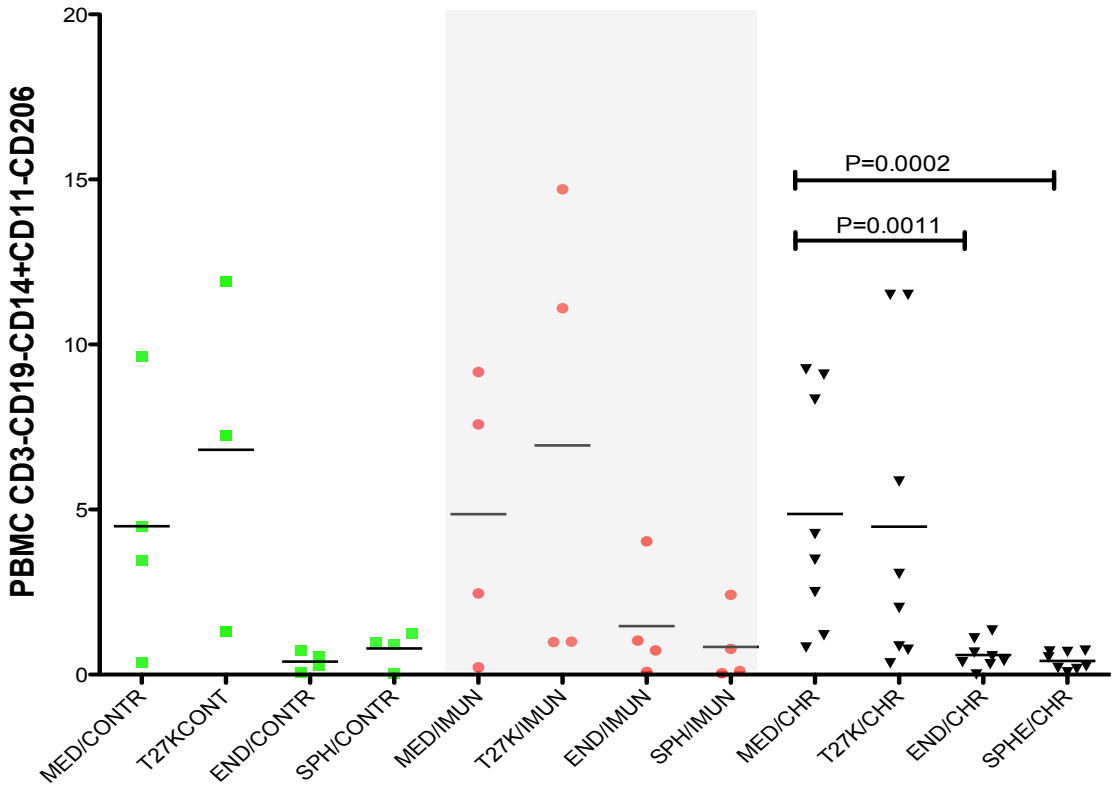

Figure 2. CD206 (Mannose receptor) reduction of cell surface expression by CD14+CD11c- cells of chronic patients: The p values are showing the significance after stimulation with endospore and spherules (p=0.0011 and p=0.0002, respectively, with the t-student). Stimuli : MED=Media (without stimuli), T27K=T27K antigen, END=endospore, SPH=spherules. Patients : CONT=healthy non immune patients, IMUN= healthy immune patients, CHR= chronic patients.

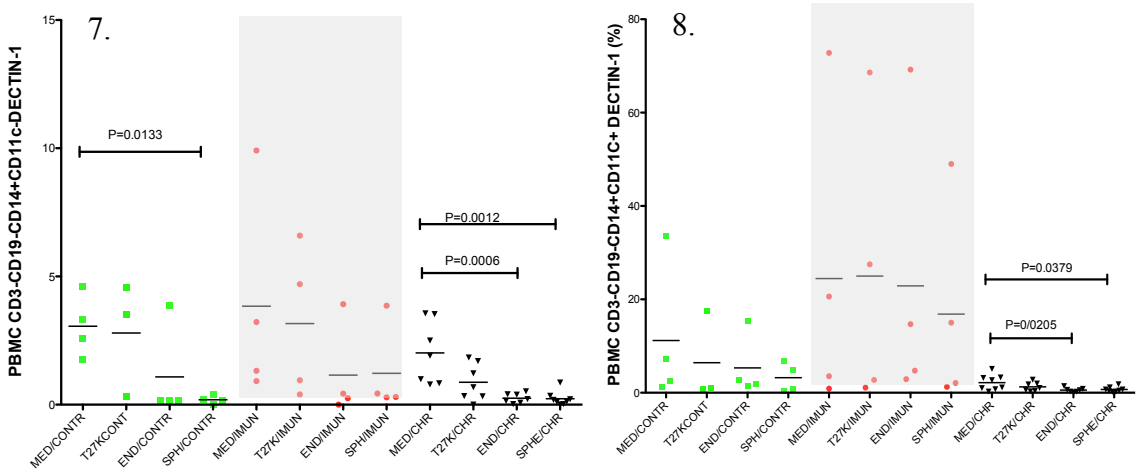

Figure 3. A. DECTIN-1 reduction of cell surface expression by CD14+CD11c- cells of chronic and healthy non-immune patients: The p value is showing the significance after healthy non-immune patients cells stimulation with spherules (p=0.0133) and chronic patients cells stimulation with endospores and spherules (p=0.0012 and p=0.0006, respectively). **B. DECTIN-1 reduction of cell surface expression by CD14+CD11c+ cells of chronic patients**: The p values are showing the significance after stimulation with endospore and spherules (p=0.0011 and p=0.0002, respectively). Stimuli : MED=Media (without stimuli), T27K=T27K antigen, END=endospore, SPH=spherules. Patients : CONT=healthy non immune patients, IMUN= healthy immune patients, CHR= chronic patients. All comparisons were evaluated by t-student test.

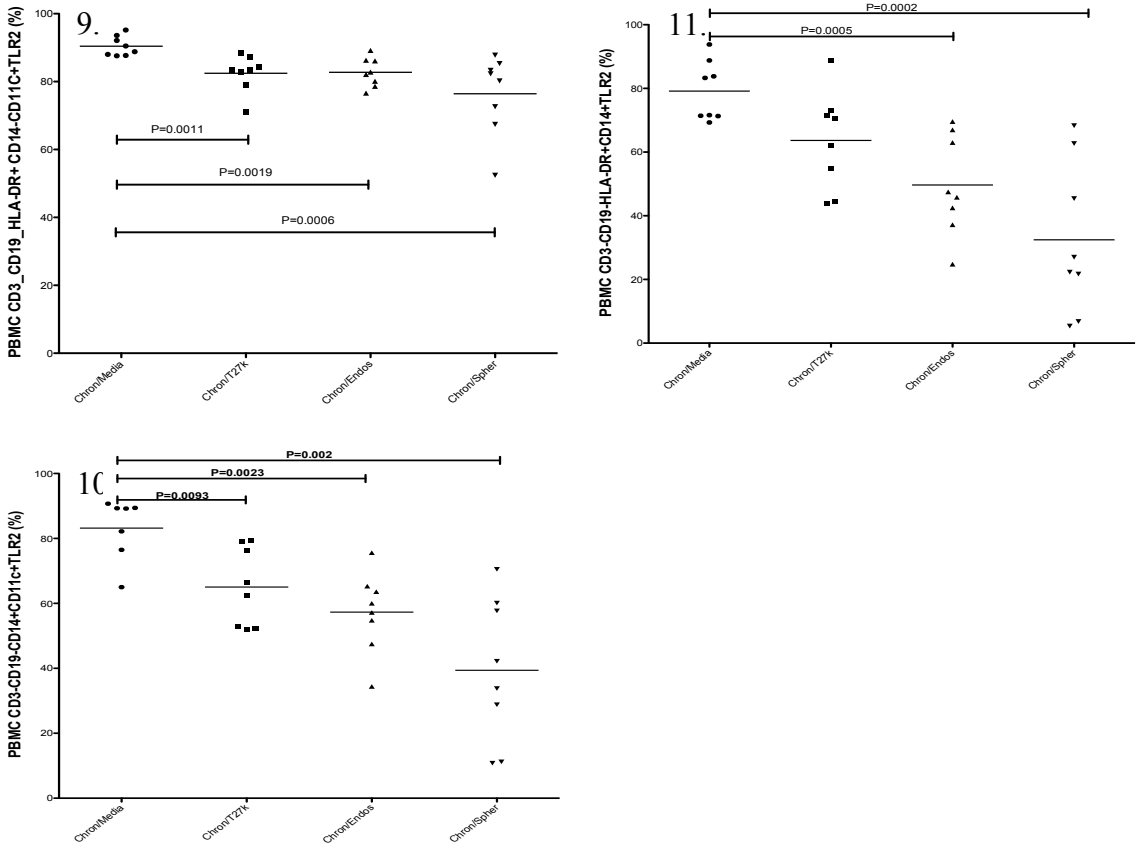

Figure. 4A. TLR2 reduction of cell surface expression by HLA-DR+CD14-CD11c+ cells of chronic patients: The p values are showing the significance after stimulation with T27K, endospore and spherules (p=0.0011, p=0.0019 AND p=0.0006, respectively). **B. TLR2 reduction of cell surface expression by HLA-DR+CD14+ cells of chronic patients**: The p values are showing the significance after stimulation with endospore and spherules (p=0.0005 AND P=0.0002, respectively). **C. TLR2 reduction of cell surface expression by CD14+CD11c+ cells of chronic patients:** The p values are showing the significance after stimulation with T27K, endospore and spherules (p=0.0093, p=0.0023 AND P=0.002, respectively). Stimuli: MED=Media (without stimuli), T27K=T27K antigen, END=endospore, SPH=spherules. Patients: CHR= chronic patients.

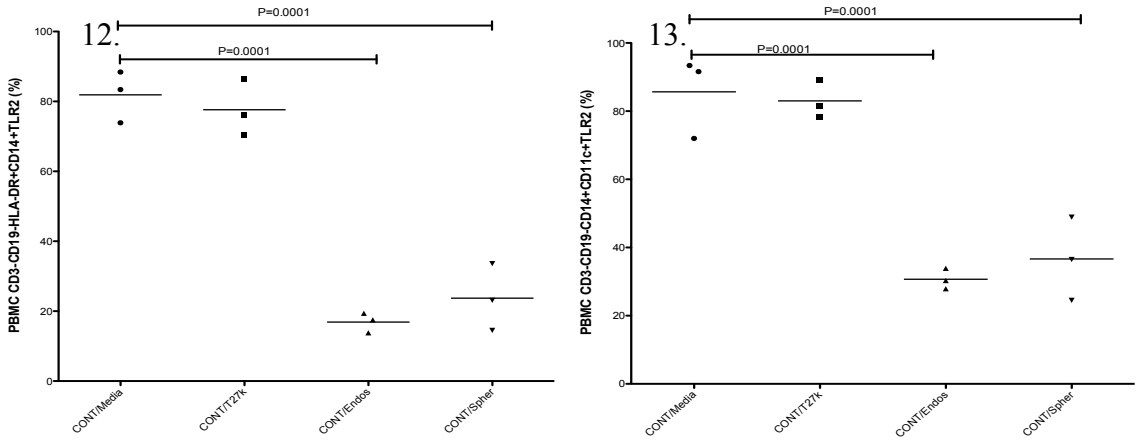

Figure 5. TLR2 lack of cell surface expression by A. HLA-DR+CD14+ cells and B. CD14+CD11c+ cells from healthy non-immune patients. The p values are showing the significance after stimulation with endospore and spherules (p=0.0001) for both types of cells. Stimuli: MED=Media (without stimuli), T27K=T27K antigen, END=endospore, SPH=spherules. Patients: CONT=healthy non immune patients.

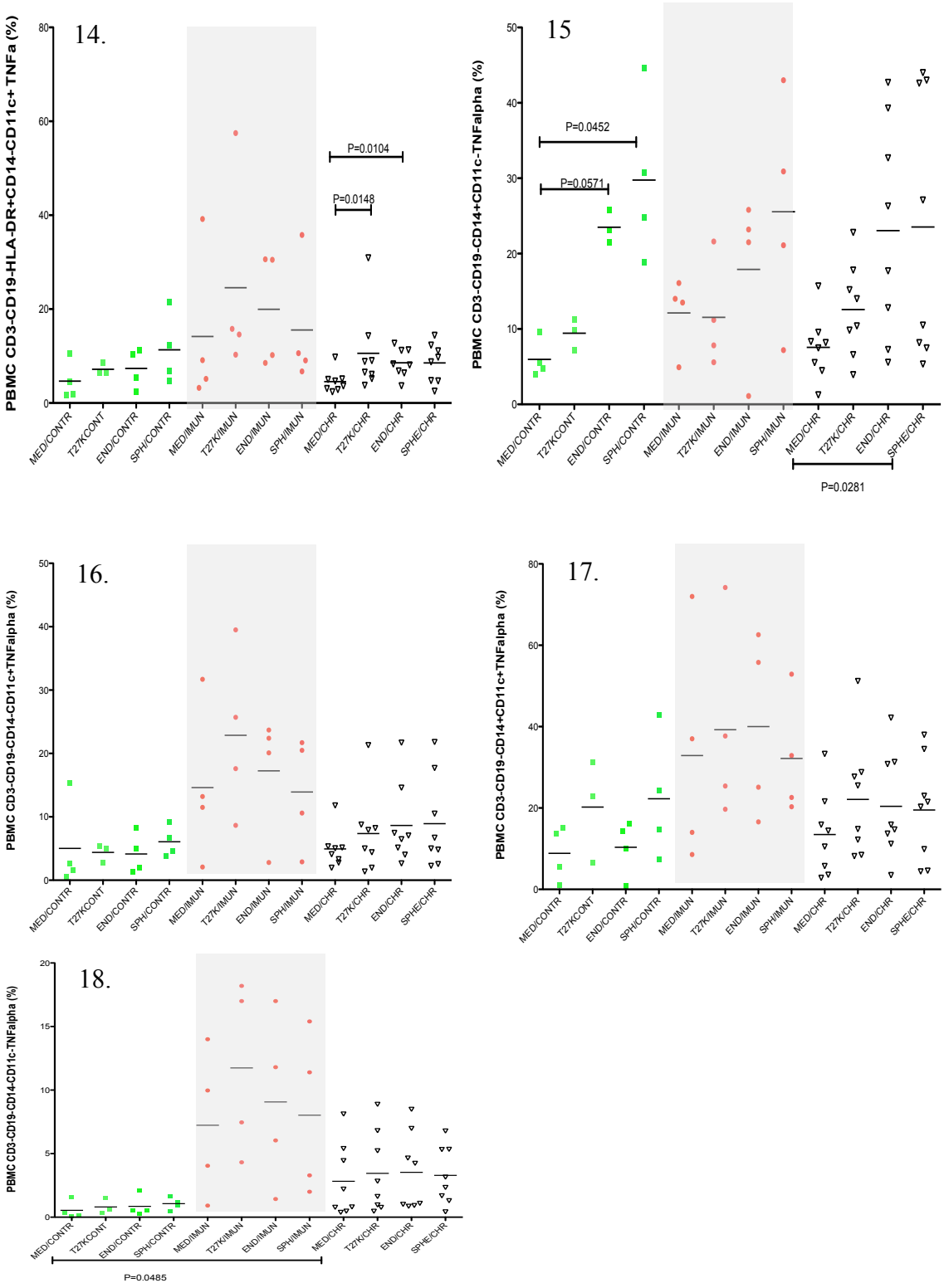

Figure 6. ***TNF alpha production by APCs*****. A.** HLADR+CD14-CD11c+ cells from chronic patients expressing increasing levels of TNF alpha after stimulation with T27K and endospores (p values of 0.0148 and 0.0104, respectively). CD14+ CD11c- cells from healthy non-immune donors increasing production levels of TNF alpha after stimulaton with endospore (p=0.0471)

and spherules (P=0.0452). Also, chronic patients had their levels of TNF alpha significantly increased (p = 0.0281). **C, D and E.** Other APCs (CD14-CD11c+, CD14+CD11c+ and CD14-CD11c-, respectively) that did not present significance between the stimulations but indicated a pattern of higher TNF alpha production on healthy immune donors comparing with other groups of patients.