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"JÚLIO DE MESQUITA FILHO"
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Reginaldo Keller Fernandes

Alterações Transcricionais em Células Dendríticas e Células T CD4^{+} Humanas em Resposta ao *Paracoccidioides brasiliensis*

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutor em Patologia.

Orientadora: Profª Drª Ângela Maria Victoriano de Campos Soares

Coorientador: Profº Drº João Pessoa de Araújo Júnior

Botucatu

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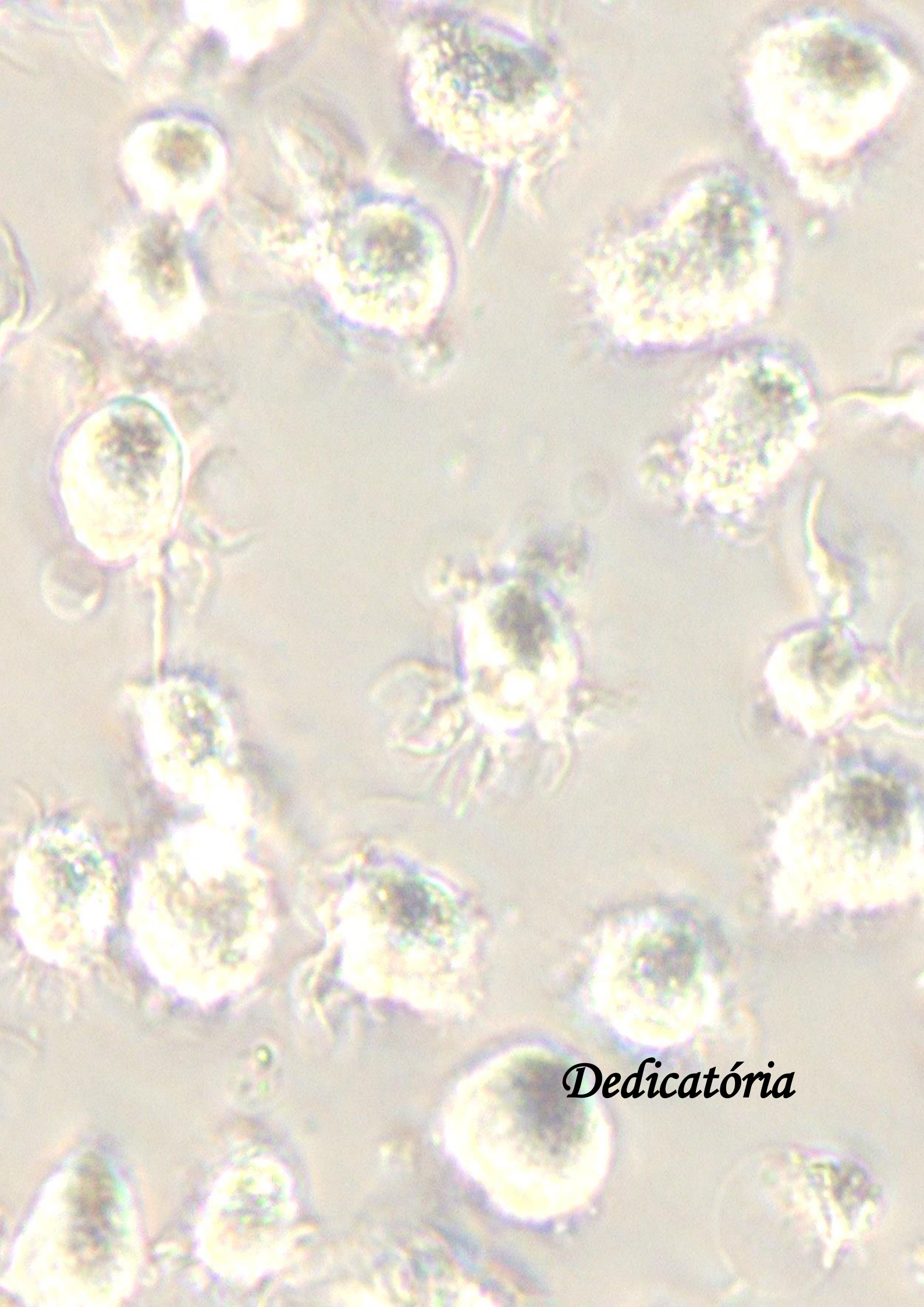

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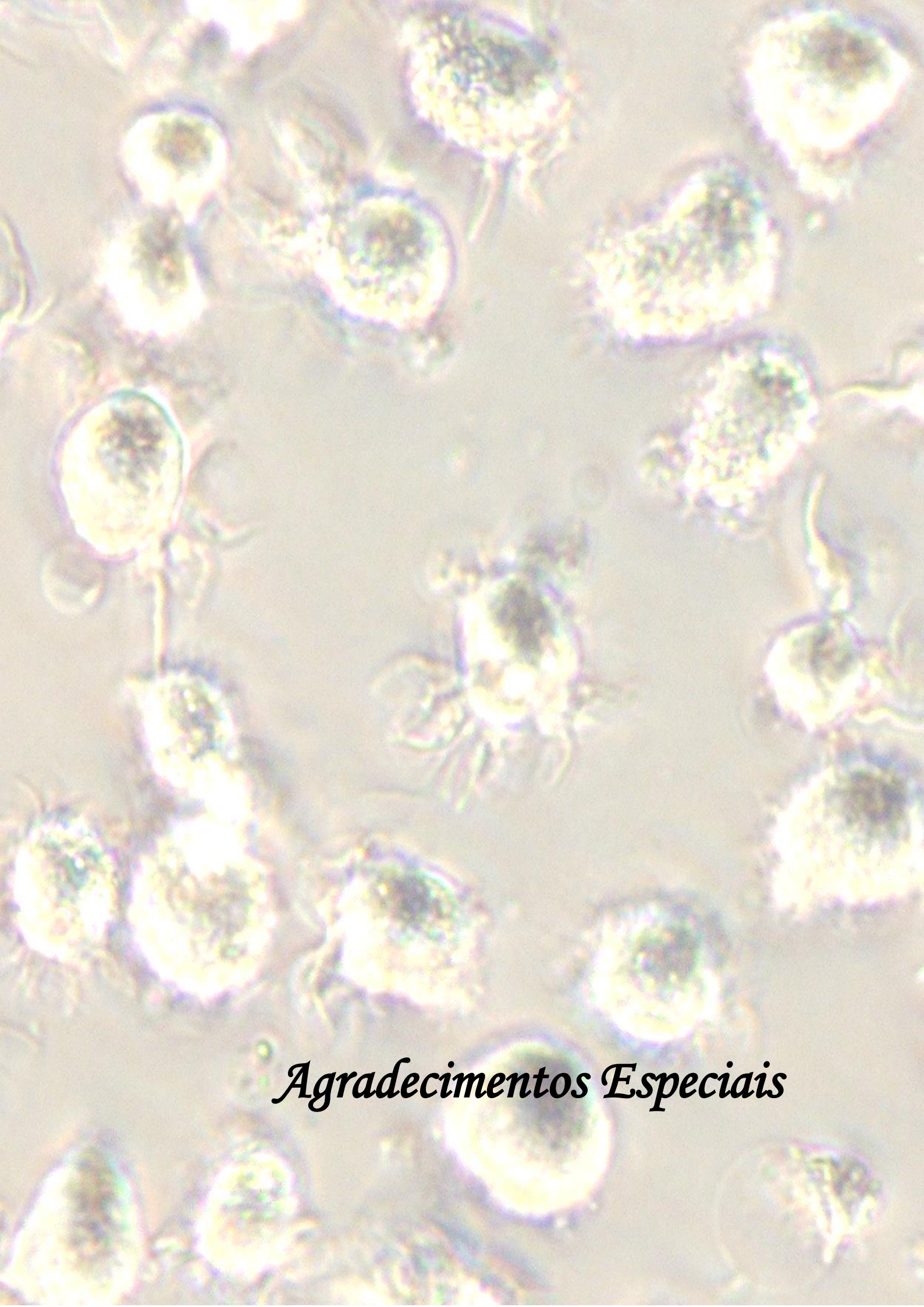

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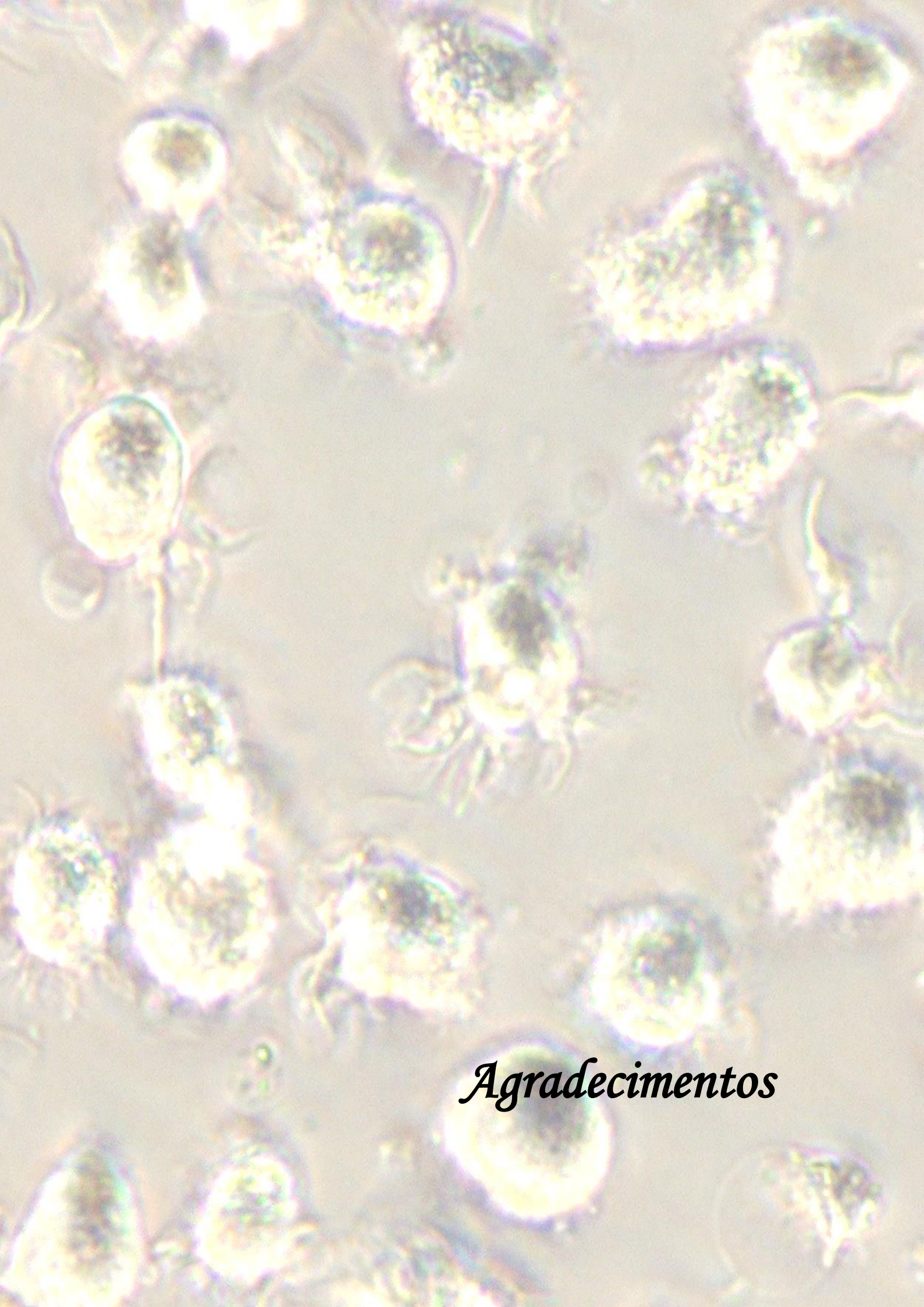

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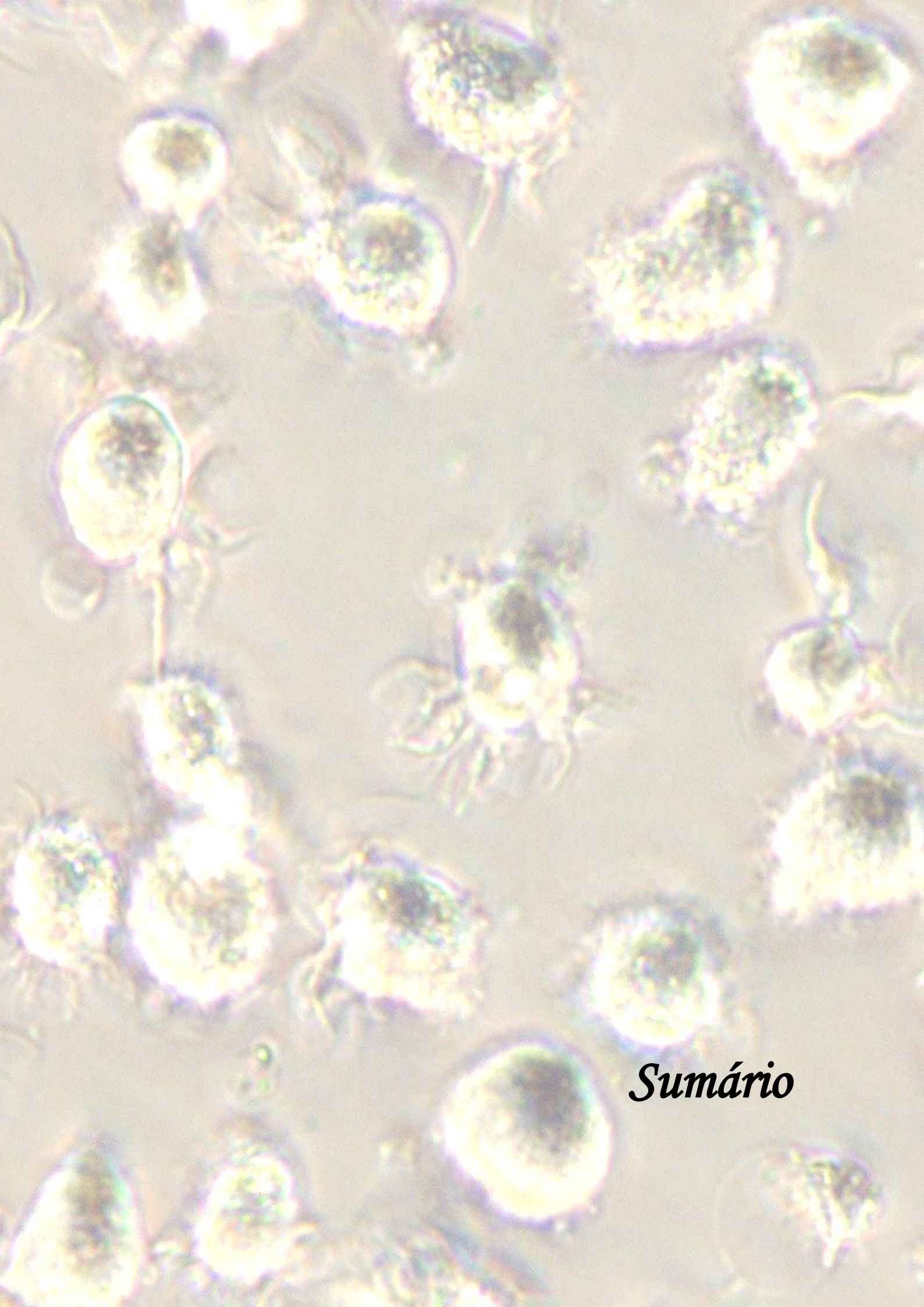
"O Planeta não precisa mais de pessoas bem sucedidas. Precisa desesperadamente de mais pacificadores, curadores, restauradores, contadores de histórias e amantes de todo tipo. Precisa de mais pessoas que vivem bem em seus lugares. Precisa de pessoas de coragem e moral dispostas a se juntar para tornar o mundo habitável e humano. E essas qualidades têm pouco a ver com o que temos definido como sucesso " (David Orr).



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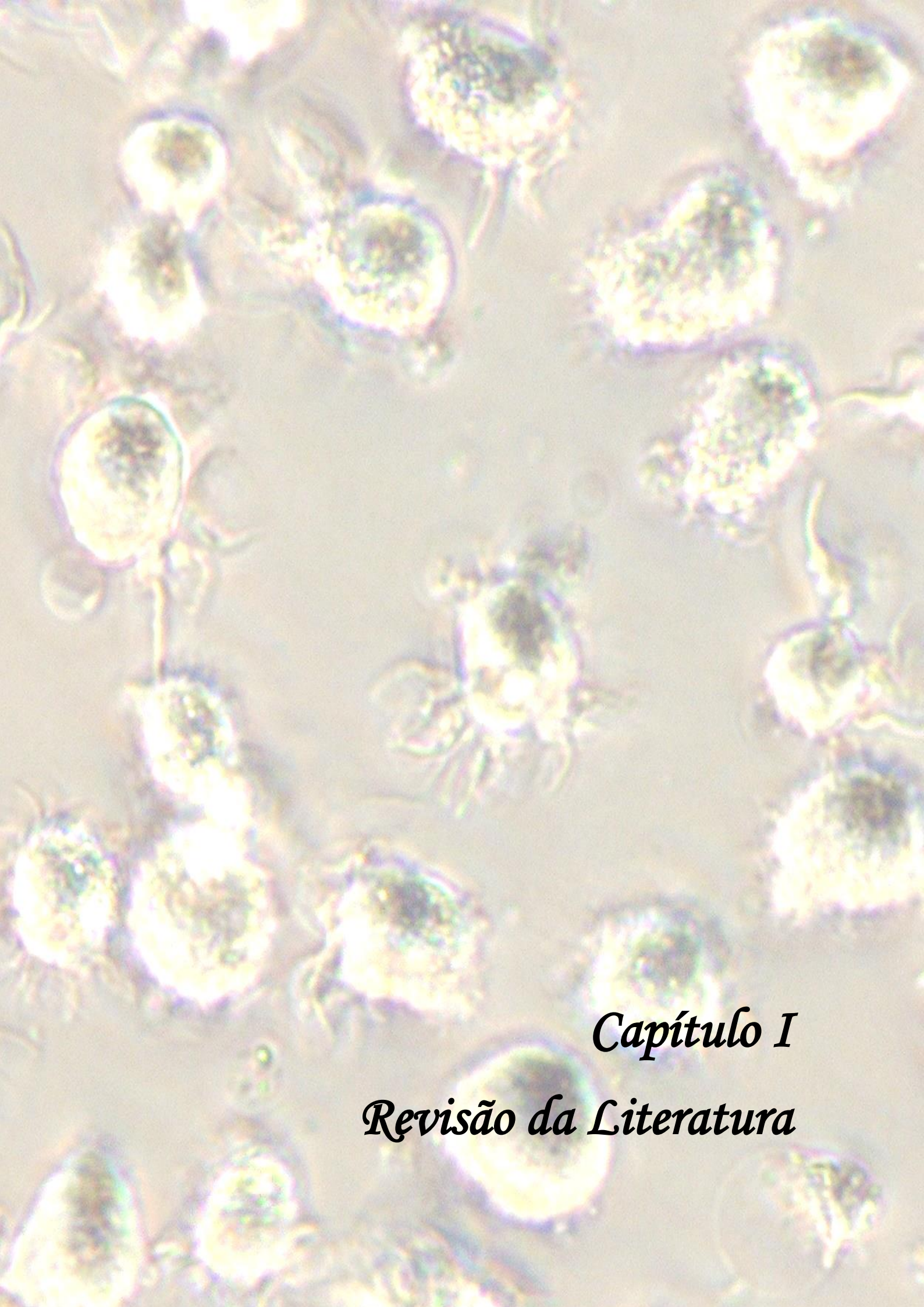

Capítulo I

Revisão da Literatura

1. Revisão da Literatura

1.1. Paracoccidioidomicose: Imunidade Inata e Adaptativa

A paracoccidioidomicose (PCM) é uma micose sistêmica que se manifesta endemicamente na maioria dos países da América Latina, especialmente Brasil, Argentina, Colômbia e Venezuela. Seu agente etiológico é um fungo imperfeito e dimórfico do gênero Paracoccidioides que pode ser dividido em duas espécies: *Paracoccidioides brasiliensis* (Pb) que ainda pode ser dividido em 3 espécies crípticas (S1, PS2, PS3) e *Paracoccidioides lutzii*. Eles se apresentam sob a forma de levedura *in vivo* e quando cultivados a 37ºC em meios de cultura enriquecidos, e na forma de micélio à temperatura ambiente com variação de 4 à 28ºC (Restrepo & Tobón, 2005; Matute *et al*., 2006, Carrero *et al*., 2008; Marini et al., 2010).

Os agentes infectantes do Pb seriam propágulos micelianos presentes principalmente no solo e, hipoteticamente, na água e nas plantas, que penetram no hospedeiro pelas vias aéreas, atingindo primeiramente os pulmões, provocando um complexo primário pulmonar. Esse processo pode evoluir para a cura ou tornar-se latente caracterizando a PCM-infecção, identificada pela ausência de sinais ou sintomas clínicos, mas com o desenvolvimento de uma resposta imune adaptativa, que pode ser evidenciada pelo teste intradérmico com paracoccidioidina (Oliveira, 2002). Ao contrário, o processo pode progredir para a PCM-doença com consequente disseminação para outros órgãos como fígado, baço e adrenais pela via linfo-hematogênica (Franco *et al.*, 1987). As manifestações clínicas da micose podem ser agrupadas em dois padrões que definem as formas aguda (juvenil) e crônica (adulta) da doença. A forma aguda é habitualmente grave, de evolução rápida e compromete o sistema fagocítico mononuclear (baço, fígado, linfonodos e medula óssea). A forma crônica tem duração prolongada, instalação lenta e gradual e as lesões permanecem localizadas ou envolvem mais de um órgão ou sistema (Franco *et al.*, 1993; Mendes, 1994).

O estabelecimento da PCM-infecção ou PCM-doença nas suas formas mais ou menos graves depende principalmente das complexas interações parasita-hospedeiro que podem resultar em uma resposta imune mais ou menos eficaz por parte deste último. Neste contexto, muitos estudos clínicos e experimentais realizados com o objetivo de caracterizar a resposta imune do hospedeiro infectado mostraram uma importante

participação tanto da resposta imune inata como da adaptativa (Benard, 2008; Calich *et al*., 2008).

1.2. Imunidade Inata

O sistema imunológico inato compreende a primeira linha de defesa contra agentes patogênicos e efetivamente protege contra microorganismos invasores. Os mecanismos da resposta imune inata desempenham um importante papel no combate a fungos patogênicos. Dentre eles, destacam-se as células fagocíticas como neutrófilos, monócitos, macrófagos e DCs que participam como efetoras diretas exercendo atividade fungicida/fungistática e como moduladoras da própria resposta inata assim como da resposta adaptativa que será desenvolvida posteriormente (Andrade *et al*., 2008; Brown, 2011).

Na PCM cuja infecção atinge primeiramente os pulmões, acredita-se que a resposta inicial seja mediada pelos macrófagos alveolares. Estudos têm mostrado que após serem inalados, os conídios do fungo são fagocitados por essas células e posteriormente diferenciam-se em leveduras (McEwen *et al*., 1987). Estas células podem fagocitar o Pb através de receptores opsônicos e não opsônicos. Nesse sentido, estudos têm mostrado que o reconhecimento e a fagocitose podem ocorrer através do receptor para complemento (CR3) presente nos macrófagos (Calich *et al*., 1979), uma vez que o Pb é capaz de ativar esse sistema pela via alternativa (Munk & Da Silva, 1992) ou pela via da lectina (Toledo *et al*., 2010). O reconhecimento pode ainda ocorrer via receptores do tipo *Toll Like* (TLRs) (Bonfim *et al*., 2009; Calich *et al*., 2008b) ou ainda do tipo manose (Popi *et al*., 2002).

Após o processo de fagocitose, as atividades efetoras diretas dessas células, assim como as atividades moduladoras, dependerão das complexas interações que serão estabelecidas com o fungo e com as outras células presentes no infiltrado inflamatório. Assim, vários estudos têm mostrado que as citocinas IFN-γ, TNF-α, GM-CSF e IL-15 estão envolvidas na ativação de monócitos, macrófagos e neutrófilos murinos e humanos para que essas células exerçam atividade fungicida / fungistática eficiente contra o Pb (Brummer *et al.*,1988 e 1989; Cano *et al.*, 1992 a e b, Moscardi-Bacchi *et al.*, 1994; Cano *et al.*, 1998; Kurita *et al.*, 1999; Gonzalez *et al.*, 2000; Kurita *et al.*, 2000; Souto *et al.*, 2000; Calvi *et al.*, 2003 a e b; Kurita *et al.*, 2005 , Carmo *et al.*, 2006; Rodrigues *et al.*, 2007; Moreira *et al.*, 2008; Tavian *et al.*, 2008, Bannwart *et al*., 2010). Adicionalmente,

os estudos mostraram que o mecanismo através do qual essas citocinas aumentam essa atividade, envolve a ativação do metabolismo oxidativo com consequente produção de H_2O_2 e NO os principais metabólitos envolvidos. Por outro lado, fatores que levam a inibição da atividade fungicida dessas células têm sido relatados, como a IL-10 que bloqueia a atividade fungicida de neutrófilos humanos e macrófagos murinos ativados com IFN-γ ou TNF-α (Costa *et al.*, 2007, Moreira *et al.*, 2010). Outras citocinas como a IL-6 e a IL-18, quando em contato com monócitos, além de não induzirem atividade fungicida, estimulam o crescimento do fungo no interior dessas células (Siqueira *et a.l*, 2009; Dias-Melicio *et al.*, 2015). Estes estudos reforçam a ideia de que as células fagocíticas de uma forma autócrina, conseguem modular as suas próprias atividades antifúngicas e provavelmente, as de outras células. De fato, estudos têm mostrado que monócitos liberam tanto citocinas pró como anti-inflamatórias em resposta ao fungo (Kurokawa *et al*., 2007). As células fagocíticas podem ainda modular as suas atividades atuando sobre outras células, que são fontes de importantes citocinas, como as NK, produtoras de IFN-γ.

Além das citocinas, outros estudos mostram a importancia de mediadores lipídicos sobre a atividade dessas células. Em estudo prévio, nosso grupo mostrou que cepas de alta e baixa virulência do fungo induzem a produção de PGE_2 por monócitos humanos, inibindo sua atividade fungicida de forma autócrina (Soares *et al*., 2001; Bordon *et al*., 2007; Bordon-Graciani *et al*., 2012). Outro estudo envolvendo mediador inflamatório destacou que em macrófagos de modelos murinos com PCM e macrófagos murinos infectados *in vitro* com Pb, as lipoxinas exercem função prejudicial ao hospedeiro com inibição de PRRs essenciais para interação de macrófagos com o fungo (Ribeiro *et al*., 2015).

Os trabalhos acima deixam claro que uma modulação positiva ou negativa pelas células fagocíticas, dependerá do balanço entre a liberação de citocinas ativadoras como o TNF-α e o GM-CSF e IFN-γ e as desativadoras como a IL-10. Esse processo parece estar vinculado principalmente à cepa do fungo e a sua capacidade de induzir a liberação de um ou outro tipo de citocina (Calvi *et al*., 2003a). Adicionalmente, estudos têm mostrado de forma clara, que a eficiência das atividades das células fagocitárias dependerá do tipo de receptor de reconhecimento de padrão molecular (PRR) ao qual o fungo preferencialmente irá se ligar. Calich *et al* (2008b), usando camundongos TLR4 deficientes e *knockout* para TLR2, mostraram que tanto o TLR2 como o TLR4 estão

envolvidos no reconhecimento do fungo. Esse reconhecimento resulta em um aumento da capacidade fagocítica com infecção de macrófagos e secreção de óxido nítrico (NO). No entanto, esse processo parece não levar a uma eficiente atividade macrofágica e diminuição significativa da carga fúngica, pois tanto animais normais quanto os deficientes apresentaram a mesma taxa de sobrevida à infecção. Estudos em nosso laboratório, avaliando o papel dos receptores TLR2 e TLR4 na atividade de neutrófilos humanos desafiados com a cepa virulenta do Pb (Pb18), sugerem que a interação do fungo com os dois receptores pode ser considerada um mecanismo de patogenicidade, uma vez que esse fungo usa o TLR2 e principalmente o TLR4 para a sua entrada nas células e escapar das funções efetoras destas através da produção de IL-8 e IL-10 (Acorci-Valério *et al.*, 2010). Nakaira-Takahagi *et al* (2011), mostraram que tanto o fungo, como a gp43, seu principal antígeno, são capazes de modular a expressão de TLR2 e TLR4 e consequentemente a produção de citocinas pró e anti-inflamatórias por monócitos humanos. Além disso, foi mostrado que a fração gp43 se liga ao receptor de manose (MR) para inibir a capacidade fagocítica e fungicida de macrófagos peritoneais de camundongos resistentes e suscetíveis (Popi *et al.*, 2002). Esta descoberta levou os autores a postular que a secreção de gp43 é um dos mecanismos de escape apresentados pelo Pb e que essa proteína exerce seus efeitos ligando-se ao MR.

Entretanto, uma outra célula que tem papel importante contra as infecções fúngicas são as células *natural killer* (NK) que tem papel importante na imunidade inata por meio da secreção de citocinas e quimiocinas, lise de células infectadas e mesmo por eliminação direta das leveduras (Lieberman & Mandelboim, 2000; Vivier *et al*., 2008). Na PCM, os estudos envolvendo as células NK ainda são escassos. Apesar disso, alguns grupos de estudo mostraram resultados interessantes. Um estudo focado na capacidade citotóxica dessas células destacou o comprometimento desta atividade em indivíduos com PCM (Peraçoli *et al*., 1991). Por outro lado, um outro estudo envolvendo células NK de camundongo, demonstrou que essas células apresentam atividade citotóxica contra células infectadas com leveduras do Pb e ainda foram capazes de impedir o crescimento das leveduras *in vitro* (Jimenez & Murphy, 1984). Mais recentemente, Longhi *et al*. (2012), mostraram que as células NK podem eliminar diretamente leveduras do Pb via liberação de granulisina ou então reconhecer e atuar efetivamente na destruição de outras células infectadas. Ao mesmo tempo, as células NK podem apresentar um papel imunomodulatório na imunidade adaptativa por meio da liberação de citocinas como IFNγ e TNF-α.

Desse modo, células NK de indivíduos saudáveis com PCM controlada, de indivíduos saudáveis infectados e que não apresentaram a doença e de indivíduos saudáveis não infectados, liberaram níveis significativos de IFN-γ, IL-4 e IL-10 ao mesmo tempo em que não tiveram comprometimento quanto ao número dessas células, quanto à capacidade de expansão e a produção de citocinas (Batista *et al*., 2011).

1.3. Imunidade Adaptativa

Muitos estudos nos últimos anos foram realizados com o objetivo de caracterizar a resposta imune adaptativa do hospedeiro infectado. Os trabalhos pioneiros mostraram de uma forma muito clara, que os pacientes, particularmente aqueles com doença mais grave, assim como camundongos suscetíveis à infecção, apresentam depressão da resposta imune celular (Castaneda *et al*., 1988; Fazioli *et al*., 1994) associada a uma exacerbação da resposta humoral (Arango *et al*., 1982; Mota *et al*., 1985; Calich *et al*., 1985; Calich *et al*.,1994; Cano *et al*., 1995). Estudos posteriores realizados com objetivo de conhecer os mecanismos envolvidos no desequilíbrio da resposta imune adaptativa mostraram que na doença mais grave humana e experimental ocorre a indução preferencial de uma resposta de padrão Th_2 em detrimento de uma resposta Th_1 relacionada a mecanismos de proteção. Nesse sentido, pacientes e camundongos apresentam produção aumentada de IgG4, IgE, IgA, eosinofilia periférica e baixos níveis de IL-8, IgA, IgM, IgG1 e IgG2b específicos para o fungo (Calich *et al*., 1985; Cano *et al*., 1995; Baida *et al*., 1999; Mamoni *et al*., 2002). Adicionalmente, estudos sobre o padrão de citocinas caracterizaram que o grupo de indivíduos com paracoccidioidomicose-infecção apresenta resposta imune associada ao padrão Th_1, com alta resposta linfoproliferativa a antígenos do fungo, teste cutâneo de HT positivo, ausência de anticorpos e níveis indetectáveis de IL-4, IL-5 e IL-10, e aumentados de IFN-γ, enquanto pacientes com a forma aguda ou subaguda (juvenil), apresentaram depressão da resposta linfoproliferativa e maiores níveis de IL-4 e IL-5, citocinas representativas do padrão Th_2, quando comparados aos indivíduos somente infectados e àqueles com a forma crônica unifical ou multifocal (adulta) da doença (Oliveira *et al*., 2002; Shikanai-Yasuda, 2015). Nos pacientes foi também observada a associação entre baixos níveis de IL-2 e IFN-γ e depressão da resposta imune celular (Benard *et al*., 2001), assim como altos níveis de IL-4, IL-5 e IL-1β (Mello *et al*., 2002). Outros estudos ainda mostraram que indivíduos com PCM-infecção expressam altos e precoces níveis de RNAm para IFN-

γ, TNF-α e CXCL-9 e CXCL-10 quando comparados aos indivíduos com as formas mais graves da infecção. As citocinas de padrão Th_2: IL-4, IL-10, IL-5 e TGF-β foram sempre maiores nas duas formas da doença em relação aos indivíduos do grupo paracoccidioidomicose-infecção (Mamoni & Blotta, 2005). Confirmando a indução preferencial de uma resposta Th_2, estudos mostraram que pacientes liberam baixas concentrações de IL-12 (Romano *et al*., 2002), assim como expressam baixos níveis dos receptores para essa citocina (Romano *et al*., 2005). Os trabalhos com animais de experimentação, mais uma vez reforçaram os achados humanos. Camundongos suscetíveis liberam um padrão de citocinas de padrão Th_2 e os resistentes um padrão Th_1 (Calich *et al*., 1994; de Almeida *et al*., 1998; Calich *et al*., 1998; Kashino *et al*., 2000; Livonesi *et al*., 2008). Outros trabalhos associaram mais uma vez indivíduos paracoccidioidomicose-infecção ao perfil Th_1 de resposta, uma vez que estes possuem maiores números de células CD8 produtoras de IFN-γ e CD4 produtoras de IL-2 e TNF-α, assim como maior número de células CD3 expressando CXCL-9, CXCL-10 e CXCR4, e um baixo número de monócitos expressando IL-10 (Mamoni & Blotta, 2006).

A partir de um estudo sobre a relação entre os diversos perfis de reposta de células CD4 e as diferentes formas clinicas da doença, de Castro *et al*., 2013, propuseram um modelo para explicar as diversas associações. A resistência à infecção detectada em indivíduos assintomáticos (PCM-infecção) é mediada pela predominância de uma resposta Th_1, cujas citocinas como o IFN- γ, são responsáveis pela ativação de macrófagos, processo que, semelhante ao que ocorre durante a resposta imune inata, é fundamentalmente importante para a resistência à infecção. O perfil de resposta associada à forma mais grave da doença (aguda/juvenil), envolve a predominância de um padrão Th_2 com produção de IL-4 e IL-5 e Th_9 que produz IL-9 e IL-21. As citocinas IL-4 e IL-9 são responsáveis pela indução da produção de anticorpos IgG4 e IgE e IL-5 pela ativação de eosinófilos. A IL-4 contribui para a desativação de macrófagos. Já a forma intermediária da doença (adulta/crônica) apresenta uma resposta mista mediada por predominância de Th_{17} e Th_{22}, e também uma contribuição dos outros subtipos Th_1 /Th_2 /Th_9. Essa resposta mista contribui parcialmente para a resistência a infecção e, ao mesmo tempo, está envolvida na exacerbação da resposta mediada por Th_{17} com consequente ativação de neutrófilos, lesão tecidual e desenvolvimento de fibrose, processo típico dessa forma clínica. Ainda nesse grupo, haveria uma participação das T_{regs} que, ao mesmo

tempo em que teriam o papel de controlar essa resposta inflamatória exagerada, participaria do processo de inibição da ativação macrofágica.

Apesar dos trabalhos citados mostrarem de forma bastante clara que a imunopatogênese na PCM pode ser explicada por mecanismos que inibem uma resposta protetora do tipo Th_1 ou induzem preferencialmente uma resposta não protetora Th_2, o conhecimento nos últimos anos de novas subpopulações de células $CD4^+$ têm estimulado a reflexão de outros possíveis mecanismos. Nesse sentido, os estudos mais recentes mostraram forte associação entre a persistência de um processo inflamatório, a incapacidade de destruição fúngica e a cronicidade das infecções, levando-nos a considerar que a resposta imune a ser desencadeada pelo hospedeiro seja adequada no sentido de permitir a sobrevivência do mesmo e estabelecer um comensalismo com o patógeno, isto é, sem a ocorrência de uma resposta inflamatória excessiva e patológica. Assim, deve-se considerar que embora a inflamação seja um componente essencial da resposta protetora contra o fungo, o seu descontrole pode levar ao desenvolvimento de doenças fúngicas muito graves e limitar as respostas protetoras (Romani *et al*., 2008). Desse modo, o papel de células Th_{17} e T_{regs} parece ser fundamental para a intensidade da resposta contra o fungo.

As células Th_{17} que produzem preferencialmente IL-17 são consideradas como uma população distinta de células T que promovem inflamação caracterizada por um grande infiltrado neutrofílico. Embora vários estudos tenham mostrado que as Th_{17} estejam envolvidas na resistência a vários microorganismos, outros têm mostrado o seu envolvimento nos mecanismos imunopatológicos em várias outras infecções (Stockinger & Veldhoen, 2007). Assim considera-se que as Th_{17} são uma linhagem de células efetoras Th que contribuem para a imunopatogênese previamente atribuída a uma hiperativação de células Th_1 (Dong, 2006). O processo de diferenciação de células T naive em células Th_{17} envolve a liberação de algumas citocinas específicas. Assim, células T naives ativadas na presença de TGF-β mais IL-6, leva a um aumento da expressão do fator de transcrição RORγt (retinoid-related orphan receptor gamma t) e consequente geração para Th_{17}, cuja estabilização é promovida pela IL-23 liberada pelas DCs (Veldhoen *et al*., 2006; Bettelli *et al*., 2007).

Na PCM, embora o seu papel não esteja bem definido, as células Th_{17} tem sido têm sido observadas em lesões cutâneas e de mucosa de pacientes e tem sido associadas a granulomas organizados e destruição fúngica o que associa essas células aos mecanismos de proteção (Pagliari *et al*., 2011).

Em modelo experimental foi mostrado que uma resposta imune Th_{17} prevalente resulta em um controle do crescimento fúngico, mas ao mesmo tempo induz lesão tecidual mediada por neutrófilos. Isso ocorreria porque o TLR2 desempenha um papel protetor durante a infecção pulmonar com o Pb, uma vez que esse receptor controla negativamente a resposta inflamatória exacerbada mediada por Th_{17}, altamente deletéria ao tecido pulmonar. Da mesma forma, mecanismos sinalizadores iniciados por TLR4 também levam a infecção pulmonar grave associada com resposta proinflamatória induzida por Th_{17} e diminuição de células T_{reg} (Loures *et al*., 2009, Loures *et al.*, 2010). Outros estudos mostraram que o receptor dectina-1 está associado a mecanismos de proteção na doença por estar envolvido na diferenciação de células Th_{17} (Loures *et al*., 2014).

Muitas células podem participar da regulação da resposta imune particularmente pela capacidade de produzir IL-10. No entanto, existe um consenso que as células T reguladoras (T_{reg}) estão entre as principais células produtoras dessa citocina. Estas células que têm uma importância fundamental na tolerância a antígenos próprios e podem desempenhar um importante papel no controle de outros padrões de resposta imune, incluindo as desenvolvidas em resposta a fungos (O' Garra & Vieira, 2004). A capacidade das T_{regs} em inibir aspectos da resposta imune inata e adaptativa antifúngica é necessária para a indução da chamada tolerância protetora aos fungos (Romani, 2004; Romani & Puccetti, 2006; Romani & Puccetti, 2007; Romani *et al*., 2008) que implica na defesa do hospedeiro que deve ser adequada para a proteção, sem necessariamente eliminar esses microorganismos. Essa eliminação poderia diminuir o desenvolvimento de uma memória imunológica ou causar um nível de lesão tecidual inaceitável (Romani & Puccetti, 2006). Neste contexto, células T_{regs} com atividade tolerogênica têm sido descritas em infecções fúngicas murinas e humanas (Hori *et al*., 2002; Montagnoli *et al*., 2002; McKinkley *et al*., 2006; Montagnoli *et al*., 2006; Romani & Puccetti, 2008).

Na PCM, tem sido demonstrada a participação de células T com fenótipo regulador associadas a imunossupressão e suscetibilidade à doença. Células com essa característica têm sido detectadas tanto em pacientes (Cavassini *et al*., 2006; Ferreira *et al*., 2010; Cardoso *et al*., 2014; Silva *et al*., 2013) como em modelos experimentais da doença (Moreira *et al*., 2008b; Noal *et al*., 2016; Tristão *et al*., 2013; Felonato et al., 2012; Loures *et al*., 2014).

Estudos mais recentes, no entanto, têm questionado o papel regulador dessas células como sendo vinculado somente a suscetibilidade à infecção. Segundo os estudos, as células T_{reg} teriam um papel modulador sobre uma resposta efetora exacerbada que

poderia levar a lesões teciduais associadas a uma maior suscetibilidade à infecção (Pina *et al*., 2013; de Amorim *et al*., 2013; Felonato *et al*., 2010; Loures *et al*., 2010) Em outro estudo recente Bazan *et al.* (2015), deixaram claro o duplo papel das células T_{regs} durante a evolução da PCM pulmonar em camundongos, isto é, um papel deletério por diminuir a resposta imune efetora e a erradicação do fungo, mas também o seu papel protetor suprimindo a inflamação tecidual exacerbada.

Os estudos deixam clara a necessidade de se entender melhor os mecanismos que direcionam o desenvolvimento de uma ou outra subpopulação de células $CD4^+$ efetoras na PCM. Nesse sentido, a fina regulação da resposta imune adaptativa ao Pb deve-se, em grande parte, à interação inicial entre o agente infeccioso e as células apresentadoras de antígeno que atuam como uma ponte entre a imunidade inata e a adaptativa, estabelecendo o tipo de resposta imune a ser desenvolvida. As células fagocíticas como macrófagos, monócitos e mesmo neutrófilos podem participar desse processo, mas as DCs merecem destaque por constituírem a população celular mais adaptada ao processo de ligar, de fagocitar, de destruir e de processar microrganismos e então migrar aos órgãos linfóides periféricos, locais onde elas maturam para desenvolver o processo de apresentação de antígeno, desencadear e instruir a resposta imune adaptativa (Cella *et al.*, 1997; Steinman, 1991 , Banchereau & Steinman, 1998).

As consequencias da relação DCs/microrganismos são definidas, entre outros fatores, pela ligação a diferentes receptores de reconhecimento padrão (PRRs). A ligação do microorganismo a um ou outro PRR influenciará sobremaneira as várias etapas de ativação das DCs e consequentemente o direcionamento da resposta de células T. Nesse sentido, estudos têm mostrado que tanto os receptores do tipo *Toll* (TLRs), quanto os do tipo lectina, como o receptor de manose, o dectina-1,2 e o DC-SIGN, podem estar envolvidos no reconhecimento dos fungos pelas DCs, possibilitando o desenvolvimento de diferentes tipos de resposta (Ramirez-Ortiz & Means, 2012).

No que se refere ao Pb, recentemente foi demonstrado, que após a infecção ocorrem alterações importantes nas DCs pulmonares que incluem a expressão de receptores para quimiocinas e consequente migração dessas células para os linfonodos regionais. Adicionalmente, as DCs derivadas da medula óssea estimuladas com Pb migram para os linfonodos e ativam respostas $CD4^+$ preferencialmente do tipo Th_2 (Silvana dos Santos *et al*., 2011). Esses resultados corroboram com outros do mesmo grupo, mostrando que DCs de animais suscetíveis à infecção, apresentam uma baixa capacidade de induzir uma resposta do tipo Th_1 (Almeida & Lopes, 2001) e que a

interação das DCs com o fungo ou seu antígeno imunodominante, a gp43, leva a uma inibição das moléculas MHC de classe II, das propriedades de adesão dessas células e da produção de IL-12 e TNF-α (Ferreira *et al*., 2004). Adicionalmente, em animais suscetíveis, o fungo promove a indução de DCs regulatórias produtoras de IL-10, através da ligação ao TLR2 e dectina-1 (Ferreira *et al*., 2007). Por outro lado, um importante estudo objetivando avaliar o perfil transcricional de DCs de camundongos em resposta a infecção pelo fungo, mostrou que os genes codificadores das citocinas IL-12 e TNF-α e as quimiocinas CCL22, CCL27 e CXCL10 estão positivamente regulados mostrando que o fungo induz uma potente resposta proinflamatória (Tavares *et al*., 2012).

Células dendríticas de animais suscetíveis à infecção apresentaram um fenótipo de DCs mielóides proinflamatórias com secreção de altos níveis de IL-12 e TNF-α que, no entanto, ocasionaram anergia de células T efetoras. Por outro lado, em animais resistentes foram detectadas DCs de fenótipo misto, isto é, tanto mielóide como plasmocitóide, com a produção de citocinas proinflamatórias acompanhada de altos níveis de TGF-β. Nesses animais foi detectada proliferação de células T efetoras produtoras de IFN-γ, IL-4 e IL-17, concomitante com indução acentuada de células T reguladoras. As DCs mielóides são também chamadas de clássicas ou convencionais e são caracterizadas por apresentarem morfologia estrelada, alta expressão da molécula apresentadora de antígeno MHC-II e com função efetora de fagocitar, migrar aos linfonodos e induzir a resposta imune adaptativa (Steinman, 2012), já as DCs plasmocitóides podem ter papel na imunidade ou na tolerância e são capazes de secretar altos níveis de IFN tipo I e TGF-β (Colonna *et al*., 2004).

Apesar dos trabalhos acima terem elucidado alguns aspectos da interação DCs-Pb, outros mecanismos decisivos na modulação desse processo merecem ser melhor investigados, como o papel da enzima indoleamina 2,3-dioxigenase (IDO). A IDO é uma enzima que degrada o aminoácido essencial triptofano, isto é, catalisa a conversão desse aminoácido para *n-formyl-kineurine*, que é então convertido em vários outros metabólitos (Stone & Darlington, 2002). A IDO tem um papel bastante complexo no processo de imunoregulação durante a gravidez, na autoimunidade, em transplantes, em neoplasias e nas infecções (Grohmann *et al*., 2003). No caso das infecções, considera-se que essa enzima faça parte da resposta imune inata do hospedeiro. A grande maioria dos microorganismos pode sintetizar o seu próprio triptofano, mas alguns dependem do triptofano externo. Assim, tais microorganismos são altamente sensíveis à ação deletora de triptofano induzida pela IDO que acaba inibindo a replicação dos mesmos

(Pfefferkorn, 1984; Carlin *et al*., 1989; Gupta *et al*., 1994; Mackenzie *et al*., 1998; Bodaghi *et al*., 1999; Hayashi *et al*., 2001; Adams *et a*l., 2004). Apesar desse efeito favorável ao hospedeiro, há um questionamento em relação à real contribuição da IDO para os mecanismos de defesa, particularmente *in vivo*, uma vez que vários trabalhos têm mostrado que a expressão de IDO em tumores é associada à indução de tolerância de células T (Mellor *et al*., 2002; Uyttenhove *et al*., 2003). Essa indução de tolerância está associada ao elevado nível de expressão de IDO pelas DCs (Hwu *et al*., 2000; Munn *et al*., 2002; Munn *et al*., 2004a).

As DCs não somente induzem imunidade, como podem participar ativamente da indução de tolerância de células T (Gabrilovich, 2004). Assim, o estado inflamatório ou antiinflamatório das DCs é controlado pela expressão de IDO. As células que expressam IDO são consideradas regulatórias, indutoras de tolerância (Munn *et al*., 1999; Grohmann *et al*., 2000; Terness *et al*., 2002; Von Bubnoff *et al*., 2002; Munn *et al*., 2004b) por meio da indução de mecanismos que incluem principalmente a geração de células T_{Regs} $CD4^+$ $CD25^+$ (Fallarino *et al*., 2006). Além disso, outros trabalhos têm mostrado que, ao mesmo tempo em que a IDO está envolvida na indução do fator de transcrição Foxp3 para T_{Reg}, também é capaz de suprimir o gene que codifica o RORγt, o fator de transcrição para Th_{17} (De Luca *et al*., 2007).

Os sinais envolvidos na indução de IDO pelas DCs não são bem estabelecidos. No entanto, estudos têm mostrado um papel essencial da prostaglandina E_2 (PGE_2). As prostaglandinas (PGs) são moléculas derivadas do ácido araquidônico (AA) por ação das enzimas ciclooxigenases (COX-1 e COX-2), sendo a COX-1 a forma constitutiva e a COX-2 a forma induzida, condicionada a vários estímulos inflamatórios. Além do seu papel em processos inflamatórios como febre, hiperalgesia e edema, as PGs, particularmente a PGE_2, regulam uma variedade de eventos da resposta imune desempenhando um importante papel na modulação dessa resposta em diferentes infecções (Kalinski, 2012). Vários estudos têm avaliado a ação moduladora das PGs sobre as DCs com apresentação de resultados contraditórios, isto é, exercendo ações tanto pró como anti-inflamatórias. De fato, a PGE_2 inibe a produção das quimiocinas e citocinas TNF-α, IL-6, IL-12, CCL-3, CCL-4, CCL-10 em resposta ao LPS. Ao contrário, induz um aumento de IL-10 (Harizi *et al*., 2001; Kalinski *et al*., 2001; Harizi *et al*., 2002, Harizi & Gualde, 2002; Harizi *et al*., 2003; Kabashima *et al*., 2003). Assim, principalmente pela acentuada capacidade de inibir IL-12, a PGE_2 está associada a um direcionamento pelas DCs a uma resposta do tipo Th_2 (Kalinski *et al*., 1997; Berger *et al*., 2006). Por outro

lado, há a participação da PGE_2 no processo de maturação das DCs, por meio da indução da migração dessas células em resposta a quimiocinas e outros quimioatraentes e no processo de apresentação de antígeno (Jonuleit *et al*., 1997; Scandella *et al*., 2002, Kabashima *et al*., 2003; Scandella *et al*., 2004; Legler *et al*., 2006). Durante esse processo de maturação, a PGE_2 também aumenta a expressão da IDO. Nesse sentido, devemos considerar que o aumento da expressão da IDO nas DCs em resposta a microorganismos que induzem a produção de PGs, pode resultar na geração de células T_{regs} e inibição de uma resposta do tipo Th_{17}. Os trabalhos citados deixam claro a importância de se desenvolver trabalhos com o objetivo de melhor entender a relação DCs / Pb e as suas consequencias para o desenvolvimento da resposta imune adaptativa.

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3. Racional do estudo e objetivos

Estudos enfocando a relação DCs/Pb ainda são escassos. Em estudo prévio, constatamos que cepas de alta e baixa virulência do fungo induzem a produção de PGE_2 por monócitos humanos, inibindo sua atividade fungicida de forma autócrina (Soares *et al*., 2001; Bordon *et al*., 2007; Bordon-Graciani *et al*., 2012). Tal resultado nos levou a avaliar se DCs humanas produzem PGE_2 quando desafiadas com diferentes cepas do Pb, buscando um melhor entendimento do papel imunomodulador desse eicosanóide na relação entre as DCs e o fungo. Contrariamente ao que observamos com monócitos, as duas cepas via ligação ao receptor de manose inibiram a produção endógena de PGE_2 pelas DCs, essa inibição resultou na produção de baixos niveis de TNF-α e consequente inibição da maturação dessas células (Fernandes *et al*., 2015). Esses resultados bastante intrigantes exigiram a continuidade dos estudos para que possamos entender as consequências da inibição da produção de PGs sobre a resposta das DCs ao fungo, particularmente sobre a instrução da resposta imune adaptativa. Assim, um dos objetivos do presente estudo foi avaliar o padrão de resposta de células T $CD4^+$ após cocultura com DCs sensibilizadas com cepas de alta e baixa virulência do fungo através da expressão intracelular das citocinas e fatores de transcrição envolvidos na diferenciação das subpopulações.

No entanto, o desafio das DCs com o fungo induz a expressão de uma variedade de genes envolvidos na modulação da resposta dessas células à infecção. Esses genes incluem os codificadores de proteínas, citocinas e quimiocinas diversas, bem como fatores de transcrição envolvidos na diferenciação das populações de linfócitos, além de outros mecanismos cujo estudo pode ampliar de forma consistente o nosso conhecimento sobre a natureza da interação DCs/Pb. Assim, uma ampla análise das alterações transcricionais em DCs expostas ao fungo pode esclarecer muitos dos fenômenos envolvidos na interação das células imunocompetentes com o agente patogênico. Neste contexto, um segundo objetivo do presente projeto foi analisar, pela técnica de RNAseq, o perfil transcricional das DCs em resposta ao Pb. Além do perfil transcricional das DCs em resposta ao fungo, consideramos extremamente importante a avaliação do perfil transcricional de linfócitos $CD4^+$ cocultivados com DCs sensibilizadas com o Pb, com especial atenção para os genes envolvidos na produção de citocinas e na expressão dos fatores de transcrição associados à diferenciação das populações de linfócitos Th_1 (t-bet), Th_2 (gata-3), Th_{17} (RORγt) e T_{reg} (Foxp3). A avaliação foi realizada por meio do sequenciamento de alto rendimento (RNAseq) do mRNA.

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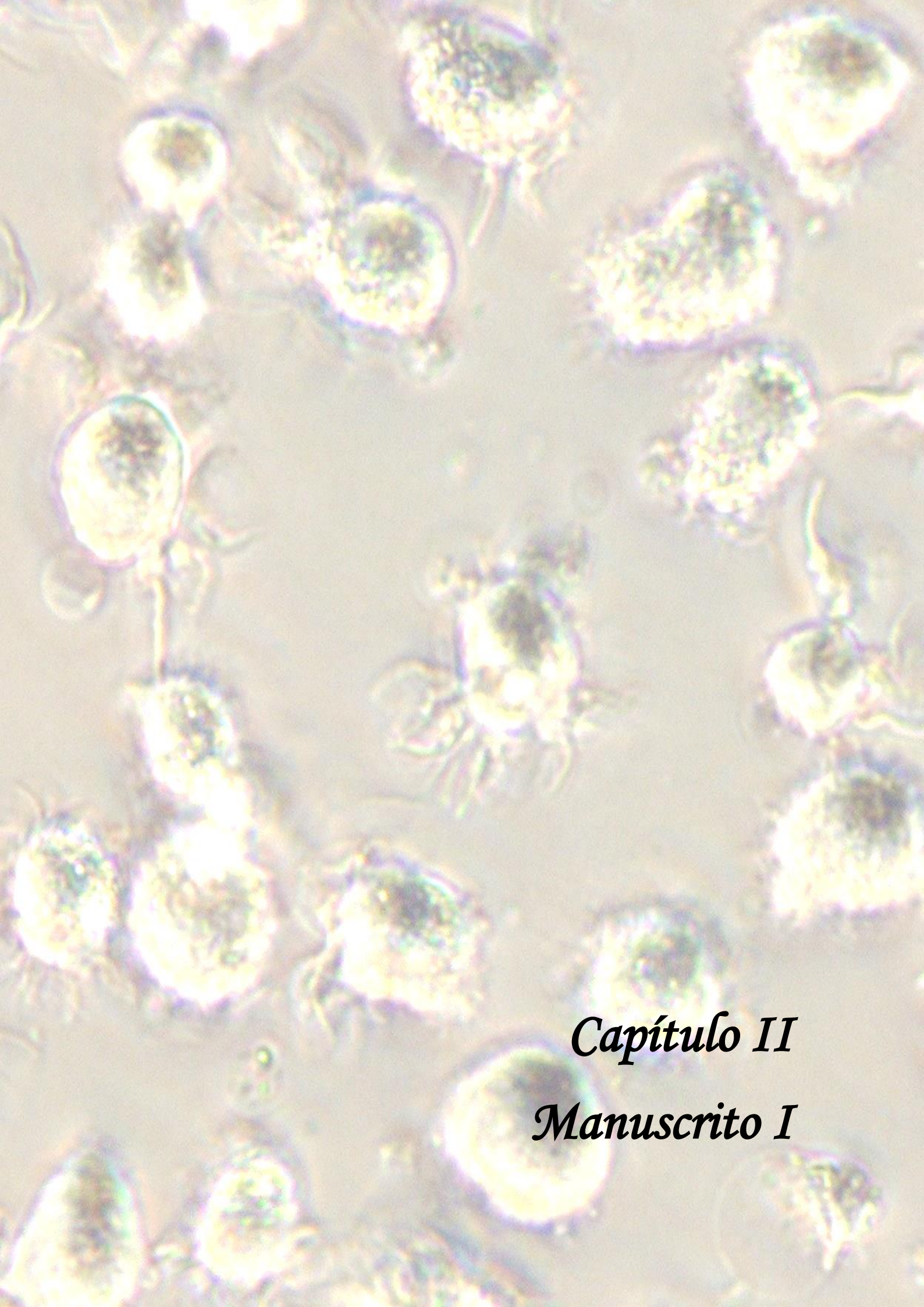

Capítulo II

Manuscrito I

Cytokine

Dendritic Cells Challenged with *Paracoccidioides brasiliensis* Promote T_{reg} Cell Response.

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Abstract

Paracoccidioidomycosis is a systemic mycosis, endemic to most Latin American countries, especially in Brazil, whose etiologic agent is the thermodimorphic fungus of genus *Paracoccidioides*, comprising cryptic species of *Paracoccidioides brasiliensis* (Pb), S1, PS2, PS3 and *Paracoccidioides lutzii*. The consequences of the fungus interaction with cells of the innate immune response, such as dendritic cells (DCs), highlighting the ability of these cells to instruct the adaptive immune response, are not fully understood. In a previous study, we found that human monocytes derived DCs fail to adequately mature in response to Pb. Thus, we aimed to evaluate whether these cells are able to induce CD4 proliferation and what is the predominant profile of the proliferated cells. Human DCs obtained from in vitro differentiation of monocytes were challenged with more (Pb18) and less virulent (Pb265) fungus strains during 48h, followed by coculture with $CD4^+$ cells by 48h to 120h. Our results showed which dentritic cells challenged with *Paracoccidioides brasiliensis* induce T_{reg} response. These findings may provide novel information for the understanding of the complex interplay between the host and this fungus.

Keywords: Dendritic cells; *Paracoccidiides brasiliensis*; T_{reg} cell; lymphoproliferation

1. Introduction

Paracoccidioidomycosis (PCM) is a systemic mycosis, endemic in most Latin American countries, especially in Brazil, whose etiologic agent is the thermodimorphic fungus of the genus *Paracoccidioides*, comprising 3 cryptic species of *Paracoccidioides brasiliensis* (Pb), S1, PS2, PS3 and *Paracoccidioides lutzii* [1-3]. Hosts are infected through the respiratory tract by mycelium propagules found in soil that reach the alveoli where conidia convert to yeast, the infective form [4, 5]. Thereafter, yeasts can disseminate by lympho-haematogenous route, inducing a disease with a wide spectrum of symptoms in a small number of individuals suggesting that in most exposed people innate and adaptive mechanisms efficiently assure resistance [6, 7]. Studies in human and

animals have shown that resistance to Pb is determined by a Th_1 response [8-15] in which TNF-α and IFN-γ play an essential role [16], while the susceptibility involves a Th_2 response with main participation of IL-4, IL-5, IL-10 and TGF-β [17, 18]. Recently, an important study showed that individuals with PCM infection (PI) present a predominant Th_1 response while those with chronic/adult form (AF) develop a Th_{17}/Th_{22} pattern. The acute, subacute/juvenile form (JF) is the most severe form of the disease being characterized by Th_2/Th_9 type response [19]. Although aforementioned studies have shown that resistance/susceptibility in PCM can be explained by the involvement of different subpopulations of $CD4^+$ cells, the mechanisms leading to preferential induction of any subpopulation are still unclear.

Dendritic cells (DCs) have the primary function of bind, capture, kill and process microorganisms. These cells migrate to peripheral lymphoid organs where they mature for efficient triggering and instructing a T cell immune response [20-22]. The nature of interaction of these cells with each microorganism will define how T cells will be instructed. Some studies have focused on the interaction DCs/Pb showing low efficiency of DCs and macrophages from susceptible mice and human DCs to inducing a Th_1 response, [23-27]. However, studies assessing which profile of $CD4^+$ cells subsets are differantiated after interaction with human DCs are scarce. Our previous studies showed that Pb, by binding to mannose receptor, inhibits PGE_2 production by DCs results in lower TNF-α levels and consequent inhibition of DCs maturation. Here, we aimed to investigate the profile of effector $CD4^+$ cells subsets triggered after interaction with human DCs challenged with high and low virulency Pb strains. We found that this interaction results preferentiatly in the induction of T_{reg} cells.

2. Material and Methods

2.1. Subjects

Healthy blood donors signed the written informed consent and were included in this study after approval by the Research Ethics Committee of the University Hospital of the Botucatu School of Medicine, São Paulo State University (UNESP) (registration number: 32809914.2.0000.5411)

2.2. Fungus

Yeast cells suspensions of high and low virulency strains of *Paracoccidioides brasiliensis* (Pb18 and Pb265, respectively) were used. Cultivation, obtention of individual cells, viability determination and concentrations adjustment were performed as previously [28].

2.3. Monocytes Purification and in vitro differentiation into DCs

Human monocytes - derived DCs were generated as follow: mononuclear cells were obtained by centrifugation of peripheral blood at 405 xg for 30 min on a Ficoll-Paque density gradient (GE Healthcare, Uppsala, Sweden). Later, cells were harvested and remaining erythrocytes were lysed by treatment with hypotonic lysis for 5 min. Cells were washed twice with RPMI 1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA). Cell pellet was ressuspended in RPMI 1640 supplemented with 2 mM L-glutamine, 40 mg/mL gentamicin and 10% inactivated fetal bovine serum (complete culture medium), and adjusted to 10^7 mononuclear cells/mL. These mononuclear cells were then centrifuged at 800 xg for 20 min at a Percoll density gradient (GE Healthcare, Uppsala, Sweden) to isolate monocytes from lymphocytes. Lymphocytes were frozen at -80º C, and monocytes ($CD14^+$) were isolated by negative magnetic selection technique (MACS Magnetic-activated cell sorting) (Human cocktail microbeads, Miltenyi Biotec kit). Initially, the cells were resuspended in MACS buffer (PBS solution with 0.5% bovine serum albumin and 2 mM EDTA) and cell viability was checked by trypan blue exclusion test. Later, monocytes ($CD14^+$) were centrifuged at 300 xg for 10 min, the supernatant was discarded and 30 μL of MACS buffer, 10 μL FcR blocking reagent, and 10 μL of Biotin-Antibody Cocktail for each 1.10^7 cells were added, followed by incubation for 10 min at 4° C. Then, 30 μL of MACS buffer and 20 μL Anti-Biotin microbeads were added for every 1.10^7 cells followed by incubation for 15 min at 4° C. Cells were centrifuged at 300 xg for 10 min for removal of unbound microspheres and 1.10^8 cells were suspended with 500 μL of MACS buffer. This suspension was applied to magnetic column model LS coupled to MACS platform, followed by 3 washes with 3 mL of MACS buffer. Cells linked to microspheres were retained on the column and discarded while unlabeled cells ($CD14^+$ monocytes) passed through the column and were collected in sterile tube. The depurated monocytes were resuspended in complete culture medium and culture with human recombinat IL-4 (80 ng/mL) and GM-CSF (80 ng/mL) in 6-wells culture plates (2

mL per well) for 7 days. After this period, the loosely adhered cells were collected with a pipette, washed with complete medium and cell concentration was adjusted to 1.10^5 dendritic cells / mL and plated in 96-well plates (100 μL/well) for the completion of proposed tests. Collected cells were phenotyped by flow cytometry, and identified as $CD14^{low}/CD1a^{high}/CD83^{low}$, which is characteristic of immature DC.

2.4. DCs Immunophenotyping and viability

DCs generated as described in item 2.3, were transferred to Falcon tubes for flow cytometer (BD-Becton, Dickinson and Company, San Diego, CA, USA) and centrifuged at 520 xg for 10 min at 4º C. After centrifugation, cells were resuspended in 1 mL of electrolyte solution (ISOTON II) followed by incubation with anti-CD14 monoclonal antibodies conjugated to PerCP-Cy 5.5 (Peridinin Chlorophyll Protein Complex) (0.3 μL), FITC (Fluorescein) - conjugated anti-CD1a (2 μL), PE (Phycoerythrin) - conjugated anti-CD83 (2 μL), and APC (Allophycocyanin) - conjugated anti-CD11c (2 μL) (BD - Becton, Dickinson and Company) for 30 min. Then, cells were washed at 520 xg for 10 min, resuspended in 450 μL of ISOTON II, and fixed with 50 μL of fixing solution containing 5% formaldehyde (BD-Becton Dickinson and Company). DCs were labeled with 1 μL/mL Live / Dead Kit (Life Technologies, Eugene, OR, USA) for assessment of viability and analyzed by flow cytometer model FACSCanto (BD-Becton, Dickinson and Company).

2.5. Purification of $CD4^+$ cells by negative magnetic selection

Total lymphocytes were defrost, washed, and counted with subsequent evaluation of viability by trypan blue exclusion test. These cells were incubated with monoclonal antibodies coupled to magnetic beads contained in the negative selection MACS® magnetic $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec Inc., Auburn, CA). Kits contain biotinylated antibody cocktail that specifically bind to their target molecules, however, cocktail for $CD4^+$ T cell isolation contains antibodies against other molecules, therefore, $CD4^+$ T lymphocytes are not marked with these biotinylated antibodies. Initially, cells were suspended in MACS Buffer (PBS solution with 0.5% bovine serum albumin and 2 mM EDTA) and and viability and counting assessed by staining with trypan blue. After centrifugation at 300 xg for 10 min, supernatant was discarded, and 40 μL of MACS buffer and 10 μL of Biotin-Antibody Cocktail $CD4^+$ were added to each 10^7 cells, and incubated for 5 min at 4° C. Then, it was added 30 uL of MACS buffer and 20

μL of CD4 microbeads cocktail for every 10^7 cells followed by incubation for 10 min at 4° C. Unbound microspheres were removed by centrifugetion at 300 xg for 10 min and cells were suspended in 3 mL of MACS buffer. This suspension was applied to magnetic column model LS coupled to MACS platform followed by 2 washes with 3 mL of MACS buffer. Antibody-biotin interaction allowed the labeled cells dropped retained when subjected to the magnetic field of the platform. Thus, $CD4^+$ cells not retained the column were collected in tubes, centrifuged, assessed for viability by trypan blue and subjected to the tests proposed.

2.6. Analysis of purity and viability of $CD4^+$ cells

$CD4^+$ cells separated according to item 2.5, were transferred to Falcon tubes for flow cytometer (BD - Becton, Dickinson and Company) and centrifuged at 520 xg for 10 min at 4^o C. After centrifugation, cells were suspended in 1 mL of electrolyte solution (ISOTON II) and incubated with anti-CD4 monoclonal antibody conjugated to PE-Cy7 (phycoerythrin) (0.6 μL), anti-CD3 conjugated to FITC (Fluorescein) (2 μL) and anti-CD14 PerCP conjugated with CY5.5 (Peridinin Chlorophyll Protein Complex) (0.3 μL) (BD - Becton, Dickinson and Company) for 30 min. Unlabeled cells were used as autofluorescense control of each sample. After incubation with respective antibodies, cells were centrifuged at 520 xg for 10 min for washing, suspended in 450 μL of ISOTON II, fixed with 50 μL of fixing solution containing 5% formaldehyde (BD-Becton Dickinson and Company). Concomitantly, we assessed the viability of these cells by Live / Dead kit (1 μL/mL of $CD4^+$ cell suspension) followed by analysis by flow cytometry.

2.7. In vitro DCs challenged with *Paracoccidiois brasiliensis*

After in vitro differentiation into DCs as described in item 2.3, cells were challenged with yeast cells of high (Pb18) and low virulence (Pb265) Pb strains (2.10^4 / mL) for 48h.

2.8. Lymphoproliferation Assay

Autologous $CD4^+$ cells isolated from peripheral blood of healthy donors as described in item 2.5. $CD4^+$ T cells were labeled with 1 μM Far Red, adjusted to 1.10^6 / mL, and cocultured with 1.10^5 / mL DCs (CD4:DC ratio - 10:1) challenged or not with Pb18 or Pb265 (DCs:Pb ratio - 5:1). After incubation with respective antibodies, cells

were centrifuged at 520 xg for 10 min for washing, suspended in 450 µL of ISOTON II, fixed with 50 µL of fixing solution containing 5% formaldehyde (BD-Becton Dickinson and Company). Unlabeled cells were used as autofluorescense control of each sample. We used 8 mg / mL of the mitogen phytohemagglutinin (PHA) as a positive control.

2.9. Intracelular Expression of IFN-γ, IL-4, and IL-17

DCs (1.10^5 / ml) were sensitized with Pb (2.10^4 / ml) for 48 h and cocultured with $CD4^+$ T cells (1.10^6 / mL) for 96 h and 120 h. 6 hours before each period Brefeldin solution (1000X - 5mg / mL) (BioLegend) were added to the cultures to prevent cytokines release from the cytoplasm. After the period, supernatants were collected from the 96-well plates, transferred to Eppendorf-type microfuge tubes and centrifuged at 10.000 rpm for 60 s. Supernatant was disposed and cells washed again with 200 µL of 1% BSA. Then, cells were labeled with anti-CD4 monoclonal antibody conjugated to PE-Cy7 (phycoerythrin) (0.6 µL), and anti-CD3 conjugated to FITC (Fluorescein) (2 µL) (BD-Becton, Dickinson and Company) and incubated for 20 min. At the end of this period 100 µL of Reagent A fixing Fix & Perm kit (Nordic MUbio) was added, and incubated for 20 min at room temperature, followed by washing with 0.5% BSA, and addittion of 100 µL of Reagent B permeabilization Fix & Perm kit (Nordic MUbio). After cells were incubated with antibodies anti-IFN-γ conjugated with PE (phycoerythrin), anti-IL-4 conjugated to PE and anti-IL17 conjugated with Alexa 488 (5 µL) incubated for 20 min at room temperature. Finally, cells were washed with 500 µL of PBS + 0.5% BSA, and suspended in bufffer solution and analysed for intracytoplasmic expression of IFN-γ, IL-4 and IL-17 by flow cytometry.

2.10. Intracell Expression of Foxp3 Transcription Factor

$CD4^+$ cells were submitted to the same treatments described in item 2.9 until the incubation with the surface marker antibodies. Then, after this phase, it was added 300 µL of wash buffer (eBiosciense - San Diego, CA), and cellular suspension was homogenized, centrifuged in a microcentrifuge at 10.000 rpm for 60 s, and 300 µL Perm reagent / FIX (eBiosciense - San Diego, CA) and incubated with 300 µL of Perm reagent / FIX (eBiosciense - San Diego, CA) for 30 min in free light refrigerator. After the period, 500 µL of wash buffer was added and the suspension was 2X centrifuged for 5 min at 405 xg and 1X for 60 s at 9500 xg. The supernatant was discarded by inversion and cells were incubated with 3 µL of anti-Foxp3 antibody conjugated to PE (phycoerythrin) for 30 min

at room temperature. Finally, cells were ressuspended in 1 mL wash buffer, centrifuged and ressuspended in 300 µL of electrolyte solution Isotonic + 1% BSA for analysis flow cytometer.

3. Results

3.1. DC phenotype and viability

Our initial experiments aimed to immunophenotype both DCs derived from $CD14^+$ monocytes and $CD4^+$ cells isolated from human peripheral blood by negative magnetic selection (Fig.1 and Fig.2). Figure 1 clearly shows that DCs obtained from monocytes are $CD11c^+/CD1a^+/CD83^-/CD14^-$, a phenotype tipical of featuring a immature DCs profile. Results proving $CD4^+$ cells purity were shown in Figure 2. The cells were positive for CD4 marker and negative for CD8.

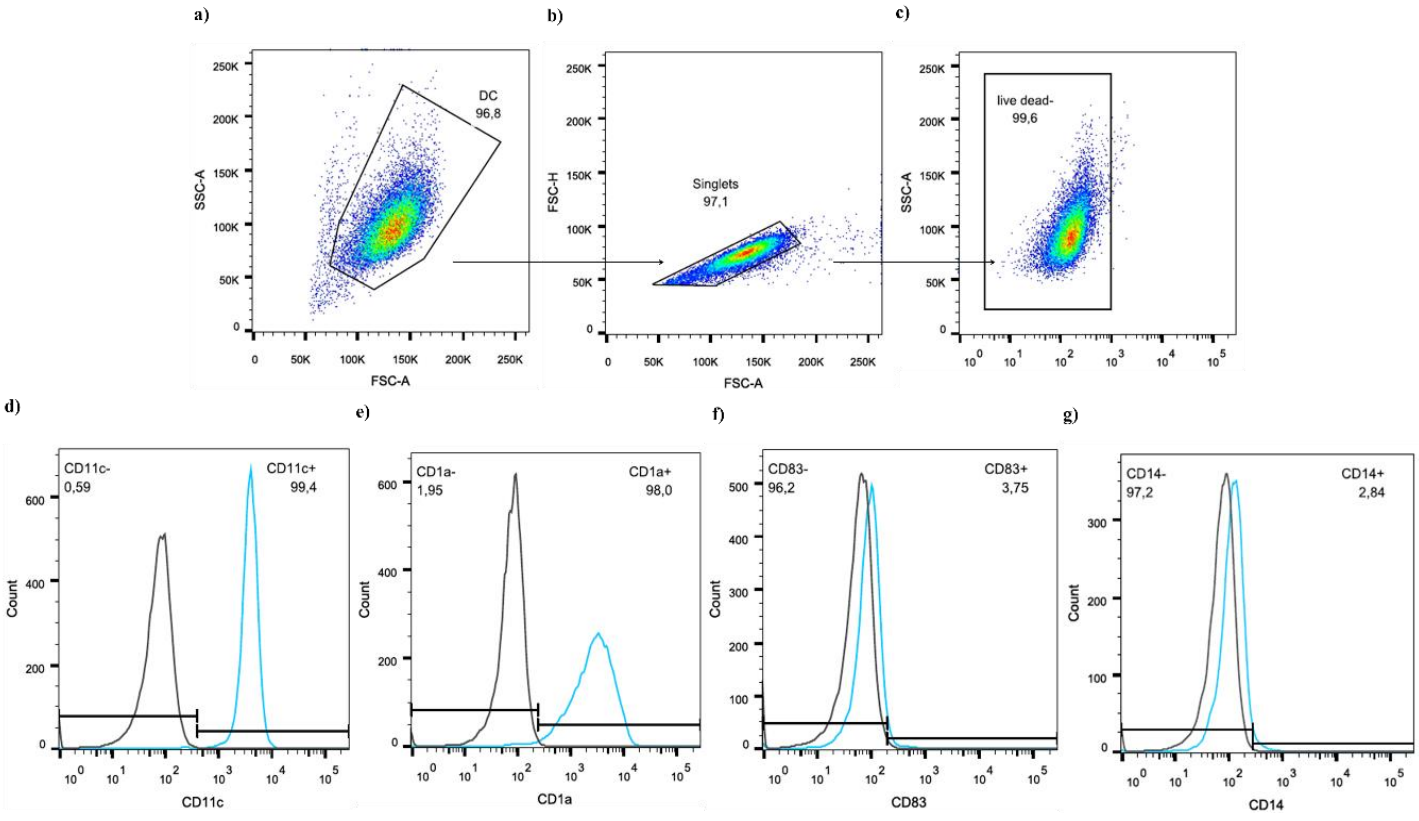

Fig. 1: Dot Plots (a, b, c) and representative histograms of the population of DCs analyzed for expression of surface molecules CD11c (d), CD1a (e) CD83 (f) and CD14 (g).

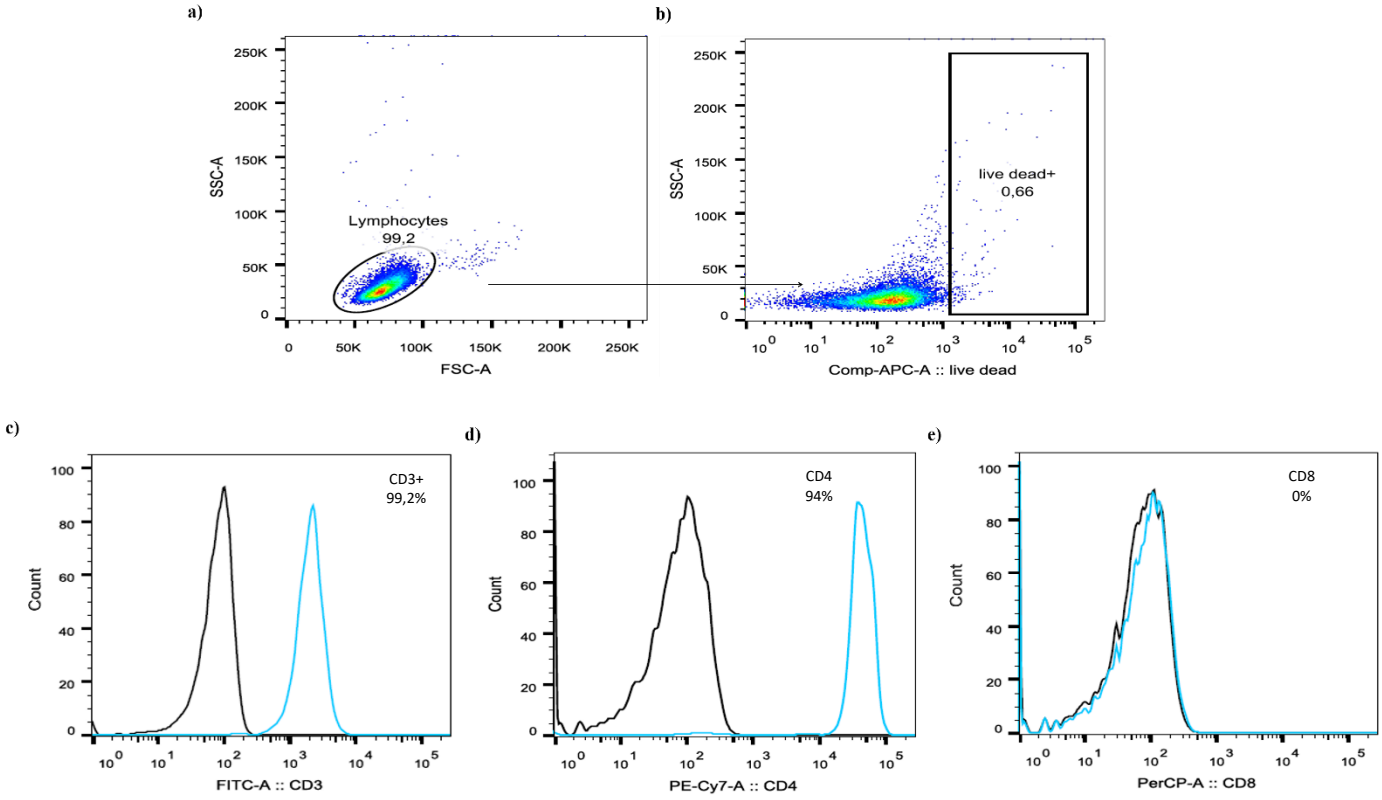

Fig. 2: Dot Plots (a and b) and representative histograms of the population of $CD4^+$ cells analyzed for expression of surface molecules CD3 (c), CD4 (d), and CD8 (e).

3.2. Lymphoproliferative response

After ensuring the immunophenotype and viability of the cells, we performed assays to evaluate the capacity of DCs to induce $CD4^+$ cells proliferation. As a positive lymphoproliferation control, we used 8 mg/mL of phytohemagglutinin mitogen (PHA) (results not showed). Figure 3 shows lymphoproliferation results at the periods of 48-120h. PHA induced a discret lymphocyte proliferation at 48h. However, a significant lymphoproliferation was detected at 48h, 96h, and 120h. $CD4^+$ cells co-cultured with DCs challenged with high (Pb18) and low (Pb265) virulence strains for 96 showed a proliferative capacity that was significantly higher than $CD4^+$-DCs cultured without fungus challenge. However, the cocultivation of DCs sensitized to both strains with $CD4^+$ cells for 120h resulted in a higher proliferative capacity when compared with 96h coculture (Figs 3-5).

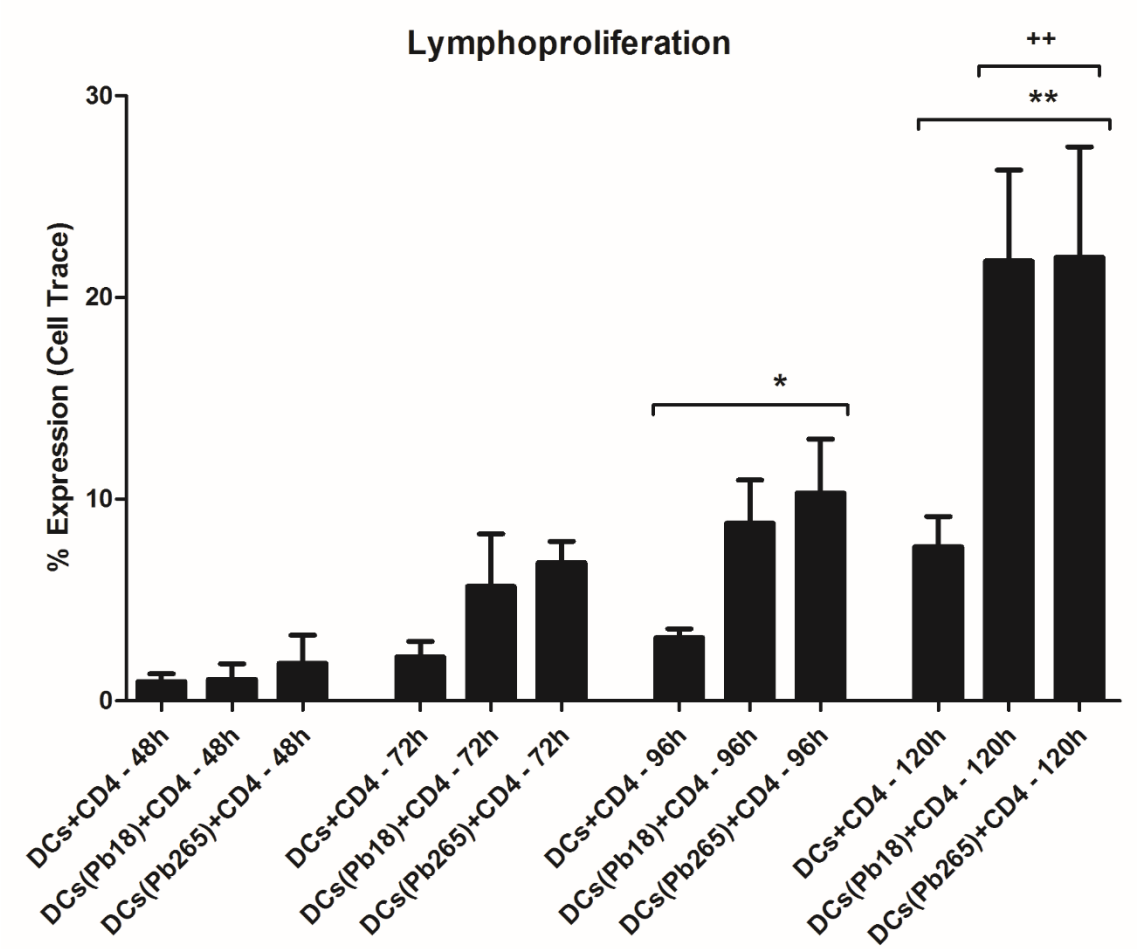

Fig. 3: Percentage of $CD4^+$ T cells lymphoproliferation after cocultivation for 48h-120h with DCs challenged or not with Pb18 or Pb265 for 48h. The results obtained with cells from 5 individuals are expressed as mean ± standard deviation. Statistically significant differences between results obtained in each group of experiments are indicated: *p <0.05, ** <0.001 x respective cocultures DCs + CD4 at the same period; ++ p< 0.001 x DCs (Pb18) + CD4 or DCs (Pb265) + CD4 at 96h.

3.3. $CD4^+$ cells subsets profile

Once $CD4^+$ cells proliferated in response to DCs sensitized with the fungus (co-culture periods of 72h, 96h and 120h), we performed experiments to evaluate the profile of adaptive response (Figs 4-7). For this, we chose to use the co-culture periods of 96h and 120h (Figs 4 and 6). We observed that $CD4^+$ T cells does not express intracell cytokines associated with the subpopulations Th_1 (IFN-γ) Th_2 (IL-4), Th_{17} (IL-17). However, foxp3 transcrition factor was significantly expressed by these cells, which indicate a phenotype of T_{regs} cells. This phenotype was confirmed by the expression of the surface molecule CD25, but not CD127 by these cells (Figs 5 and 7).

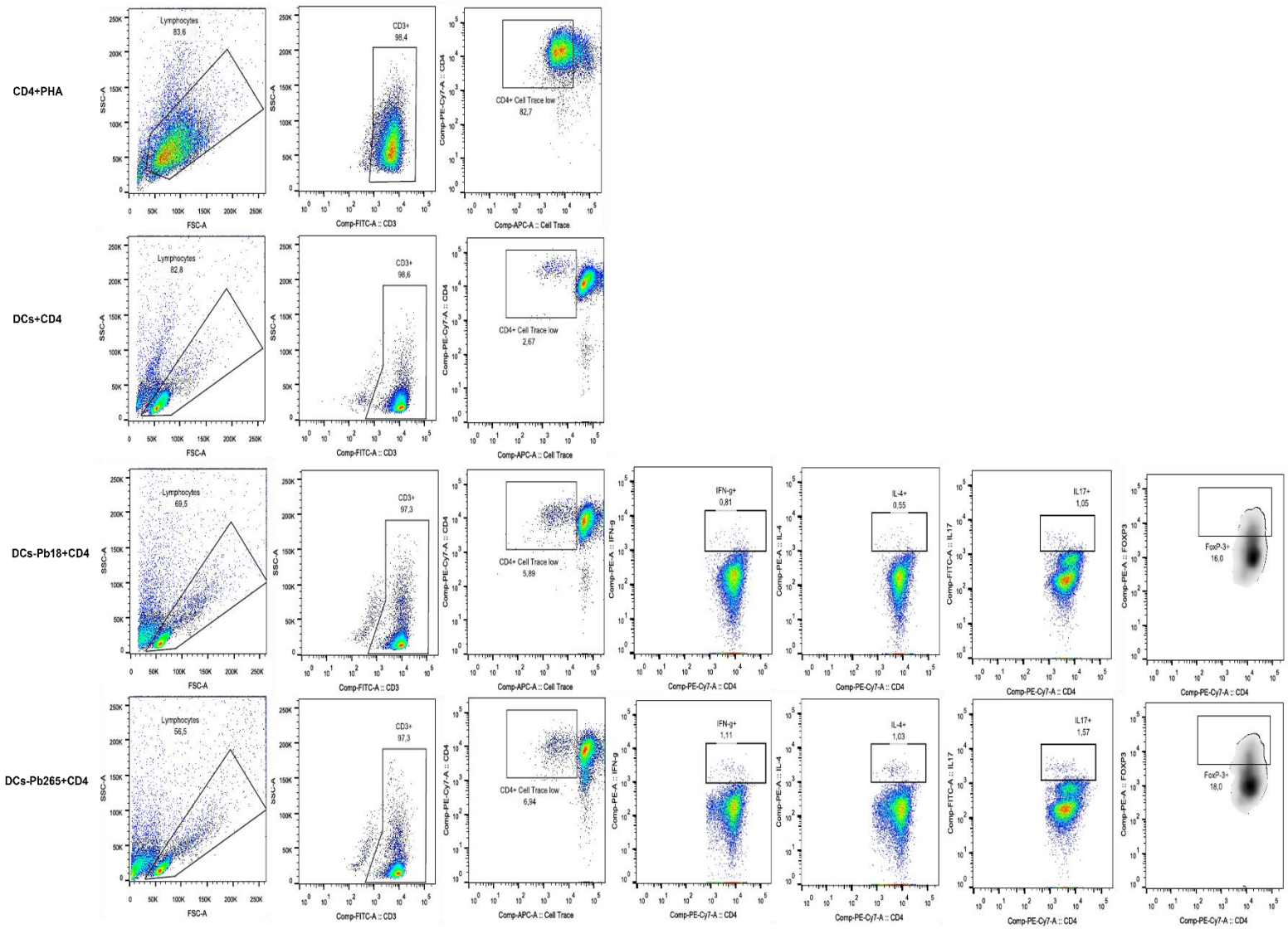

Fig. 4: Dot plots representative of the $CD4^+$ cells proliferation with positive control (CD4+PHA), negative control (DCs+CD4), cocultivated with DCs challenged with Pb18 and Pb265 (DCs-Pb18+CD4, and DCs-Pb265+CD4) for 96h.

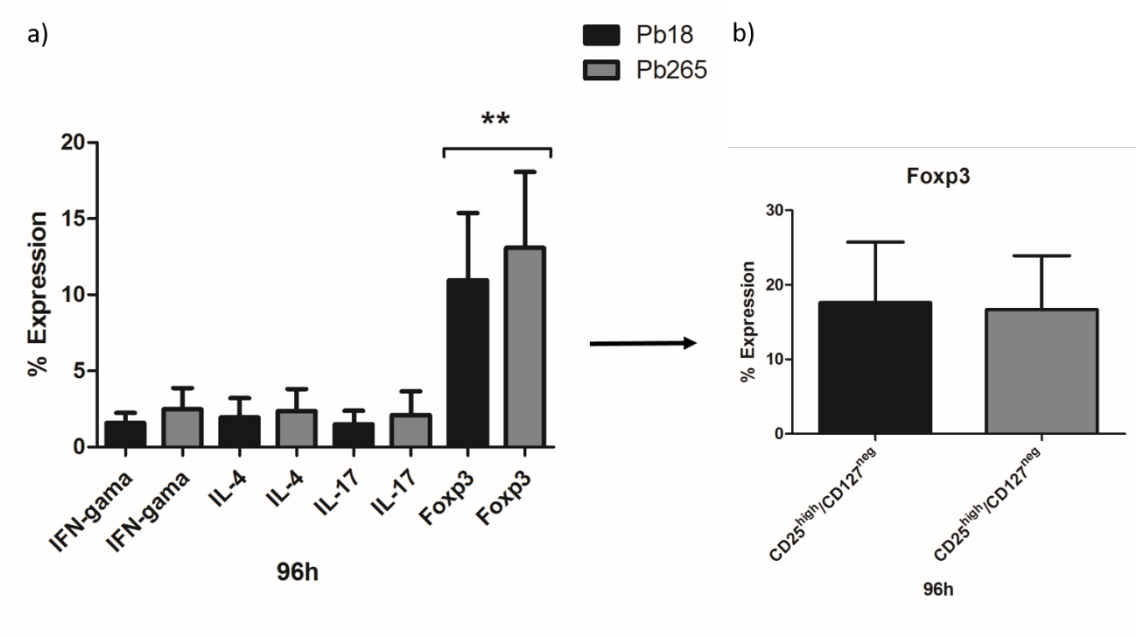

Fig. 5: Expression of intracytoplasmic cytokines and the transcription factor Foxp3 (a) and CD25 and CD127 expression (b) by $CD4^+$ T cells after cocultivation (96h) with DCs challenged with Pb18 or Pb265 and expression. The results obtained with cells from 5 individuals are expressed as mean ± standard deviation. ** $p<0.001$ compared with other groups.

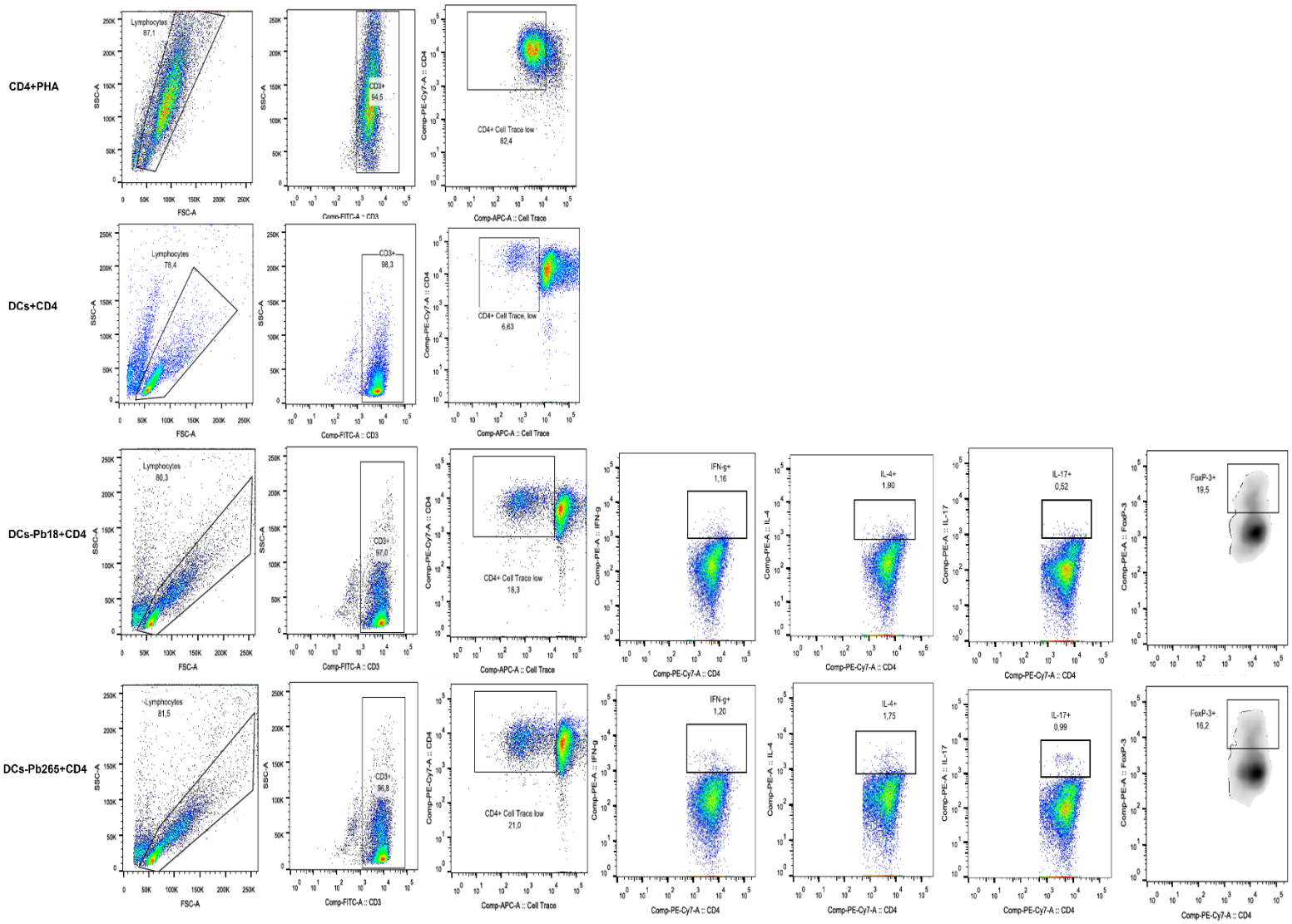

Fig. 6: Dot plots representative of the $CD4^+$ cells proliferation with positive control (CD4+PHA), negative control (DCs+CD4), cocultivated with DCs challenged with Pb18 and Pb265 (DCs-Pb18+CD4, and DCs-Pb265+CD4) for 120h.

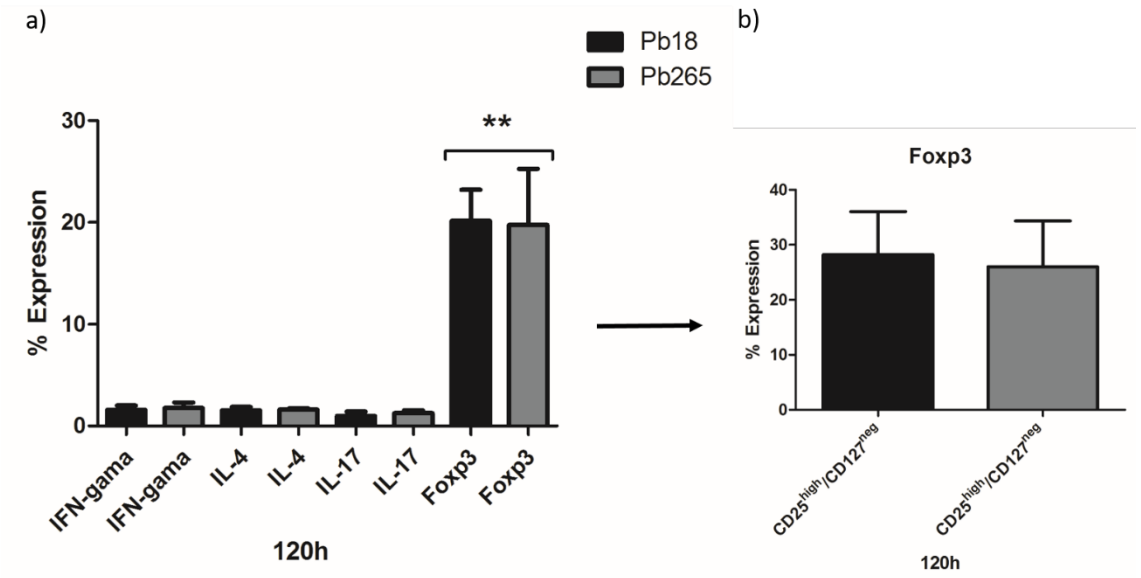

Fig. 7: Expression of intracytoplasmic cytokines and the transcription factor Foxp3 (a) and CD25 and CD127 expression (b) by $CD4^+$ T cells after cocultivation (120h) with DCs challenged with Pb18 or Pb265 and expression. The results obtained with cells from 5 individuals are expressed as mean ± standard deviation. ** $p<0.001$ compared with other groups.

4. Discussion

In this study, we aimed to investigate the profile of effector $CD4^+$ cells subsets triggered after interaction with human DCs challenged with high and low virulence Pb strains. In previous studies, we found that DCs challenged with Pb do not produce prostaglandin E2 (PGE_2) and remain in an immature stage [29]. Considering these results an important question arises relative to the profile of $CD4^+$ cells that is triggered after interaction of these cells with DCs challenged with high low virulence fungus strains.

Our results, for both fungus strains, revealed that this interaction does not not result in Th_1, Th_2, and Th_{17} differenttiation. On the other hand, there was induction of T_{reg} response, caracterized by the detection of the transcription factor Foxp3, expression of the surface molecule CD25, but absence of CD127. Our results agree with others showing that immature DCs is able to instuct CD4 cells to differenttiate in T_{reg} cells [30-34]. Thus, the mechanism proposed for our results is that inhibition of PGE_2, avoiding DC maturation is a fungus escape mechanism, as this process instructs imune response to a regulatory pattern, that is not protective to host. T_{reg} cells play a key role in immune homeostasis, as they secrete the regulatory cytokines IL-10 and TGF-β, that can inhibit

CD4 effector cells activity [35, 36]. A high frequency of T_{reg} cells was identified in patients with severe forms of the PCM [37, 38]. Furthermore, in susceptible animals the fungus induces regulatory DCs to produce IL-10 by binding to TLR2 and dectin-1 [25]. Thereafter, the same author showed that in vivo depletion of T_{reg} with anti-CD25 decreased the severity of disease [39]. Likewise, T_{reg} cells facilitated increased fungal burden in other fungal infections such as *Candida albicans*, *Pneumocystis carinii*, and *Aspergillus fumigatus* [40-42]. After infection by *Aspergillus fumigatus*, T_{reg} cells plays a role negative regulatory due to its ability to inhibit the Th_1 inflammatory response [43].

For now, our study highlights that human monocytes-derived dendritic cells are able of promote T_{reg} adaptive immune response after challenge with high and low virulence strains of the fungus *Paracoccidioides brasiliensis*.

Acknowledgements

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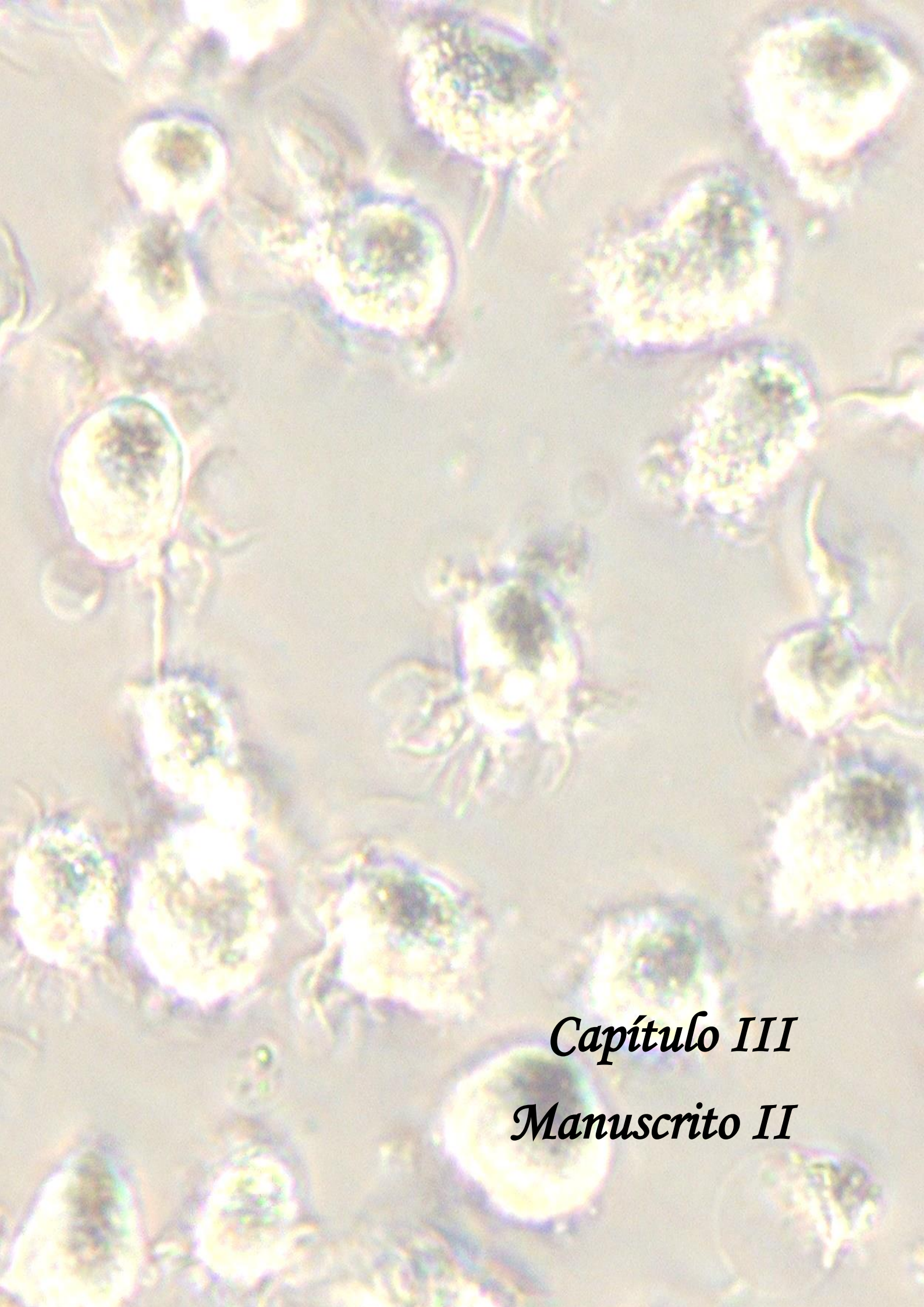

Capítulo III

Manuscrito II

Transcriptional Changes in Dendritic Cells and CD4$^+$ Cells in Response to *Paracoccidioides brasiliensis*

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Abstract

Paracoccidioidomycosis (PCM) is an endemic systemic mycosis in Latin America mainly Brazil, Venezuela, and Argentina that promotes an important impact on public health. Its etiological agent is a thermodimorphic fungus belonging to the *Paracoccidioide*s genus which comprises the *Paracocidioides brasiliensis* (Pb) and its cryptic species S1, PS2, PS3, and *Paracoccidioides lutzii*. The consequences of the fungus interaction with cells of the innate immune response, such as dendritic cells (DCs), highlighting the ability of these cells to instruct the adaptive immune response, are not fully understood. In a previous study, we found that DCs does not mature in response to challenge with Pb. This fail was associated to PGE_2 inhibition by the fungus, as this eicosanoid is an important factor for cells maturation. Here, in an attempt to better understanding this process, and its consequences to the instruction of the CD4 adaptive response, we aimed to analyze the transcriptional profile of DCs in response to Pb as well as that of CD4$^+$ lymphocytes cocultured with DCs sensitized with the fungus. For these analyses, we used the RNA-seq metodology, that allows high-throughput sequencing and systematic counting of all transcripts. After analyses, the genes that were upregulated and down regulated in both cells (DCs and CD4) were listed and the functions of the proteins codified by them identified. The overall analysis of the proteins that were high or low expressed, such as diverse cytokines and chemokines, inflammatory mediators, as well as transcription factors involved in the differentiation of lymphocyte populations, allow us to determine the profile of effector CD4 subsets that was differenttiated after interaction of DCs with CD4 cells, as well some mechanisms that lead to this profile.

1. Introduction

Paracoccidioidomycosis (PCM) is an endemic systemic mycosis in Latin America, mainly Brazil, Venezuela, and Argentina (Coutinho *et al*., 2002; Bocca *et al*., 2013) with an important impact on public health (Colombo *et al*., 2011). The etiologic agent of this mycosis is a thermodimorphic fungus that belongs to the *Paracoccidioides* genus wich comprises the *Paracoccidioides brasiliensis* (Pb) and its cryptic species S1, PS2, PS3, and *Paracoccidioides lutzii* (Brummer *et al*.,1993; Matute *et al*., 2006; Teixeira *et al*., 2009). The infectious agents of Pb would be mycelial propagules in the soil, water,

and plants that penetrate in the host through the airways, first reaching the lungs causing a pulmonary primary complex. This process can progress to cure or become latent characterizing the PCM-infection identified by the absence of the clinical signs or symptoms, but with the development of an adaptive immune response, which can be evidenced by the intradermal test with paracoccidioidin (Oliveira, 2002). Rather, the process can progress to PCM- disease with subsequent spread to other organs such as liver, spleen, and adrenal glands bylymphatic and hematogenous pathway (Franco *et al*., 1987). Clinical manifestations of the systemic mycosis can be grouped into two patterns that define the acute and chronic forms of the disease. The acute form (juvenile form) is typically severe with rapid evolution and compromises the mononuclear phagocytic system (spleen, liver, lymphnodes, and bone marrow). The chronic form (adult form) is prolonged, has slow and gradual installation and the lesions remain localized or involve more than one organ or system (Franco *et al*, 1987; Mendes, 1994; Shikanai-Yasuda *et al*., 2006)

Regarding the immune response in paracoccidioidomycosis, studies have clarified the association of different subpopulations of CD4 cells with the different clinical forms of the disease (de Castro *et al*., 2013). However, mechanisms that drive the differentiation of one or the other subpopulation of effector $CD4^+$ cells in PCM are not fully understood. The fine regulation of the adaptive immune response is greatly dependent on the initial interaction between the infectious agent and the antigen presenting cells that act as a bridge between innate and adaptive immunity, instructing the type of immune response to be developed. Phagocytic cells such as macrophages, monocytes and even neutrophils may participate in this process, but dendritic cells (DCs) are the most specialized cells in binding, phagocytosing, destroying and processing microorganisms and then migrating to local peripheral lymphoid organs where they mature, present antigens to CD4 cells, triggering and instructing the adaptive immune response (Cella *et al*., 1997; Steinman, 1991; Banchereau & Steinman, 1998).

Some studies have elucidated some aspects of DCs/Pb interaction (Silvana dos Santos *et al*., 2011; Almeida & Lopes, 2001; Ferreira *et al*., 2004, 2007; Tavares *et al*., 2012) However, there is a lack of studies focusing on this interaction using human DCs. In a previous study, we found that interaction human DCs/Pb does not result in maturation of these cells. This fail was atributed to the finding that Pb, by binding to mannose receptor, inhibits PGE_2 production by DCs, which results in lower TNF-α levels and consequent inhibition of DCs maturation. In an attempt to better understanding this

process and its consequences to the instruction of the CD4 adaptative response, our objective was to analyze the transcriptional profile of DCs in response to Pb and the transcriptional profile of $CD4^+$ lymphocytes cocultured with DCs sensitized with the fungus. We used an approach that allowed high-throughput sequencing (mRNAseq) and systematic counting of all transcripts. In particular, RNA-seq does not require initial DNA sequence information from the genes, has a larger range of analysis and is more sensitive than the microarray essay, since the quantification of the transcript of each gene is based directly on the number of readings.

We found that DCs challenge with Pb, as well as CD4 sensititization with DCs challenged with Pb result in an up or dowregulation of a variety of genes codifing diverse cytokines and chemokines, inflammatory mediators, as well as transcription factors involved in the differentiation of lymphocyte populations, and others. Overall, the analysis of this expression alowed us to beter understanding the nature of the interaction human DCs/Pb, with focus on the instruction of CD4 adaptive immune response.

2. Material and Methods

2.1. Subjects

Healthy blood donors signed the written informed consent and were included in this study after approval by the Research Ethics Committee of the University Hospital of the Botucatu School of Medicine, São Paulo State University (UNESP) (registration number: 32809914.2.0000.5411)

2.2. Fungus

Yeast cells suspensions of high and low virulency strains of *Paracoccidioides brasiliensis* (Pb18 and Pb265, respectively) were used. Cultivation, obtention of individual cells, viability determination and concentrations adjustment were performed as previously (Soares *et al*., 2001).

2.3. Monocytes Purification and in vitro differentiation into DCs

Human monocytes - derived DCs were generated as follow: mononuclear cells were obtained by centrifugation of peripheral blood at 405 xg for 30 min on a Ficoll-

Paque density gradient (GE Healthcare, Uppsala, Sweden). Later, cells were harvested and remaining erythrocytes were lysed by treatment with hypotonic lysis for 5 min. Cells were washed twice with RPMI 1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA). Cell pellet was ressuspended in RPMI 1640 supplemented with 2 mM L-glutamine, 40 mg/mL gentamicin and 10% inactivated fetal bovine serum (complete culture medium), and adjusted to 10^7 mononuclear cells/mL. These mononuclear cells were then centrifuged at 800 xg for 20 min at a Percoll density gradient (GE Healthcare, Uppsala, Sweden) to isolate monocytes from lymphocytes. Lymphocytes were frozen at -80º C, and monocytes ($CD14^+$) were isolated by negative magnetic selection technique (MACS Magnetic-activated cell sorting) (Human cocktail microbeads, Miltenyi Biotec kit). Initially, the cells were resuspended in MACS buffer (PBS solution with 0.5% bovine serum albumin and 2 mM EDTA) and cell viability was checked by trypan blue exclusion test. Later, monocytes ($CD14^+$) were centrifuged at 300 xg for 10 min, the supernatant was discarded and 30 µL of MACS buffer, 10 µL FcR blocking reagent, and 10 µL of Biotin-Antibody Cocktail for each 1.10^7 cells were added, followed by incubation for 10 min at 4° C. Then, 30 µL of MACS buffer and 20 µL Anti-Biotin microbeads were added for every 1.10^7 cells followed by incubation for 15 min at 4° C. Cells were centrifuged at 300 xg for 10 min for removal of unbound microspheres and 1.10^8 cells were suspended with 500 µL of MACS buffer. This suspension was applied to magnetic column model LS coupled to MACS platform, followed by 3 washes with 3 mL of MACS buffer. Cells linked to microspheres were retained on the column and discarded while unlabeled cells ($CD14^+$ monocytes) passed through the column and were collected in sterile tube. The depurated monocytes were resuspended in complete culture medium and culture with human recombinat IL-4 (80 ng/mL) and GM-CSF (80 ng/mL) in 6-wells culture plates (2 mL per well) for 7 days. After this period, the loosely adhered cells were collected with a pipette, washed with complete medium and cell concentration was adjusted to 1.10^5 dendritic cells / mL and plated in 96-well plates (100 µL/well) for the completion of proposed tests. Collected cells were phenotyped by flow cytometry, and identified as $CD14^{low}/CD1a^{high}/CD83^{low}$, which is characteristic of immature DC.

2.4. DCs Immunophenotyping and viability

DCs generated as described in item 2.3, were transferred to Falcon tubes for flow cytometer (BD-Becton, Dickinson and Company, San Diego, CA, USA) and centrifuged at 520 xg for 10 min at 4º C. After centrifugation, cells were resuspended in 1 mL of electrolyte solution (ISOTON II) followed by incubation with anti-CD14 monoclonal antibodies conjugated to PerCP-Cy 5.5 (Peridinin Chlorophyll Protein Complex) (0.3 µL), FITC (Fluorescein) - conjugated anti-CD1a (2 µL), PE (Phycoerythrin) - conjugated anti-CD83 (2 µL), and APC (Allophycocyanin) - conjugated anti-CD11c (2 µL) (BD - Becton, Dickinson and Company) for 30 min. Then, cells were washed at 520 xg for 10 min, resuspended in 450 µL of ISOTON II, and fixed with 50 µL of fixing solution containing 5% formaldehyde (BD-Becton Dickinson and Company). DCs were labeled with 1 µL/mL Live / Dead Kit (Life Technologies, Eugene, OR, USA) for assessment of viability and analyzed by flow cytometer model FACSCanto (BD-Becton, Dickinson and Company).

2.5. Purification of $CD4^+$ cells by negative magnetic selection

Total lymphocytes were defrost, washed, and counted with subsequent evaluation of viability by trypan blue exclusion test. These cells were incubated with monoclonal antibodies coupled to magnetic beads contained in the negative selection MACS® magnetic $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec Inc., Auburn, CA). Kits contain biotinylated antibody cocktail that specifically bind to their target molecules, however, cocktail for $CD4^+$ T cell isolation contains antibodies against other molecules, therefore, $CD4^+$ T lymphocytes are not marked with these biotinylated antibodies. Initially, cells were suspended in MACS Buffer (PBS solution with 0.5% bovine serum albumin and 2 mM EDTA) and and viability and counting assessed by staining with trypan blue. After centrifugation at 300 xg for 10 min, supernatant was discarded, and 40 µL of MACS buffer and 10 µL of Biotin-Antibody Cocktail $CD4^+$ were added to each 10^7 cells, and incubated for 5 min at 4° C. Then, it was added 30 uL of MACS buffer and 20 µL of CD4 microbeads cocktail for every 10^7 cells followed by incubation for 10 min at 4° C. Unbound microspheres were removed by centrifugetion at 300 xg for 10 min and cells were suspended in 3 mL of MACS buffer. This suspension was applied to magnetic column model LS coupled to MACS platform followed by 2 washes with 3 mL of MACS buffer. Antibody-biotin interaction allowed the labeled cells dropped retained when

subjected to the magnetic field of the platform. Thus, $CD4^+$ cells not retained the column were collected in tubes, centrifuged, assessed for viability by trypan blue and subjected to the tests proposed.

2.6. Analysis of purity and viability of $CD4^+$ cells

$CD4^+$ cells separated according to item 2.5, were transferred to Falcon tubes for flow cytometer (BD - Becton, Dickinson and Company) and centrifuged at 520 xg for 10 min at 4º C. After centrifugation, cells were suspended in 1 mL of electrolyte solution (ISOTON II) and incubated with anti-CD4 monoclonal antibody conjugated to PE-Cy7 (phycoerythrin) (0.6 µL), anti-CD3 conjugated to FITC (Fluorescein) (2 µL) and anti-CD14 PerCP conjugated with CY5.5 (Peridinin Chlorophyll Protein Complex) (0.3 µL) (BD - Becton, Dickinson and Company) for 30 min. Unlabeled cells were used as autofluorescense control of each sample. After incubation with respective antibodies, cells were centrifuged at 520 xg for 10 min for washing, suspended in 450 µL of ISOTON II, fixed with 50 µL of fixing solution containing 5% formaldehyde (BD-Becton Dickinson and Company). Concomitantly, we assessed the viability of these cells by Live / Dead kit (1 µL/mL of $CD4^+$ cell suspension) followed by analysis by flow cytometry.

2.7. In vitro DCs challenged with *Paracoccidioides brasiliensis*

After in vitro differentiation into DCs as described in item 2.3, cells were challenged with yeast cells of high (Pb18) and low virulence (Pb265) Pb strains (2.10^4 / mL) for 48h.

2.8. Lymphoproliferation Assay

Autologous $CD4^+$ cells isolated from peripheral blood of healthy donors as described in item 2.5. $CD4^+$ T cells were labeled with 1 µM Far Red, adjusted to 1.10^6 / mL, and cocultured with 1.10^5 / mL DCs (CD4:DC ratio - 10:1) challenged or not with Pb18 or Pb265 (DCs:Pb ratio - 5:1). After incubation with respective antibodies, cells were centrifuged at 520 xg for 10 min for washing, suspended in 450 µL of ISOTON II, fixed with 50 µL of fixing solution containing 5% formaldehyde (BD-Becton Dickinson and Company). Unlabeled cells were used as autofluorescense control of each sample. We used 8 mg / mL of the mitogen phytohemagglutinin (PHA) as a positive control.

2.9. Total RNA extraction

DCs generated according to item 2.3 and challenged or not with Pb18 and Pb265 (item 2.7) were incubated with $CD4^+$ T lymphocytes (CD4/DC ratio - 10:1) for 120h. After this time, the total RNA of the cells was extracted with the Total RNA Purification Kit (Norgen Biotek Corp.) following to the manufacturer's instructions. RNA quality and concentration were assessed using the NanoDrop 2000c Spectrophotometer (Thermo Scientific) and Qubit 2.0 Fluorometer (Invitrogen). The quantified material was immediately frozen at -80°C for further library preparation by the SureSelect Strand-Specific RNA Library Preparation Kit for Illuminated Multiplexed Sequencing (Agilent Technologies).

2.10. Library construction

After extraction and quantification of total RNA according to item 2.9, the samples were submitted to the library preparation protocol using the SureSelect Strand-Specific RNA Library Preparation Kit for Illuminated Multiplexed Sequencing (Agilent Technologies). The poly (A) RNA of all samples was microparticle purified, fragmented and the first cDNA strand was synthesized. After generation of the first strand, it was purified by means of AMPure XP beads followed by the synthesis and purification of the double strand of cDNA. Subsequently the cDNA was adenylated, then the adapters were added followed by purification of the cDNA libraries with the adapters linked by means of the AMPure XP beads kit (Beckmann & Coulter). The next step was to add the appropriate index primers and incubate the tubes with the thermocycler samples for amplification of the cDNA libraries by PCR. Further purification of the libraries was performed using AMPure XP beads and quantification was performed on RTq-PCR (7500 fast Real Time PCR Systems - Applied Biosystems) using Kappa library quantification kit according to the manufacturer's protocol. After, the libraries were frozen at -80°C for further evaluation of the transcriptome by means of the RNAseq - Truseq Stranded mRNA Sample Preparation Kit. Finally, the samples were submitted to the Illumina MiSeq sequencer (Illumina, Inc., San Diego, USA) using flowCell of 50 cycles.

2.11. Lybrary sequencing

For sequencing, the libraries were defrost and prepared with TruSeq RNA Sample Prep Kiy v.2 (RS-122-2001, Illumina) according to the manufacturer's instructions. The pool of samples was then submitted to the Illumina MiSeq sequencer (Illumina, Inc., San Diego, USA) using the cycle 50 flowCell.

2.12. Sequencing analysis

The total number of Reads (55.965.285) was imported into the CLC Genomic Workbench (GWB) (Qiagen Company). Sequences were trimmed to the exclusion of poor-quality sequences and adapters, and only sequences greater than 40 base pairs were accepted (55.017.312). To identify the sequences, we counterposed them to the human reference genome NC_000001.11 (GRCh 38). Afterwards, we started the analysis of the differential expression of the transcripts by normalization by RPKM (readings per kilobases of reads mapped per million) in conjunction with the statistical analysis using the Kal's test. Subsequently, we used data filtering with False Discovery Rate (FRD) $\leq$ 0.05, values of RPKM $\geq$ 2, Fold Change> 2 and Bonferroni correction (P value $\leq$ 0.05).

3. Results

3.1. Number of Reads after the lybrary sequencing in the Illumina MiSeq and the gene differential expression between the groups.

All the tested cultures totalized 48 samples. After analysis of these samples a total of 55.965.285 Reads was obtained that after trimmed was diminished to 55.017.312 reads. The Figures 1a and 1b show the number of reads obtained in the cultures with DCs and CD4, respectively.

Subsequently we identified the number of genes that presented differential expression in the cultures with DCs (Figure 2a) and CD4 (Figure 2b).

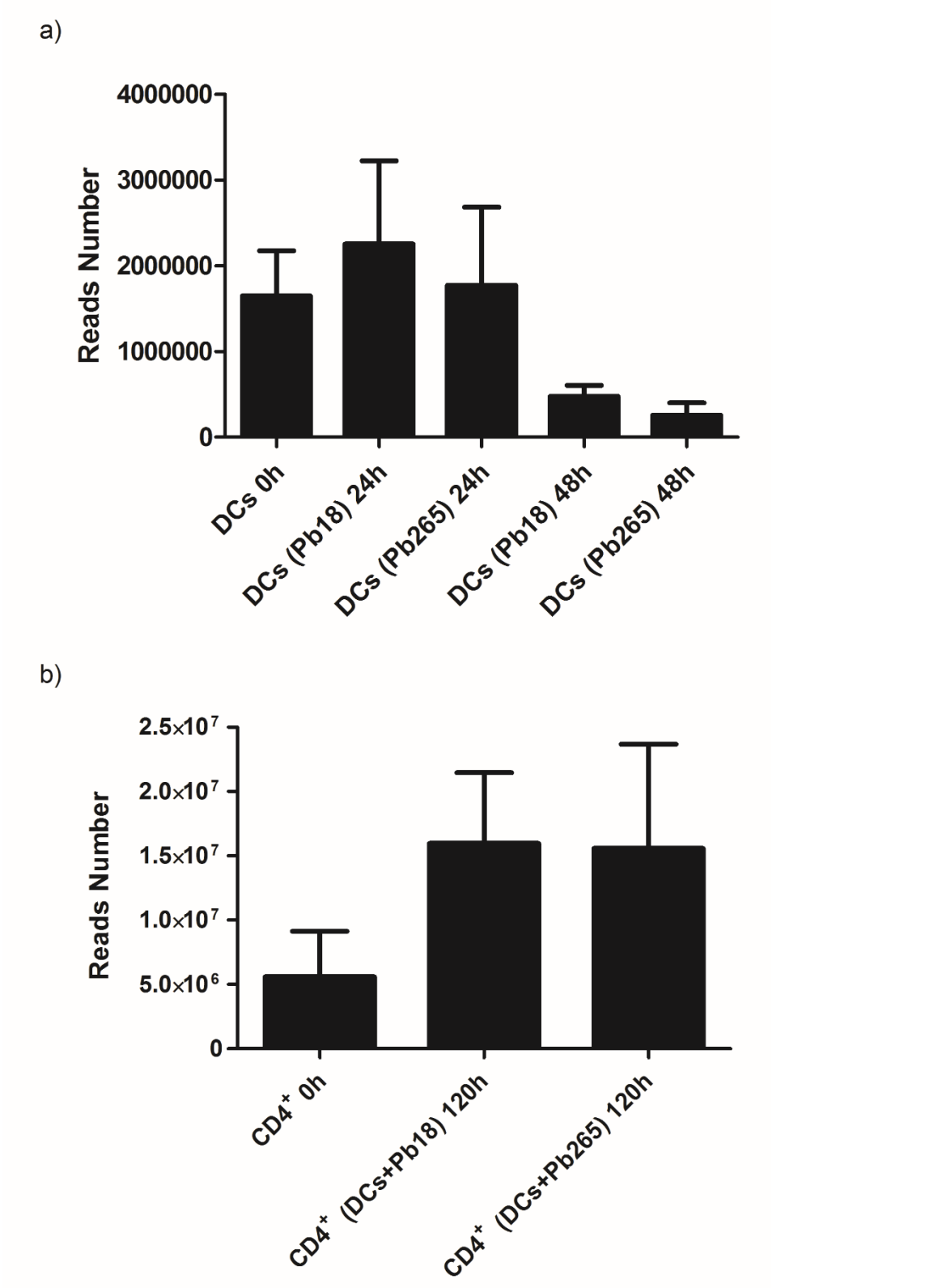
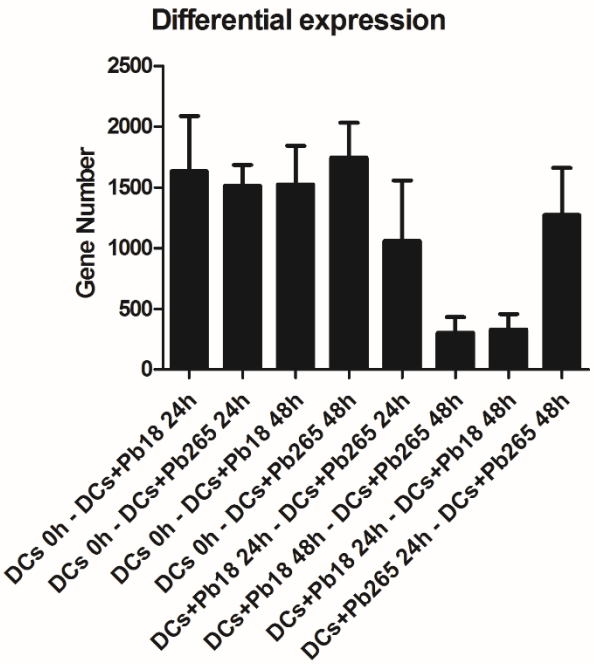


Fig. 1. Read numbers after the sequencing of the cDNAs in the Illumina MiSeq of DCs in the 0h period and DCs challenged with Pb18 and / or Pb265 for 24h and 48h (a) and of $CD4^+$ cells at 0h and $CD4^+$ cultured with DCs sensitized with Pb18 and / or Pb265 for 120 h (b). The results are expressed as mean ± standard deviation and were obtained from cells of 6 healthy subjects.

a)



b)

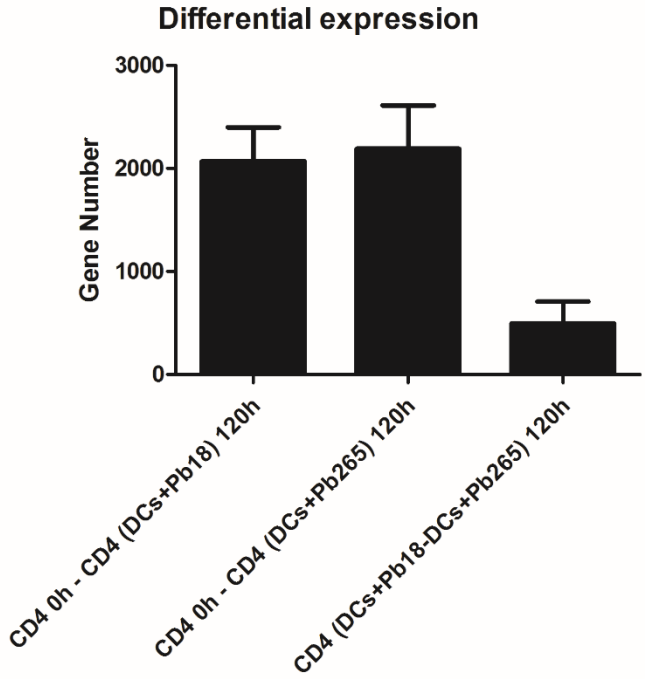


Fig. 2. Genes number showing differential expression in various DCs cultures (a) and CD4/DCs cocultures. The results are expressed as mean ± standard deviation and were obtained from cells of 6 healthy subjects.

3.3. Identification and function of positively and negative regulated genes in DCs challenged with Pb18 and Pb265

To evaluate the ratio of the positively or negative modulated genes in the DCs by the fungus, we submitted the cells of the various groups to the CLC Genomic Workbench and a plateau for the positively and negative regulated genes was stablished. Then, it was considered the genes with Fold Change ≥10X + or Fold Change ≥ 10X - in relation to the control (Figs 3 and 4). Exception for this criterion (outside the Fold Change ≥ 10X + and Fold Change ≥ 10X) was used for the gene that codifies the molecules associated to HLA-II complex.

We observed that the genes modulated positively by Pb18 in the DCs at the 24h period were those codifing the following proteins: IL-10, TRAF1, FSCN1, CCL4, CCL22, CXCL2, TNF, CCR7, CCL3, CXCL1, CXCL8, and IL1B, while the negatively modulated genes were those codifing: PTGS1, CCL13, CCL24, CD209, CCL23, CLEC4G, ALOX15, and CLEC10A (Fig 3a). Similar results were obtained with DCs at the 48h period, except for the genes codifing CXCL2 and CLEC4G that were not regulated (Fig 3b). The genes modulated positively by Pb265 in the DCs at the 24h period were the same regulated by Pb18 plus the gene for CXCL3. On the other hand, the negatively modulated genes were only PTGS1, CLEC4G, CD209, and ALOX 15 (Fig 4a). At the 48h period, the genes positively regulated were almost the same detected at 24h, exception for ALOX15B that was regulated only at 48h and CCL4 and CXCL2 genes that were only regulated at 24h. On the other hand, the genes negatively regulated by this strain at 24h were: ALOX 15, CD209, CLEC4G, and PTGS1. At 48h only the genes for CD209 and ALOX15 were negatively modulated by this strain. The results obtained specifically for the genes associated to HLA- II complex in the cultures of DCs challlenged with: Pb18 and Pb265 for 24 h and Pb18 and Pb265 for 48h were shown in the Figures 5a to 5d, respectively.

The products of the genes that were positively or negatively regulated present direct or indirect action on the immune response processes. The functions of these genes were listed in table 1 and 2, respectively.

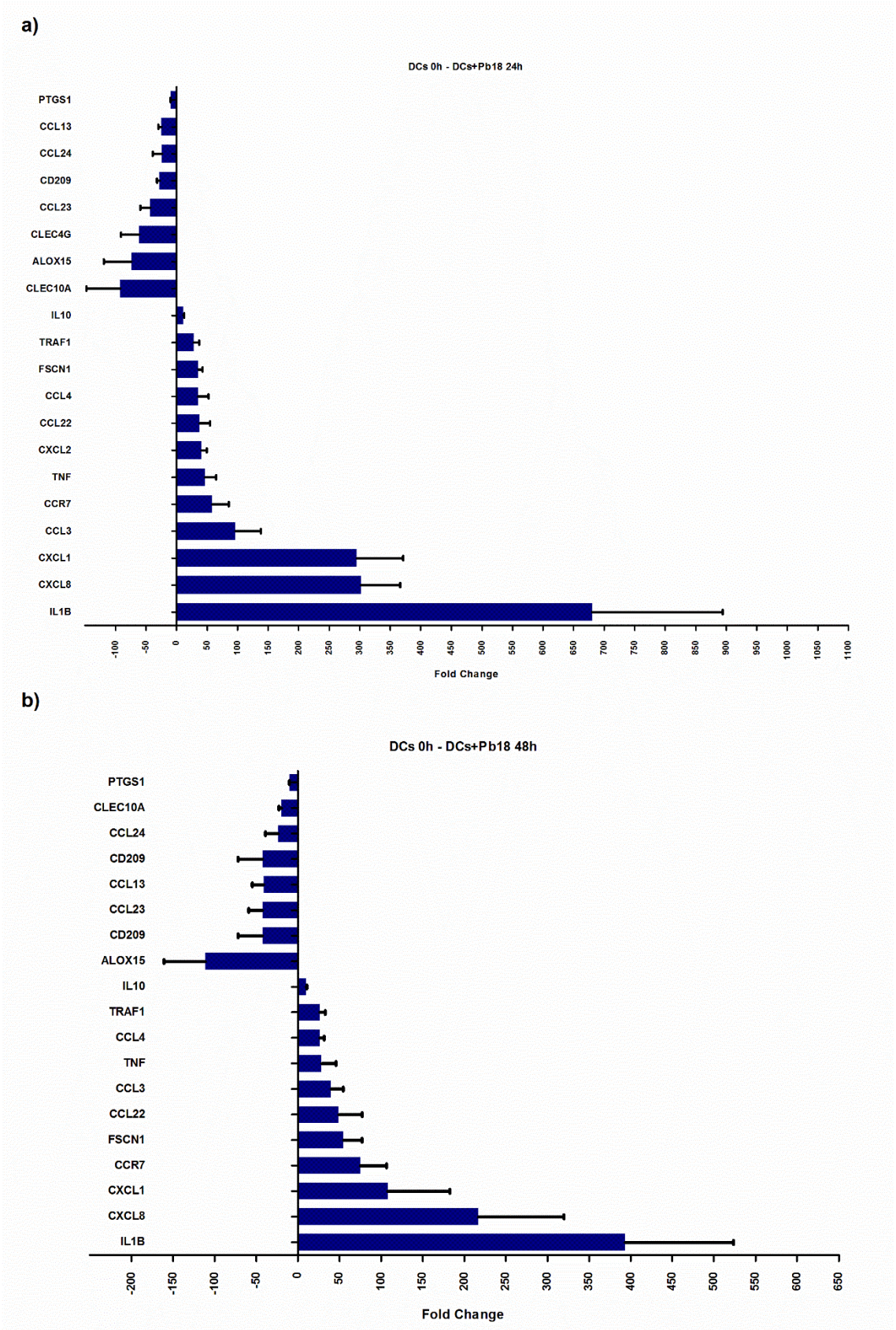


Fig. 3. Genes positively or negatively regulated in the culture of DCs challenged with Pb18 for 24h (a) and 48h (b) Genes with Fold Change ≥10X + or Fold Change ≥ 10X - in relation to the control (DCs 0h) were considered positively or negatively regulated,

respectively. The results are expressed as mean ± standard deviation and were obtained from cells of 6 healthy subjects.

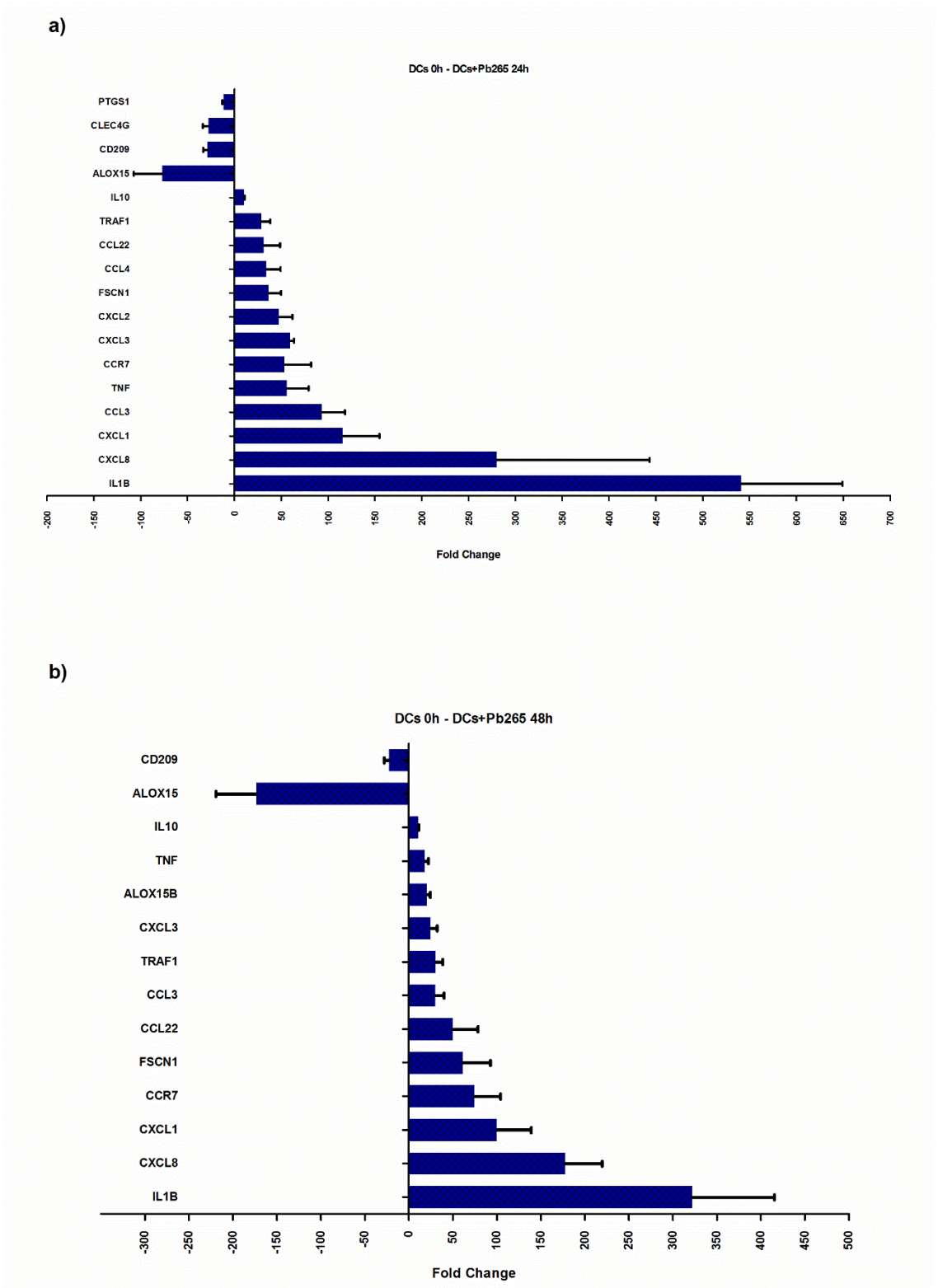


Fig. 4. Genes positively or negatively regulated in the culture of DCs challenged with Pb 265 for 24h (a) and 48h (b) Genes with Fold Change ≥10X + or Fold Change ≥ 10X - in relation to the control (DCs 0h) were considered positively or negatively regulated,

respectively. The results are expressed as mean ± standard deviation and were obtained from cells of 6 healthy subjects.

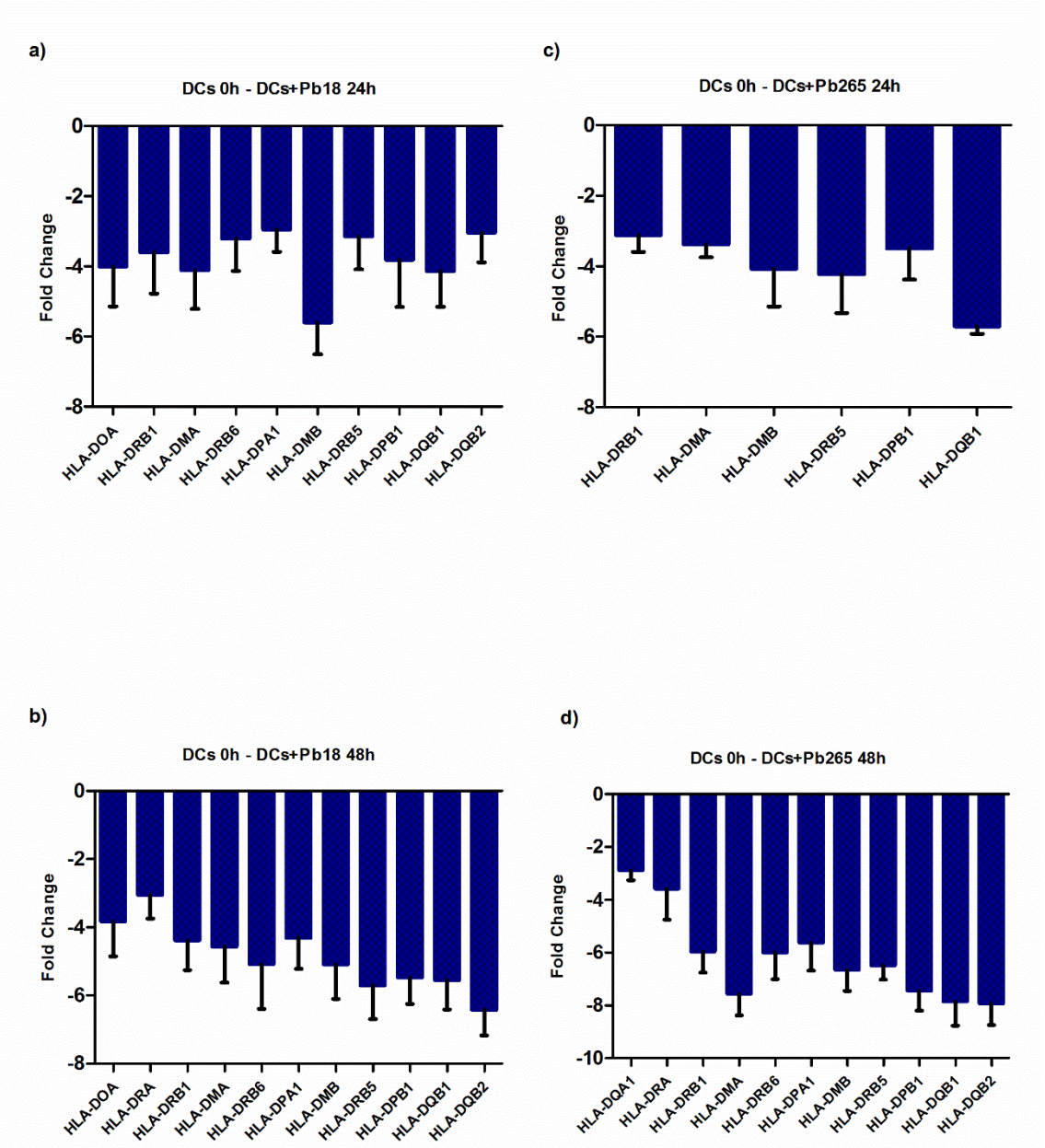


Fig. 5. Fold Changes relative to the genes associated to the molecules of the complex of HLA-II obtained in the cultures of DCs challenged with: Pb18 (a) and Pb265 (b) for 24 h and Pb18 (c) and Pb265 (d) for 48h. The results are expressed as mean ± standard deviation and were obtained from cells of 6 healthy subjects.

Genes	Function
IL-1B	It is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis.
IL10	It codes a cytokine produced mainly by monocytes and, in smaller production, by lymphocytes. It has pleiotropic effects on immunoregulation and inflammation and negatively regulates the expression of Th1 cytokines, MHC class II and costimulatory molecules. It also increases B cell proliferation.
TNF	This gene encodes a multifunctional proinflammatory cytokine tumor necrosis factor (TNF). It is involved in the regulation of a variety of biological processes, including cell proliferation, differentiation, apoptosis, lipid metabolism and coagulation.
CCL3	It plays a role in inflammatory responses through binding to the receptors CCR1, CCR4 and CCR5
CCL4	The encoded protein has inflammatory and chemokinetic functions
CCL22	The cytokine encoded by this gene shows chemotactic activity for monocytes, DCs, NK cells and for activated T lymphocytes. The product of this gene binds to the chemokine receptor CCR4.
CXCL1	This protein plays a role in inflammation and is chemotactic for neutrophils.
CXCL2	Involved in inflammatory and immunoregulatory processes. This chemokine is a member of the CXC subfamily, is expressed at sites of inflammation and may suppress the proliferation of hematopoietic progenitor cells.
CXCL3	This protein plays a role in inflammation and is chemotactic for neutrophils.
CXCL8	This chemokine is one of the major mediators of the inflammatory response and can be produced by several cell types. It has chemotactic function.
CCR7	This receptor is expressed in various lymphoid tissues and activated B and T lymphocytes. It is able to control the migration of memory T cells to the inflamed tissues as well as stimulate the maturation of DCs. CCL19 chemokine is a specific ligand of that receptor. Signals mediated by this receptor regulate T-cell homeostasis in lymphnodes, and may also participate in T-cell activation and polarization.
FSCN1	The encoded protein plays a crucial role in cell migration, motility and adhesion.
TRAF1	The protein encoded by this gene is a member of the TNF receptor family (TNFR) associated with TRAF protein. This protein forms a heterodimeric complex, which is required for the activation of MAPK8 / JNK-mediated TNF-alpha and NF-kappaB. The protein complex formed by this protein also mediates the anti-apoptotic signals of TNF receptors.

Table 1. Functional role of positively modulated genes in DCs.

CCL13	This gene is one of several genes involved in inflammatory and immunoregulatory processes. The cytokine encoded by this gene shows chemotactic activity for monocytes, lymphocytes, basophils, and eosinophils, but not neutrophils.
CCL23	It encodes one of the chemokines involved in inflammatory and immunoregulatory processes. This chemokine exhibits significant chemotactic activity for non-activated T lymphocytes and monocytes, minor activity for neutrophils and no activity for activated T lymphocytes.
CCL24	It encodes one of the chemokines involved in inflammatory and immunoregulatory processes. Exhibits significant chemotactic activity for non-activated T lymphocytes, minor activity for neutrophils, and no activity for monocytes and activated T lymphocytes.
HLA-II	It plays a central role in the immune system through the presentation of peptides derived from extracellular proteins. Class II molecules are expressed on antigen presenting cells (B lymphocytes, DCs, macrophages).
CD209	This gene encodes the DC-SIGN receptor present on the surface of dendritic cells and macrophages. The encoded protein is involved in the innate immune system and recognizes numerous pathogens. The extracellular region consists of the type C lectin domain and has a dual function as a pathogen recognition receptor and a cell adhesion receptor.
CLEC4G	This gene encodes a type C lectin glycine binding receptor which plays a role in the T cell immune response
CLEC10A	This gene encodes a member of the C-type lectin domain. It has several functions, such as cell adhesion, cell-cell signaling, and functions in inflammation and immune response.
PTGS1	This is one of two genes encoding similar enzymes that catalyze the conversion of arachidonic acid to prostaglandin.
ALOX15	It encodes protein 15 lipoxygenase arachidonate responsible for the synthesis of leukotrienes.

Table 2. Functional Role of Negatively Modulated Genes in DCs.

3.4. Identification and function of positively and negative regulated genes in $CD4^+$ cells co-cultured with DCs challenged with Pb18 and Pb265

The same criteria adopted for DCs analysis (Fold Change ≥10X + or Fold Change ≥ 10X - in relation to the control) were used for CD4 cells. Here, exception for this criterion (outside the Fold Change ≥ 10X + and Fold Change ≥ 10X) was used for the gene that codifies the molecules associated to the transcription factors Foxp3 and GATA3.

After coculture of $CD4^+$ cells with autologous DCs sensitized with Pb18, the positively modulated genes were CCL17, CCL22, IL2RA, LAMP3, MT2A, and FSCN1

(Fig 6a). Similarly, the Pb265 positively regulated most of these genes, except for LAMP3 and FSCN1 (Fig 6b). In contrast, the FOS, KLF2, TNFAIP3, TXNIP, CXCR4, FAM65B, JUN, and PTGER4 genes were regulated negatively by Pb18 (Fig 6a). Pb265 negatively regulated all these genes including DUSP1, CD69, and SIGIRR, with the exception of the FAM65B gene, which was not regulated (Fig 6b). The results obtained specifically for the genes associated to Foxp3 and GATA3 were shown in the Figures 8a and b respectively. We found that the gene for Foxp3 (transcription factor for T_{reg} cells) was positively regulated in $CD4^{+}$ cells when these cells were co-cultured with DCs sensitized with Pb18 (Fig 7a). On the other hand, GATA3 was negatively regulated when these cells were co-cultured with the DCs sensitized with both strains (Fig 7b).

The functions of the genes positively and negatively regulated were listed in tables 3 and 4, respectively.

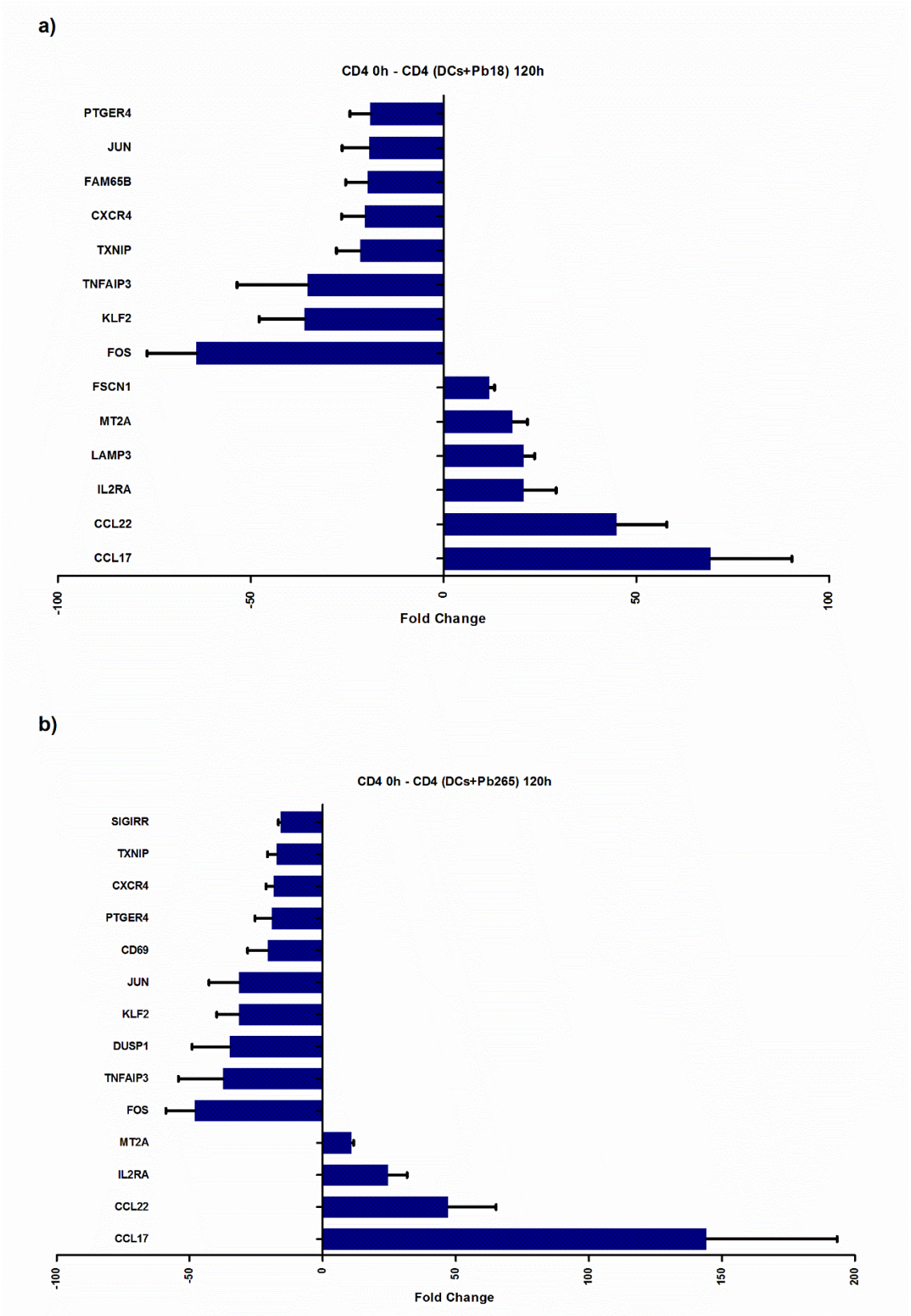


Fig. 6. Genes positively or negatively regulated in the culture of CD4 cells sensibilized with DCs challenged with Pb18 (a) and Pb265 (b) for 120h. Genes with Fold Change ≥10X + or Fold Change ≥ 10X - in relation to the control (DCs 0h) were considered positively or negatively regulated, respectively. The results are expressed as mean ± standard deviation and were obtained from cells of 6 healthy subjects.

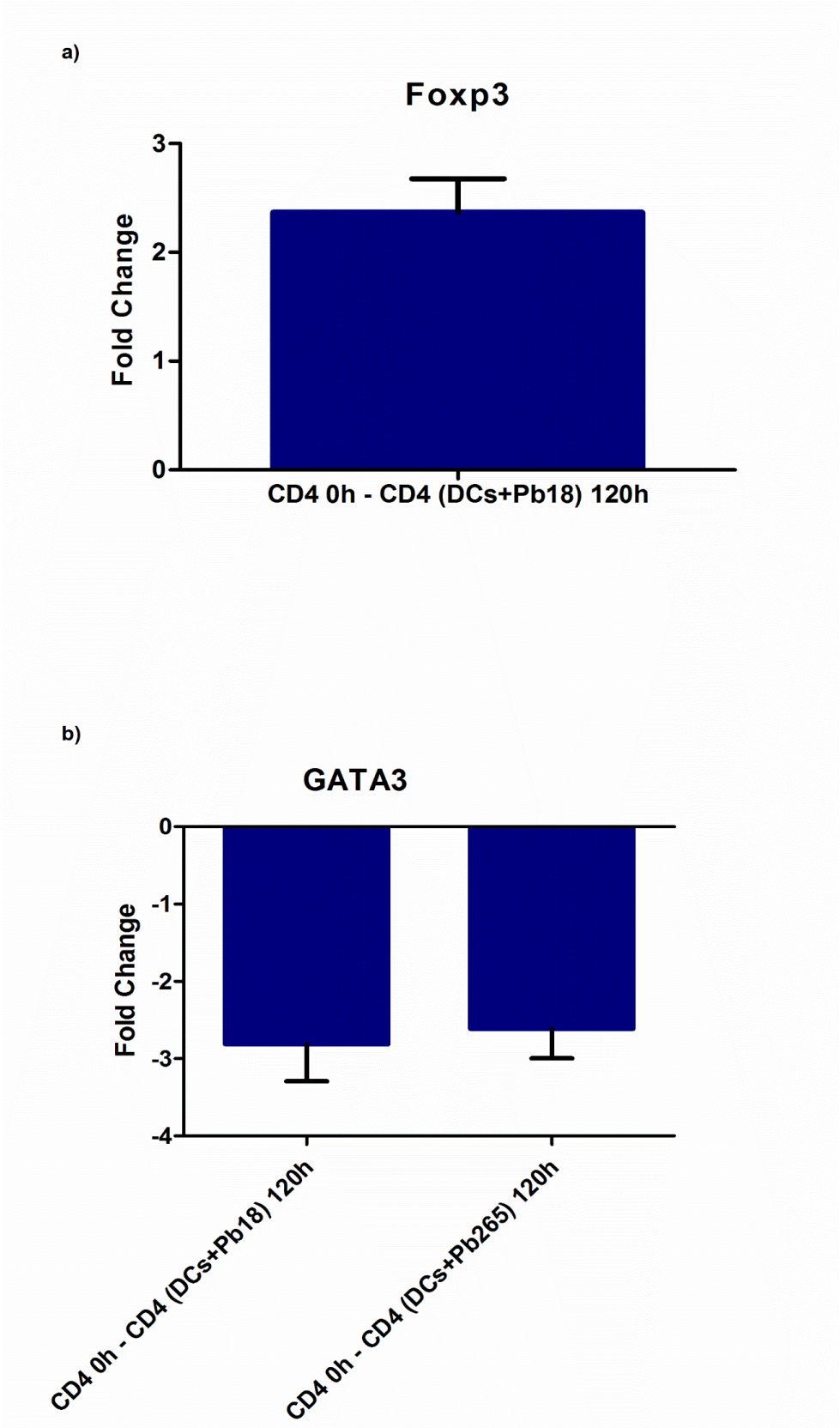


Fig. 7. Mean of the Fold Change of Foxp3 (a) and GATA3 (b) genes. The result obtained is expressed as mean ± standard deviation.

CCL17	This gene is involved in inflammatory and immunoregulatory processes. The encoded chemokine plays an important role in the development of T cells in the thymus, as well as chemotaxis and activation of mature T cells. The product of this gene binds to CCR4 and CCR8 receptors.
CCL22	The cytokine encoded by this gene shows chemotactic activity for monocytes, DCs, NK cells, and for activated T lymphocytes. The product of this gene binds to the chemokine receptor CCR4.
IL2RA	Encodes the receptor for interleukin 2 (IL-2).
MT2A	It encodes the metallothionein-2 protein involved in the antioxidant action. They are able to capture Cu+ ions, which reduces the radical production promoted by this metal. They also release Zn2+, which inhibits lipid peroxidation.
LAMP3	Lysosome-associated membrane protein 3. It may play a role in the function of DCs and in adaptive immunity.
FSCN1	The encoded protein plays a crucial role in cell migration, motility, adhesion, and cellular interactions.
Foxp3	It encodes a member of the family of transcriptional regulatory proteins and is determinant to define a regulatory adaptive T response profile.

Table 3. Functional Role of Positively Modulated Genes in $CD4^+$ cells.

CXCR4	This gene encodes a chemokine receptor-specific CXC-specific protein for stromal cell-derived factor-1, also known as CXCL12. It is endowed with potent chemotactic activity for lymphocytes.
CD69	This gene encodes a member of the lectin superfamily. Expression of the encoded protein is induced after activation of T lymphocytes, and may play a role in proliferation. In addition, the protein can act to transmit signals in NK cells.
GATA3	It encodes a member of the family of transcriptional regulatory proteins and is determinant for defining a Th2 type adaptive response profile.
PTGER4	The encoded protein is one of the four receptors identified for prostaglandin E2 (PGE2). This receptor can activate the T cell signaling factor. It regulates the stability of cyclooxygenase-2 mRNA.
TNFAIP3	This gene has been identified as a gene whose expression is induced by tumor necrosis factor (TNF). The protein encoded by this gene is a zinc finger enzyme, and has been shown to inhibit NF-kappa B activation, as well as TNF-mediated apoptosis. The encoded protein is involved in the immune and inflammatory response mediated by cytokines.
FOS	It encodes proteins with role in the regulation of cell proliferation, differentiation and transformation. In some cases, FOS gene expression has also been associated with apoptotic cell death.
TXNIP	This gene encodes a thioredoxin binding protein which is an important thiol oxidoreductase to regulate cell signaling that protects cells from oxidative stress. This protein inhibits the antioxidant function of thioredoxin resulting in the accumulation of reactive oxygen species and cellular stress.
JUN	It encodes the c-JUN protein that can exert a role in the regulation of growth factors, pro-inflammatory cytokines, cell cycle progression, and anti-apoptotic activity.
FAM65B	This gene encodes an atypical inhibitor of the small G protein RhoA. Inhibition of RhoA activity by the encoded protein mediates the polarization of T cells and neutrophils.
DUSP1	It encodes the dual-specificity 1 protein phosphatase that may play an important role in the human cellular response to environmental stress, as well as in the negative regulation of cell proliferation.
SIGIRR	It may exert function in modulating the immune response and interacting with TRAF6.
BCL2	This gene encodes a protein that blocks the apoptotic death of some cells such as lymphocytes.
KLF2	It regulates trafficking of T cells by promoting expression of the S1P1 lipid receptor and CD62L selectin.

Table 4. Functional Role of Negatively Modulated Genes in $CD4^+$ Cells.

4. Discussion

In paracocidioidomycosis studies focusing on the interaction human DCs/Pb are scarce. In an attempt to better understanding this process, particularly its consequences to the instruction of the CD4 adaptive response, we aimed to analyze the transcriptional profile of DCs in response to Pb as well as that of $CD4^+$ lymphocytes cocultured with DCs sensitized with the fungus. Here, in an attempt to better understanding this process, and its consequences to the instruction of the CD4 adaptive response, we aimed to analyze the transcriptional profile of DCs in response to Pb as well as that of $CD4^+$ lymphocytes cocultured with DCs sensitized with the fungus. Many genes with function not yet described and those that may present direct or indirect action on the immune response processes were positively or negative regulated. The genes positively regulated by Pb in DCs were IL-1, TNF, CXCL2, CXCL3, CXCL8, CCL3, CCL4, CCL22, CCR7, SLAMF7, TRAF1, FSCN1, ALOX15B, and IL-10, and those negatively regulated were CCL13, CCL23, CCL24, CD209, CLEC4G, CLEC10A, ALOX15, PTGS1, and HLA-D. The positively regulated genes in $CD4^+$ cells by DCs sensitized with Pb were CCL17, CCL22, IL2RA, LAMP3, MT2A, FSCN1, and Foxp3 and the negatively regulated genes were CXCR4, CD69, FOS, JUN, TNFAIP3, KLF2, TXNIP, FAM65B, SIGIRR, PTGER4, DUSP1, and GATA3. However, for our discussion we have chosen some genes whose functions would be more closely associated with the mechanisms associated with our main objective. In this context, one of these genes was that associated to HLA-II complex, that was negatively expressed bu DCs. This finding corroborates our previous studies showing that DCs don't mature in response to Pb as HLA molecules are essential for mature DCs to present antigens to CD4 cells (Fernandes *et al*., 2015). Other genes that deserve attention are these codifing TNF-α an CCL22 (positively regulated) and DC-SIGN and CLEC4G and CLEC10A (negatively regulated). TNF-α is essential to control fungal infection that increases the cytotoxic activity of macrophages and stimulates the release of chemokines (Roach *et al*., 2002). However, this cytokine is associated with DCs maturation. Considering this function, our present results showing uperegulation of this cytokine gene, are not in agreement with our previous study in which nonmaturation of DCs was atributed to PGE2 inhibition by the fungus, which results in lower TNF-α levels and consequent inhibition of DCs maturation. The chemokine CCL22, plays an important chemotaxis function for monocytes, T lymphocytes and even for DCs which is considered the major producer of this chemokine (Traynor & Huffnagle, 2001; Vulcano

et al., 2001), at the same time it is a key chemokine for the recruitment of T_{reg} cells into tumor tissue (Wiedemann *et al*., 2016). Thus, upregulation of the gene for this chemokine may favour the recruitment of Treg cells, during the instruction of adaptative immune response in response to Pb.

The DC-SIGN receptor recognizes essentially mannan and beta-glucan and may participate in both, DCs migration and in their interaction with $CD4^+$ cells during the initial period of the immune response (Relloso *et al*., 2002, Valera *et al*., 2008). It also favors the phagocytosis of pathogenic fungi such as *Candida albicans* and *Aspergillus fumigatus* (Cambi *et al*., 2003; Serrano-Gomez *et al*., 2004). Just like DC-SIGN, CLEC4G and CLEC10A encode lectin C-like receptors that can bind to wall components of glucocyanins, allowing fungus recognition and capture (Suzuki *et al*., 1996; Vliet *et al*., 2005; Dominguez-Soto *et al*., 2007). Thus, our findings showing that the genes coding these receptors are negatively regulated inplyes that some DCs functions mainly those of fungus binding and capture can be compromissed.

Important chemokine coding genes like CXCL1, CXCL2, CXCL8, CCL3, and CCL4 were upregulated in DCs by both strains. Similar results were found in DCs challenged with influenza virus where the virus positively regulated CCL3 and CCL4 in plasmacytoid DCs (pDCs), and CCL4 and CXCL1 in myeloid DCs (mDCs). In contrast to our results, the gene encoding CXCL1 and CXCL8 were down-regulated (Piqueras *et al*., 2006). CXCL1, CXCL2, and CXCL8 chemokines are important in the immune response since they are chemotactic for polymorphonuclear cells (PMNs) and CCL3 and CCL4 are considered inflammatory chemokines (Zlotnik & Yoshie, 2000; Cook *et al*., 1995; Scimone *et al*., 2005). Thus, the upregulation of these genes by DCs implies that these cells may have an effective participation in the mounting of an inflammatory response to Pb.

The gene encoding the CCR7 receptor was positively modulated by the fungus in DCs. This receptor is characteristic of mature DCs, as it is involved in cells migration from the infection site to secundary limphoid organs. These results, again, don't agree with our previous studies showing nonmaturation of DCs after Pb challenge. However, genes codifing important chemokines such as CCL19 and CCL21 that interact with CCR7 to ensure the migration of these cells were not expressed. This finding, allow us to consider CCR7 are not able to acts on DCs, despite be increased. In addition, there is a study that showing that the CCR7 receptor is essential for tolerance induction (Foster *et al*., 2008). Thus, increased CCR7 levels can be associated to the instruction of the

adaptative response toward T_{reg} cells.

In conjunction with the positively modulated genes, several genes were negatively modulated as the chemokine genes CCL13, CCL23 and CCL24. The CCL13 chemokine is constitutively expressed in immature DCs and its increase is proportional to the maturation process of DCs after inflammatory stimuli (Hashimoto *et al*., 1999; Sallusto & Lanzavecchia, 2000; Le *et al*., 2004). The CCL23 is related to the recruitment and activation of leukocytes in inflammatory diseases (Berahovich *et al*., 2008; Forssmann *et al*., 1997) and has the potential to increase levels of TNF-α in monocytic cells (Kim *et al*., 2010). CCL24 plays a role in T-cell recruitment and is related to the accumulation of eosinophils (Beaulieu *et al*., 2002) and participates in the activation of Th2 cells via the CCR3 receptor (Sallusto *et al*., 1997). These results, particularly those refering to CCL3 again reinforces our results showing that DCs do not mature in response to Pb.

An important result was obtained in relation to IL-10, whose gene was upregulated in DCs. IL-10 is a cytokine with potent anti-inflammatory activity that can act to prevent inflammatory and autoimmune diseases (Sabat *et al*., 2010; Kuhn *et al*., 1993), while limiting host response and favoring persistence of the infectious agent (Brooks, 2006; Roncarolo *et al*., 2006), including Pb (Araújo *et al*., 2015; Bazan *et al*., 2015). Specifically, IL-10 produced by DCs is associated with instruction of an adaptive response toward T_{reg}.

The analysis of genes expression in CD4 cell sensitized with DCs challenged with Pb, showed that the genes CCL17, CCL22, IL2RA, LAMP3, MT2A, FSCN1, and Foxp3 were positively regulated, while CXCR4, CD69, FOS, JUN, TNFAIP3, KLF2, TXNIP, FAM65B, SIGIRR, PTGER4, DUSP1, and GATA3 were negatively regulated. Among these genes, the coders for CCL17, CCL22, IL2RA, and CD25 must to be highlighted as CCL17 and CCL22 are chemokines with chemotactic effect for T_{reg} cells (Curiel *et al*., 2004). In addition, the IL-2 RA (receptor for IL-2) and CD25 molecule are constitutively expressed by these cells (Malek & Castro, 2010). However, other genes not directly related to the mechanisms discussed above deserve to be highlighted.

There was a positive regulation of the MT2A gene that can control a potent anti-inflammatory activity (Stankovic *et al*., 2007; Wang *et al*., 2014; Kozela *et al*., 2016; Krizkova *et al*., 2016) and of the LAMP3 gene that is associated to inflammatory processes and codifies a lysosomal protein associated with the risk of metastasis (Kanao *et al*., 2005; Nagelkerke *et a*l., 2013). In addition to the positively regulated genes, others

were down-regulated as CXCR4 that can be highly expressed on activated $CD4^+$ T cells and $CD4^+$ memory T cells (Bryant *et al*., 2012; Nagafuchi *et al*., 2016). The interaction CXCR4 and CXCL12 may be chemotactic for T cells and NK cells (Piao *et al*., 2015). Therein, Pb could negatively regulate CXCR4 as an escape mechanism of NK cells.

The FOS and JUN genes that play an important role in inflammatory processes (Zolotarenko *et al*., 2016) and CD69 that is a marker of crucial activation in T cells (Monaco *et al*., 2016; Sécca *et al*., 2016) were also negatively modulated by the fungus. At the same time, the TNFAIP3 gene expressed in T cells (Tewari *et al*., 1995) and that has the capacity to act preventively in the inflammation in vivo and in the survival of these cells (Lee *et al*., 2000; Matsuzawa *et al*., 2015), the KLF2 gene important for differentiation, activation and recruitment of $CD4^+$ cells (Lee *et al*., 2006; Carlson *et al*., 2006; Sebzda *et al*., 2008), the FAM65B that participates in the migration of T cells (Trinité *et al*., 2014) was negatively regulated by both strains of the fungus (Zhou *et al*., 2010; Oslowski *et al*., 2012). Concomitantly, the genes SIGIRR, PTGER4, and DUSP1 also underwent negative regulation. The first one exerts the negative regulation function for signaling IL-1R, TLR4 and TLR9 (Garlanda *et al*., 2004; Wald *et al*., 2003; Qin *et al*., 2005) and consequently a fine regulation of the inflammatory response (Lech *et al*., 2008). In mice infected with *Candida albicans*, Bozza *et al*. (2008) reported the importance of the SIGIRR gene against infection by inhibiting IL-1R and consequently the induction of a Th_{17} response. The PTGER4 gene encodes the EP4 receptor for PGE_2 and is constitutively expressed at high levels in $CD4^+$ cells. Thus, PGE_2 may favor the induction of a Th_{17} response by binding to the EP4 receptor (Boniface *et al*., 2009). In murine infection by *Cryptococcus neoformans*, inhibition of EP4 favored the survival of these animals by improving the response of alveolar macrophages (Shen & Liu, 2015). However, down-regulation of these genes in CD4 cells by DCs infected in vitro with Pb may account for a possible absence of a Th_{17} inflammatory response, but activation of a T_{reg} one. Finally, which has an important function in the activation of T cell proliferation (Zhang *et al*., 2009; Huen *et al*., 2013) has been shown to be negatively modulated. Once again, the fungus could modulate this gene in the $CD4^+$ cell as an escape mechanism of the effector functions of the adaptive immune response.

Of extreme importance to our discussion are the results with the Foxp3 and GATA3 genes that encode the determinant transcription factors for the development of adaptive T_{reg} and Th_2 responses, respectively. The positive regulation of Foxp3 associated to a negative regulation of GATA3 reinforce our hypothesis that DCs instruct the adaptive

response toward T_{reg} cells.

Overall, the evaluation of the transcriptional profile of DCs in response to Pb as well as of $CD4^+$ lymphocytes cocultured with DCs sensitized with the fungus allow to advance in the knowledge of the role of DCs in the instruction of the adaptive response to Pb. In summary, our results point out for the induction of a nonmature DCs by both strains of the fungus, that instruct adaptive response toward differentiattion of T_{reg} cells.

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Anexos

1. Parecer do Comitê de Ética em Pesquisa

FACULDADE DE MEDICINA DE BOTUCATU -UNESP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Alterações transcricionais em células dendríticas e células T CD4+ humanas em resposta ao Paracoccidioides brasiliensis

Pesquisador: Reginaldo Keller Fernandes

Área Temática:

Versão:

CAAE: 32809914.2.0000.5411

Instituição Proponente: Departamento de Microbiologia e Imunologia

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 737.619

Data da Relatoria: 04/08/2014

Apresentação do Projeto:

A paracoccidioidomicose (PCM) é uma micose sistêmica endêmica na maioria dos países da América Latina, cujo agente etiológico é o

Paracoccidioides brasiliensis. A evolução para cura, paracoccidiodomicose-infecção (forma latente) ou paracoccidioidomicose-doença (formas aguda ou crônica) depende de vários fatores, especialmente o perfil de resposta Th1 ou Th2, sendo este último associado à doença mais grave. Outros fatores vêm sendo estudados, como o tipo de receptor envolvido na interação entre fungo e célula dendrítica (CD) e a diferenciação da célula T naive em células TH17 ou Treg.

Esse estudo enfocará o perfil transcricional das CDs em resposta ao P. brasiliensis, com especial atenção aos genes envolvidos no controle da enzima indoleamine 2,3-dioxigenase (IDO) e no metabolismo do ácido araquidônico (Fosfolipase A2, COX 1 e COX 2). Analisará também o perfil transcricional de linfócitos CD4+ cocultivados com CDs sensibilizadas com o P. brasiliensis, com especial atenção para os fatores de transcrição envolvidos na diferenciação das populações de linfócitos Th1 (t-bet), Th2 (gata-3), Th17 (RORt) e TReg (FoxP3) e citocinas envolvidas no processo.

Para tanto serão coletados 50 ml de sangue periférico de 20 indivíduos saudáveis, de ambos os sexos, com idade entre 20 e 40 anos, voluntários do Instituto de Biociências, UNESP – Botucatu,

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Bairro: Rubião Junior **CEP:** 18.618-970
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após assinatura do TCLE. As CDs obtidas a partir de monócitos do sangue periférico serão desafiadas com cepas de alta e baixa virulência do P. brasiliensis por 1h a 48h e submetidas à análise transcricional pela técnica de seqüenciamentos de RNA (RNA-seq). A mesma análise transcricional será feita com células CD4+ cocultivados com CDs, que também serão avaliadas quanto ao seu fenótipo por meio da detecção intracitoplasmática das diferentes citocinas.

Objetivo da Pesquisa:

Analisar o perfil transcricional das CDs em resposta ao P. brasiliensis, com especial atenção aos genes envolvidos no controle da enzima IDO e no metabolismo do ácido araquidônico (Fosfolipase A2, COX 1 e COX 2). Analisar o perfil transcricional de linfócitos CD4+ cocultivados com DCs sensibilizadas com o P. brasiliensis, com especial atenção para os fatores de transcrição envolvidos na diferenciação das populações de linfócitos Th1 (t-bet), Th2 (gata-3), Th17 (RORt) e TReg (FoxP3) e citocinas envolvidas no processo.

Avaliação dos Riscos e Benefícios:

O projeto envolve riscos mínimos para os sujeitos, decorrentes apenas de complicações da punção venosa. Os benefícios serão indiretos e relacionados ao melhor conhecimento da imunopatogênese da doença.

Comentários e Considerações sobre a Pesquisa:

O projeto atende aos requisitos das resoluções de pesquisa em seres humanos, está claro e bem redigido, e trata de tema pertinente. O TCLE está adequado; utiliza linguagem clara e esclarece os procedimentos a que os sujeitos serão submetidos, porém o número de telefone do CEP está incorreto. O cronograma apresentado é adequado, com início previsto em setembro de 2014 e conclusão em 2,5 anos. O orçamento foi estimado em R$ 140.000,00, e será financiado com recursos próprios.

Considerações sobre os Termos de apresentação obrigatória:

No processo constam as autorizações pertinentes solicitadas por esse CEP, dentre elas a declaração de cumprimento da Resolução 466, a autorização do chefe do departamento de Microbiologia e Imunologia do IB e o termo de entrega do relatório final de atividades.

Recomendações:

-Corrigir os telefones do CEP para (14) 3880-1608 ou 3880-1609.

Conclusões ou Pendências e Lista de Inadequações:

Parecer favorável à aprovação, sem necessidade de envio à CONEP.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Projeto de pesquisa APROVADO, deliberado em reunião do CEP de 04 de agosto de 2.014, sem necessidade de envio à CONEP.

Solicitamos aos pesquisadores que apresentem ao CEP, o respectivo "Relatório Final de Atividades" ao final do estudo.

OBS: O CEP solicita aos pesquisadores que antes de aplicar o TCLE aos participantes da pesquisa, deverá ser corrigido o número de telefone do CEP para 3880-1608 e 3880-1609

BOTUCATU, 04 de Agosto de 2014

Assinado por:
SILVANA ANDREA MOLINA LIMA
(Coordenador)

Endereço: Chácara Butignolli , s/n
Bairro: Rubião Junior **CEP:** 18.618-970
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2. Termo de Consentimento Livre e Esclarecido

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

CONSENTIMENTO LIVRE E ESCLARECIDO

Convido o Senhor(a),_____,
RG nº_____, **telefone (opcional):** _____, **residente à**_____
_____
_____, **a participar do projeto de pesquisa** "Alterações transcricionais em células dendríticas e células T $CD4^+$ humanas em resposta ao *Paracoccidioides brasiliensis*". **Entendi que os pesquisadores querem testar o comportamento de células de defesa do corpo em resposta a um fungo causador de uma micose bastante grave que atinge além da pele e mucosas, alguns órgãos internos, como o pulmão. Entendi também, que para a realização desse estudo é necessário usar 50 ml do meu sangue, pois essas células serão isoladas desse sangue, cultivadas no laboratório e posteriormente estudadas.** Concordando em participar, estou ciente de que apenas as células de defesa chamadas de células brancas do meu sangue, serão usadas para a realização dessa pesquisa. Compreendi que a retirada do meu sangue pode resultar em dor local e a formação de uma mancha roxa ocasionada pela picada da agulha, que não haverá necessidade de alterar a quantidade de sangue a ser doado e que o meu nome não aparecerá quando os resultados da pesquisa forem divulgados. **Entendi também que eu não vou ser diretamente beneficiado pelos resultados, mas que as pessoas que tem essa micose poderão ser beneficiadas no futuro.**

O trabalho será desenvolvido pelo doutando **Reginaldo Keller Fernandes**, sob orientação da Prof. Titular Ângela Maria Victoriano de Campos Soares e coorientação do Prof. Livre Docente João Pessoa Araújo Júnior, ambos do Departamento de Microbiologia e Imunologia do Instituto de Biociências de Botucatu.

Assim considero-me esclarecido(a) e concordo em colaborar com o desenvolvimento do projeto, permitindo o uso das minhas células. Afirmo não ter sido pressionado(a) física ou psicologicamente para colaborar com a pesquisa ou assinar o presente termo, estando ciente que os responsáveis por este trabalho estarão disponíveis para responder a quaisquer perguntas ou dúvidas no

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

Departamento de Microbiologia e Imunologia. O não consentimento da minha parte não interferirá na qualidade do atendimento de minha saúde em qualquer setor do HC-FMB/UNESP.

Observação: O Senhor(a) receberá uma cópia deste Termo de Consentimento Livre e Esclarecido. Dúvidas adicionais poderá ser obtida no Comitê de Ética em Pesquisa por meio do telefone 3811 61 43.

Botucatu, _____ de _____ de _____.

Doador

Prof. Dr. Ângela M. V. Campos Soares
Depto. Microbiologia e Imunologia
Instituto de Biociências de Botucatu
UNESP
Orientadora

Reginaldo Keller Fernandes
Depto. Microbiologia e Imunologia
Instituto de Biociências de Botucatu
Pesquisador Responsável

Prof. Dr. João Pessoa Araújo Júnior
Depto. Microbiologia e Imunologia
Instituto de Biociências de Botucatu
UNESP
Coorientador

3. Comprovante de submissão do paper I

Cytokine
04:47

Cytokine Submission - Manuscript Number Assigned
Para: regiskeller@msn.com; acsoares@ibb.unesp.br

Ms. No.: CYTO-17-29
Title: Dendritic Cells Challenged with Paracoccidioides brasiliensis Promote Treg Cell Response.
Corresponding Author: Mr. Reginaldo Keller Fernandes
Authors: Daniela R Rodrigues, Doctor; Graziela G Romagnoli, Doctor; Ivy R Vieira, M.D; Ramon Kaneno, Doctor; Ângela M Soares, Doctor

Dear Mr. Fernandes,

Your submission, referenced above, has been assigned the following manuscript number: CYTO-17-29

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