Texto sistematizado elaborado de forma crítica

CARLA PAGLIARI

**Contribuições ao estudo da resposta imune tegumentar
em infecções fúngicas**

Texto sistematizado elaborado de forma crítica, apresentado ao Instituto de Medicina Tropical de São Paulo para concorrer ao título de Livre-Docente da Universidade de São Paulo junto ao Programa na Área de Micologia.

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Resumo

Pagliari C. Contribuições ao estudo da resposta imune tegumentar em infecções fúngicas [texto sistematizado]. São Paulo. Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo, 2017.

No presente trabalho foram apresentados e discutidos 18 artigos publicados entre 2002 e 2017 sobre os temas Paracoccidioidomicose, Cromoblastomicose e Doença de Jorge Lobo, a fim de caracterizar alguns fatores importantes na resposta imune do hospedeiro e elementos envolvidos em sua patogenia. Nossos resultados demonstram que os dendrócitos dérmicos Fator XIIIa⁺ participam da resposta imune tegumentar na paracoccidioidomicose como células apresentadoras de antígenos e/ou célula-alvo para a infecção fúngica. As células de Langerhans na Paracoccidioidomicose e Doença de Jorge Lobo têm alterações tróficas e estão diminuídas em número, que resultariam de ação lesiva do fungo. Células CD207⁺ nos epitélios das lesões e na derme/lâmina própria dos tegumentos podem ser interpretadas como células de Langerhans no processo de migração ou uma nova população de células dendríticas dos tegumentos. As células dendríticas plasmocitoides participam da resposta tecidual da Paracoccidioidomicose e Cromoblastomicose, entretanto são ausentes nas lesões da Doença de Jorge Lobo. O perfil de citocinas na Paracoccidioidomicose é concordante com a melhor/pior organização da resposta tecidual. Na Cromoblastomicose o perfil de citocinas tem relação com o tipo de lesão clínica observada: lesões em placa atrófica e perfil Th1; lesões verrucosas e perfil Th2. Na doença de Jorge Lobo o perfil de citocinas reflete a natureza espectral das lesões: lesões nodulares e perfil Th1; lesões infiltrativas e perfil Th2. Há participação de células com expressão de IL17 na Paracoccidioidomicose, inclusive em localização perivascular, o que poderia ser fator ativador do endotélio. A elevada expressão de IL17 na cromoblastomicose e Doença de Jorge Lobo poderia representar uma tentativa do sistema imune em eliminar a infecção fúngica. Linfócitos TCD8⁺ possuem função citotóxica nas três infecções, podendo representar um importante mecanismo no controle da infecção. Linfócitos T reguladores Foxp3⁺ parecem participar de mecanismos moduladores na Paracoccidioidomicose e se correlacionam às diferentes respostas granulomatosas,

sendo maior nos granulomas compactos. Na Cromoblastomicose e na Doença de Jorge Lobo, os mecanismos reguladores mediados por linfócitos T Foxp3⁺ estão diminuídos, podendo significar um desequilíbrio na resposta imune, com exacerbação da resposta inflamatória evidenciada. Os mastócitos, avaliados na Paracoccidioidomicose, participam da resposta imune local nas lesões com granulomas pouco organizados. Os queratinócitos são parasitados pelo *Paracoccidioides sp*, o que pode representar um possível processo de evasão do fungo dos mecanismos imunes locais. Na Doença de Jorge Lobo, há disfunção microvascular que reflete na progressão clínica da doença.

Descritores: Paracoccidioidomicose. Cromoblastomicose. Imunopatologia. Imunohistoquímica. Mucosa oral. Pele.

Summary

Pagliari C. Contributions to the study of the tegumentary immune response in fungal infections [systematized text]. São Paulo. Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo, 2017.

In the present work, 18 articles published between 2002 and 2017 on the topics Paracoccidioidomycosis, Chromoblastomycosis and Jorge Lobo's Disease were presented and discussed, in order to characterize some important factors in the immune response of the host and elements involved in its pathogenesis. Our results demonstrate that dermal dendrocytes Factor XIIIa + participate in the integumentary immune response in paracoccidioidomycosis as antigen presenting cells and / or target cell for fungal infection. The Langerhans cells in Paracoccidioidomycosis and Jorge Lobo's disease have trophic alterations and are diminished in number, which would result from the harmful action of the fungus. CD207 + cells in the lesion epithelia and in the dermis / lamina propria of the integuments can be interpreted as Langerhans cells in the migration process or a new dendritic cell population of the integuments. The plasmacytoid dendritic cells participate in the tissue response of Paracoccidioidomycosis and Chromoblastomycosis, but are absent in the lesions of Jorge Lobo's Disease. The cytokine profile in Paracoccidioidomycosis agrees with the best / worst organization of the tissue response. In Chromoblastomycosis the cytokine profile is related to the type of clinical lesion observed: lesions in atrophic plaque and Th1 profile; Verrucous lesions and Th2 profile. In Jorge Lobo's disease the cytokine profile reflects the spectral nature of the lesions: nodular lesions and Th1 profile; Infiltrative lesions and Th2 profile. There is participation of cells with IL17 expression in Paracoccidioidomycosis, including perivascular localization, which could be an endothelial activating factor. The high expression of IL17 in chromoblastomycosis and Jorge Lobo's disease could represent an attempt by the immune system to eliminate fungal infection. TCD8 + lymphocytes have cytotoxic function in all three infections, and may represent an important mechanism in infection control. Foxp3 + regulatory T lymphocytes appear to participate in modulatory mechanisms in Paracoccidioidomycosis and correlate to different granulomatous responses, being

greater in compact granulomas. In Chromoblastomycosis and in Jorge Lobo's disease, the regulatory mechanisms mediated by Foxp3 + T lymphocytes are diminished, which suggest an imbalance in the immune response, with exacerbation of the inflammatory response evidenced. Mast cells, evaluated in Paracoccidioidomycosis, participate in the local immune response in lesions with poorly organized granulomas. Keratinocytes are parasitized by *Paracoccidioides* sp, which may represent a possible fungus evasion process of the local immune mechanisms. In Jorge Lobo's disease, there is microvascular dysfunction that reflects on the clinical progression of the disease.

Descriptors: Paracoccidioidomycosis. Chromoblastomycosis. Immunopathology. Immunohistochemistry. Oral mucosa. Skin.

Lista de abreviaturas, siglas e símbolos

AMP	Peptídeos antimicrobianos
APC	Célula apresentadora de antígenos
CD	<i>Cluster of differentiation</i>
CTLA-4	<i>Cytotoxic T-Lymphocyte Associated Protein 4</i>
CXCR2	<i>C-X-C chemokine receptor type 2</i>
DD	Dendrócito dérmico
DJL	Doença de Jorge Lobo
<i>F. pedrosoi</i>	<i>Fonsecaea pedrosoi</i>
Foxp3	Forkhead box P3
FXIIIa	Fator XIIIa
GM-CSF	Fator estimulante de colônias de granulócitos e monócitos
HCFMUSP	Hospital das Clínicas da Faculdade de Medicina da USP
HLA-DR	Antígeno leucocitário humano
IDO	Indoleamina 2,3-dioxigenase-1
IFN	Interferon
IL	Interleucina
ILC	Células linfoides inatas
iNOS	Óxido nítrico sintetase indutível
<i>L. loboi</i>	<i>Lacazia loboi</i>
M1	Macrófagos do tipo 1
M17	Macrófagos do tipo 17
M2	Macrófagos do tipo 2
MHC	Complexo principal de histocompatibilidade
MMP	Metaloproteinase
NK	Natural killer
<i>P. brasiliensis</i>	<i>Paracoccidioides brasiliensis</i>
<i>P. lutzii</i>	<i>Paracoccidioides lutzii</i>
PAMPs	Padrões moleculares associados a patógenos
PCM	Paracoccidioidomicose
RBP-J	<i>Recombination signal binding protein for immunoglobulin kappa J region</i>

ROR γ t	<i>Retinoic acid-related orphan receptor</i>
SALT	Tecido linfoide associado à pele
SIS	Sistema imune da pele
STAT-1	Transdutores de Sinal e Ativadores da Transcrição - 1
TGF β	Fator transformador de crescimento
Th1	Linfócitos T auxiliaries do tipo 1
Th17	Linfócitos T auxiliaries do tipo 17
Th2	Linfócitos T auxiliaries do tipo 2
TLR	Receptor do tipo <i>toll</i>
TNF α	Fator de necrose tumoral alfa
Treg	Linfócitos T reguladores

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1 INTRODUÇÃO

1.1 A pele como órgão imune

Para a adequada compreensão dos temas abordados nesse texto sistematizado, é importante contextualizar a pele enquanto órgão imune. Desta forma, a seguir será feita revisão sobre os elementos constituintes da pele que participam dos eventos imunológicos locais. Na sequência, serão discutidos os resultados das pesquisas feitas ao longo de 18 anos e que resultaram em artigos científicos publicados.

A pele já há algum tempo deixou de ser considerada apenas como uma estrutura de interface entre os órgãos internos e o meio ambiente. Além de atuar como barreira mecânica para agentes físicos, químicos e micro-organismos, atua também como um órgão com capacidade imunológica, mediada por seus elementos peculiares¹. Seus componentes constitutivos têm a capacidade de interagir com aqueles da imunidade inata e adaptativa, criando uma rede de íntimo contato, induzindo a mecanismos efetivos contra uma série de agentes. Por outro lado, um desequilíbrio nessa inter-relação contribui para a resposta imune menos efetiva.

A epiderme e os anexos cutâneos são de origem epitelial. As células mais numerosas da epiderme são os queratinócitos, seguidos pelas células de Langerhans, que são de origem mielomonocítica. A derme é constituída por elementos de origem mesenquimal, como os fibroblastos, matriz extracelular, elementos vasculares sanguíneos e linfáticos e células imunes de origem mieloide e linfoide.

O termo SALT (*Skin Associated Lymphoid Tissue*), proposto por Streilen¹ inclui as células de Langerhans, linfócitos T, queratinócitos e linfonodos de drenagem da pele. O termo SIS (*Skin Immune System*), sugerido por Bos e Kapsenberg em 1986 inclui as células dendríticas apresentadoras de antígenos, queratinócitos, macrófagos, neutrófilos, mastócitos, células endoteliais vasculares e linfáticas, fibroblastos e glândulas sudoríparas².

Fazem parte da resposta imune inata os queratinócitos, células endoteliais, macrófagos, células de Langerhans e células dendríticas dérmicas. A resposta imune adaptativa é composta pelos linfócitos T de memória, linfócitos B e seus produtos³.

Será dado destaque a alguns desses elementos celulares e seus produtos, por terem sido estudados em maior profundidade por nosso grupo de pesquisa no contexto das infecções fúngicas. Entre estas darei maior enfoque, por sua importância no âmbito da Dermatologia Tropical, a **Paracoccidioidomicose**, **Cromoblastomicose** e **Doença de Jorge Lobo**.

1.1.1 Queratinócitos

Os queratinócitos são células de revestimento com capacidade de fagocitar e apresentar antígenos⁴⁻⁶. Esses elementos celulares epiteliais têm a capacidade de induzir a expressão de citocinas pelos linfócitos TCD4+ e TCD8+, tendo, portanto função nos processos inflamatórios cutâneos⁷.

Enquanto mantenedores da barreira mecânica e funcional da epiderme, apresentam junções intercelulares (*tight junctions*) que permitem a organização do estrato córneo. Possuem a capacidade de distinguir entre agentes próprios e patogênicos. Os queratinócitos possuem mecanismos de reconhecimento através de receptores dos padrões moleculares associados a patógenos (PAMPs). Entre esses, há os *Toll-like receptors* (TLR) que ao se ligarem aos patógenos ativam a imunidade inata e adaptativa. Os queratinócitos expressam de modo constitutivo TLR1, 2, 3, 4, 5, 6 e 9⁸.

Essas células são produtoras de peptídeos antimicrobianos (AMPs) quando há alguma lesão, induzindo a morte de patógenos e ativação de células imunes com produção de citocinas. Os AMPs produzidos pelos queratinócitos são as defensinas e catelicidinas⁹.

É interessante notar que citocinas de perfil Th17 potencializam a expressão de AMPs pelos queratinócitos, evidenciando a inter-relação entre essas células e a imunidade adaptativa¹⁰.

Além dos receptores citados, os queratinócitos são também produtores de algumas citocinas importantes no desencadeamento de respostas imunes, entre elas a interleucina (IL) 1 e o fator de necrose tumoral alfa (TNF- α)¹¹.

1.1.2 Células de Langerhans

As células de Langerhans são as células dendríticas clássicas da pele, mucosa oral e nasal, distribuídas ao longo da epiderme e epitélio¹²⁻¹⁵. Tais células têm localização suprabasal e constituem 2-8% do conteúdo intraepitelial. Foram descritas em 1868 por Paul Langerhans.

Essas células são derivadas da medula óssea de onde migram para os epitélios para desempenhar sua função de reconhecimento e posterior apresentação de antígenos. São caracterizadas pela expressão da molécula CD1a e pela lectina tipo C denominada langerina (CD207). Esta última é responsável pela formação do grânulo de Birbeck, constituinte exclusivo dessas células¹⁶. Há duas hipóteses para explicar a origem desses grânulos. Eles poderiam ser derivados do complexo de Golgi e migrar para a membrana celular com provável função secretora de seu conteúdo para o exterior das células, ou surgiriam pela invaginação da membrana citoplasmática se constituindo numa organela endocítica¹⁷.

As células de Langerhans interagem com patógenos via receptores de reconhecimento de patógenos (PRR) que se ligam a padrões conservados de carboidratos, lipídeos, proteínas e ácidos nucleicos dos agentes infecciosos. Subsequentemente migram para os linfonodos de drenagem onde entram em contato com células T apresentando os antígenos via complexo principal de histocompatibilidade (MHC) de classe II.

O contato da pele e mucosas com antígenos estimula a liberação de citocinas e de metaloproteinases (MMP) 2 e 9. A IL1 β estimula células dendríticas e queratinócitos a secretarem TNF- α , que por sua vez, estimula maior migração de células dendríticas. A ação das MMP2 e MMP9 sobre a membrana basal permite a passagem de células dendríticas e clivagem de colágeno tipo IV da junção dermoepidérmica¹⁸.

1.1.3 Células dendríticas dérmicas

O termo dendrócito dérmico (DD) foi proposto por Headington em 1986¹⁹ e constitui um grupo de células que expressam as moléculas Fator XIIIa, MHC II e CD36^{20,21}. Além disso, são CD14+, expressam *Dendritic Cell-Specific ICAM-Grabbing Non-integrin* (DC-SIGN) e CD163²².

Em trabalho de 2008, Ochoa *et al.*³ comentam que as células dendríticas dérmicas têm características funcionais de macrófagos e de células dendríticas, como fagocitose e apresentação de antígenos.

De acordo com Zaba *et al.*^{23,24}, esse fenótipo permitiria aos dendrócitos dérmicos Fator XIIIa+ serem classificados como macrófagos da pele ao invés de células dendríticas. Enfatizam que a derme não inflamada contém mais macrófagos que células dendríticas que expressam CD163. Segundo esses autores, tais células não co-expressam marcadores de células dendríticas, como as moléculas CD1c e CD11c.

Células dendríticas dérmicas podem ser divididas entre as que expressam CD14, mas não expressam CD1a e uma população maior, caracterizada pela expressão de CD1a mas não CD14²⁵⁻²⁷.

As células dendríticas dérmicas parecem expressar pouco a molécula CD207 (langerina) e não foram feitos muitos estudos para melhor explorá-las. As células dendríticas dérmicas que expressam CD207 representariam células de Langerhans em trânsito para os linfonodos²⁸. Entretanto, metodologias mais recentes permitiram inferir-se que as células langerina+ dérmicas poderiam ao menos em parte ser outra população celular, distinta das células de Langerhans^{15,29-31}.

1.1.4 Células dendríticas plasmocitoides

As células dendríticas plasmocitoides (pDC) são mediadoras das respostas imunes contra vírus, uma vez que produzem interferon do tipo I (IFN-I). Estão ausentes ou em número restrito na pele não inflamada, porém são observadas nos processos inflamatórios cutâneos como o lúpus eritematoso sistêmico e psoríase. As pDC são também importantes nas respostas anti-tumores e auto-imunes³².

As pDC são caracterizadas pela expressão das moléculas CD123 e CD303 (BDCA-2), cujos anticorpos específicos possibilitam sua visualização à imuno-histoquímica³³.

Além da produção de IFN-I, as células dendríticas plasmocitoides expressam moléculas de MHC e outras co-estimulatórias, capazes de apresentar antígenos virais a

linfócitos TCD4⁺ e efetuar apresentação cruzada a linfócitos TCD8⁺³⁴. São também fonte de quimiocinas pró-inflamatórias que ativam linfócitos T CD4 e CD8 aos locais de infecção³⁵. Secretam IL-12, contribuindo para a polarização de linfócitos para o perfil de citocinas Th1^{36,37}.

Estudo experimental recente evidenciou seu papel imunomodulador na paracoccidiodomicose pulmonar. Estudos *in vitro* mostram que o *Paracoccidioides brasiliensis* induz a maturação das pDC. Na infecção *in vivo* há afluxo dessas células para os pulmões e aumento de IFN- γ . Sua depleção reduz a gravidade da doença, dano tecidual e consequente maior sobrevivência dos animais infectados. Nesse quadro de depleção, há aumento no afluxo de macrófagos, neutrófilos e linfócitos expressando IFN- γ e IL17. Na infecção fúngica há aumento de pDC que expressam IDO (indoleamine 2,3-dioxygenase-1), que tem propriedades imunorreguladoras. Os resultados desse trabalho apontam para a função tolerogênica das pDC na infecção pulmonar pelo *Paracoccidioides brasiliensis*, mediada pela expressão de IDO, que aumenta a atividade T reguladora³⁸.

1.1.5 Macrófagos, mastócitos, linfócitos e citocinas

A dinâmica de monócitos e macrófagos na pele não está totalmente investigada. A caracterização dos padrões que determinam a diferenciação dos macrófagos específicos nos tecidos poderia favorecer a identificação de alvos terapêuticos³⁹. Com base em sua função, monócitos e macrófagos são classificados em macrófagos M1 (pró-inflamatórios), M2 (reguladores) e M17. Entretanto, outras possíveis populações vêm sendo exploradas.

Os macrófagos M1 participam nos processos inflamatórios agudos e crônicos da pele. Considerados, portanto como pró-inflamatórios, expressam citocinas pró-inflamatórias como IFN- γ e TNF- α , óxido nítrico sintetase indutível (iNOS) e moléculas MHC de classe II que são importantes para a morte de patógenos intracelulares.

Os macrófagos M2 apresentam um perfil imunomodulador com a diminuição na expressão das moléculas citadas acima. São identificados pela expressão de outras citocinas (IL4 e IL13) e uma ampla variedade de marcadores, tais como a arginase-1 e o receptor de

manose (CD206), sendo importantes na resposta imune contra parasitas. A população M2 parece estar relacionada à evolução para a cronicidade das doenças infecciosas⁴⁰.

Na resposta imune frente a diversos vírus, bactérias, fungos e protozoários sabe-se que macrófagos ativados consomem L-arginina, e a expressão de enzimas que degradam essa proteína é regulada por citocinas. A expressão de arginase-1 é induzida por IL4, IL10 e IL13^{41,42}. Na leishmaniose, por exemplo, verifica-se que o perfil de resistência do hospedeiro é caracterizado pela expressão de iNOS induzido por citocinas de perfil Th1, enquanto a susceptibilidade está relacionada à maior expressão de arginase, induzida por citocinas Th2^{43,44}.

Nos últimos anos, a expressão de arginase e consequente depleção de L-arginina tem sido considerada como importante fator de imunossupressão⁴⁵.

A molécula STAT-1 (*signal transducer and activator of transcription 1*) em sua forma fosforilada (pSTAT-1) liga-se a regiões promotoras de genes estimulados por IFN. STAT-1 aumenta em resposta aos IFN dos tipos I, II e III. cMAF é fator de transcrição essencial para a expressão gênica de IL10 pelos macrófagos.

A caracterização da polarização de macrófagos *in situ* foi realizada em trabalho recente, através da demonstração por técnica de imuno-histoquímica de dupla-marcação dos fatores de transcrição relacionados a essa polarização. Utilizou-se os marcadores CD68 e CD163 em associação ao pSTAT-1, RBP-J (M1) e cMAF (M2)⁴⁶.

Tatano *et al.*⁴⁷ demonstraram na infecção por micobactérias a presença de uma população de macrófagos distinta de M1 e M2. Essa população macrofágica é induzida pela expansão de Th17, e obviamente, esses autores os denominou macrófagos M17. Os M17 são funcionalmente distintos de M1 e M2 e seu fenótipo é assim caracterizado: IL12+, IL1 β ^{high}, IL6+, TNF- α +, NOS2+, CCR7^{high}, IL10^{high}, arginase-, receptor de manose^{low}, Ym1^{high}, Fizz^{low} e CD163^{high}.

Os **mastócitos** são células especializadas na vigilância imune da pele, uma vez que possuem mediadores pró-inflamatórios estocados em seus grânulos citoplasmáticos que são rapidamente liberados após contato com antígenos. Semelhante aos macrófagos, os

mastócitos têm sua plasticidade que lhes faculta a função pró- e anti-inflamatória na pele. Há determinadas condições em que produzem IL2, que estimula a geração de linfócitos T reguladores, e outras em que produzem TNF- α , que estimula a produção de IL1 pelos macrófagos M1.

Na pele normal há cerca de 2×10^{10} **linfócitos T**, praticamente o dobro do número de linfócitos T circulantes⁴⁸. No contexto de imunovigilância, há também os linfócitos T $\gamma\delta$. Tais células têm papel importante na homeostase epitelial e no reparo tecidual⁴⁹.

Os subtipos de células TCD4⁺ Th1 e Th2 são bem definidos e estão relacionados aos mecanismos de resposta imune celular e humoral, respectivamente. O IFN- γ é a principal citocina produzida pelas células Th1 e, juntamente com a IL12, estimula a diferenciação para o fenótipo Th1 ativando os fatores de transcrição STAT1 e T-bet (*T cell specific T-box transcription factor*) (via IFN- γ), e STAT4 (via IL-12). O perfil Th2 tem como características as citocinas IL4, IL5, IL13 e IL33. A IL4 é fundamental para estimular esse perfil ao ativar o fator de transcrição STAT6, que em conjunto com os sinais de receptores de células T, induz a expressão do fator de transcrição de GATA3 (*GATA binding protein 3*), que regula a diferenciação do perfil Th2⁵⁰.

Entre as células que abordaremos estão ainda as células de perfil de citocinas Th17. Essas células têm ocupado papel de destaque nas infecções fúngicas e têm como citocinas características a IL17 e IL22. Essas células são distintas das células de perfil Th1 e Th2 e necessitam de ambiente citocínico e fatores de transcrição específicos para sua diferenciação, como IL6, TGF- β , IL23 e ROR γ t.

As células Th17 têm sido descritas em diferentes doenças inflamatórias crônicas e verifica-se que fibroblastos, células endoteliais e epiteliais são alvo de IL17. Além das células T, as células *natural killer* (NK), mastócitos e neutrófilos também expressam IL17⁵¹.

Outra população importante de linfócitos são os T reguladores (Treg) CD4⁺CD25⁺, pelo seu papel na regulação da resposta imune e diminuição dos efeitos exacerbados e consequente dano tecidual. Um marcador específico para essa população celular é o Foxp3 (*Forkhead box P3*). Os linfócitos Treg atuam na inibição da proliferação de células T e células

apresentadoras de antígenos (APC), pelo contato célula-célula através de CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), ou pela produção de citocinas, como TGF- β , IL10 e IL35⁵².

Um outro grupo de células imunes na pele são as chamadas células linfoides inatas (ILC). São células produtoras de citocinas e coordenam imunidade e inflamação nos tecidos, como a pele. São divididas em três grupos: grupo 1 – ILCs e NK, grupo 2 – ILC2 e grupo 3 – ILC3 e células indutoras de tecido linfóide. As ILC2 têm papel na indução de resposta Th2 e produzem IL13, que diminui a função dos mastócitos *in vitro*. As ILC3 são as principais entre as ILCs na pele. Por exemplo, na psoríase, são fundamentais na formação das placas⁵³.

1.2 PARACOCOCCIDIOIDOMICOSE

1.2.1 A paracoccidioidomicose e seus agentes

A Paracoccidioidomicose (PCM) é a micose sistêmica mais prevalente na América Latina, de elevada incidência no Brasil, seguido por Colômbia e Venezuela⁵⁴. Essa doença foi descrita pela primeira vez em 1908 por Adolfo Lutz, ao estudar lesões de mucosa oral.

A PCM constitui-se em sério problema em saúde pública uma vez que acomete principalmente homens na fase produtiva (entre 30 e 60 anos) e é causa frequente de morbidade e incapacitação para o trabalho⁵⁵.

O agente etiológico originalmente descrito relacionado à PCM é o fungo dimórfico *Paracoccidioides brasiliensis*. Entretanto, estudos recentes têm descrito nas regiões centro-oeste e norte do Brasil o isolado Pb01-“like”, que constitui a espécie denominada *Paracoccidioides lutzii*⁵⁶.

O *Paracoccidioides sp* é um agente termo-dependente, que se apresenta sob a forma miceliana a 25 °C e como célula leveduriforme a 37 °C *in vitro* e em tecidos. A temperatura parece ser o único fator determinante dessa transformação. A forma miceliana é caracterizada por hifas septadas que dão origem a clamidósporos terminais ou intercalares. Produz conídios intercalantes que germinam e formam células leveduriformes semelhantes às teciduais.

A infecção ocorre pela inalação de conídios infectantes. As leveduras possuem forma arredondada ou oval, são multinucleadas, com parede celular espessa rodeada por esporulações múltiplas (“roda-de-leme”). Essa fase é conhecida como parasitária, por ser a forma tecidual.

Além do hospedeiro humano, o *Paracoccidioides sp* infecta tatus, frequentemente encontrados em áreas hiperendêmicas⁵⁷.

A composição genética do *P. brasiliensis* tem sido estudada em detalhes e sabe-se que o genoma nuclear tem cerca de 30 Mb, com cerca de 7500-9000 genes presentes. O projeto genoma funcional e diferencial do fungo engloba 11 instituições de pesquisa e acadêmicas no Brasil.

Estudos recentes abrangendo a análise de variabilidade genética permitiram a divisão em três grupos filogenéticos: S1, grupo parafilético com 38 isolados na Argentina, Brasil, Peru e Venezuela e um na Antártida, isolado de pinguim; PS2, grupo monofilético com cinco isolados do Brasil e um da Venezuela e PS3, grupo monofilético composto por 21 isolados da Colômbia⁵⁸.

A distribuição geográfica da paracoccidioidomicose restringe-se às Américas. Essa micose ocorre em regiões tropicais e subtropicais da América do Sul e Central, principalmente no Brasil, Colômbia, Venezuela e Argentina. A distribuição é heterogênea, com áreas de alta endemicidade próximas a áreas de baixa incidência. O termo “reservárea” diz respeito à área onde o indivíduo adquiriu a paracoccidioidomicose. É considerada como pertencente à região tropical ou subtropical, com temperatura variando de 10 °C a 28 °C e índices pluviométricos variando entre 500 e 2500 mm/ano.

A paracoccidioidomicose não é doença de notificação compulsória e os dados de incidência, prevalência e mortalidade são baseados em inquéritos epidemiológicos. De acordo com o consenso de 2006, há de 1 a 4 casos novos /milhão até 1 a 3 casos novos/ 100 mil habitantes ao ano, em áreas endêmicas. As investigações epidemiológicas no Brasil mostram elevados índices de mortalidade na região Sudeste (São Paulo, Minas Gerais e Rio de Janeiro) e região Sul (Paraná e Rio Grande do Sul)⁵⁹.

Uma vez em contato com o fungo, se estabelece o complexo primário e a doença pode regredir ou evoluir. A disseminação hematogênica favorece a constituição de focos metastáticos e as manifestações sintomáticas originam diferentes formas clínicas classificadas como: forma aguda, subaguda, crônica, unifocal, multifocal, residual ou sequelar. Essas e outras características foram discutidas no Consenso de paracoccidioidomicose, que envolveu pesquisadores de diferentes grupos⁶⁰.

Forma aguda/subaguda (tipo juvenil) – predomínio em crianças e adolescentes, semelhante nos gêneros masculino e feminino, evolução rápida. Afeta órgãos principalmente do sistema fagocítico-mononuclear (fígado, baço e medula óssea). Os pacientes mostram altos títulos de anticorpos, depressão da resposta imune celular e os granulomas da resposta tecidual são exsudativos com grande número de fungos.

Forma crônica (tipo adulto) – predomínio em adultos, em sua maioria do sexo masculino, de instalação lenta. Manifestações pulmonares são as mais frequentes. Na forma unifocal ocorrem sinais e sintomas em um único órgão ou sistema, como pulmões, orofaringe, pele, supra-renais e sistema nervoso central. Na forma multifocal, o comprometimento é em mais de um órgão ou sistema.

Formas residuais ou sequelas – caracterizadas por aspectos cicatriciais de lesões pregressas, que se seguem ao tratamento.

De extrema importância também é a forma oportunística, associada ao imunocomprometimento verificado em pacientes com câncer, síndrome da imunodeficiência adquirida (Aids) ou transplantados de órgãos. Há registros de cerca de 200 casos no Brasil.

A paracoccidioidomicose em pacientes com Aids é caracterizada por febre prolongada, emagrecimento importante, comprometimento das funções pulmonares, linfadenopatia, hepatomegalia, esplenomegalia, comprometimento neurológico e cutâneo.

O diagnóstico da paracoccidioidomicose pode ser feito pela demonstração do agente a partir de material obtido a fresco por exame micológico direto ou exame histológico, pelo isolamento do fungo em meio de cultura, ou por provas sorológicas, como imunodifusão

dupla, contraímunoeletroforese, imunofluorescência indireta, ELISA e imunoblot. O exame anátomo-patológico permite a visualização do fungo em colorações de rotina (Hematoxilina-Eosina, Grocott ou ácido periódico de Schiff), ou mais especificamente por método imuno-histoquímico, que além de detectar formas fúngicas íntegras, permite a visualização de antígenos.

1.2.2 Resposta imune na paracoccidioidomicose

O *Paracoccidioides sp* é constituído por proteínas, glicoproteínas, polissacarídeos e lipídeos com características antigênicas⁶¹. Esses elementos são reconhecidos pelos constituintes da resposta imune inata: monócitos, macrófagos, células dendríticas, neutrófilos e células NK. Alguns receptores, como o *toll like receptor* (TLR) 2 e TLR4, receptor de manose e dectina 1, quando estimulados por antígenos fúngicos, induzem a produção de citocinas pró-inflamatórias⁶².

As citocinas possuem papel determinante na regulação da resposta imune contra diversos agentes infecciosos. Muitos trabalhos têm avaliado seu papel como mediadoras da resposta imune na PCM. O TNF- α é um fator mediador da inflamação e resposta imune celular. Sua principal função é ativação de monócitos e macrófagos, a diferenciação destes em células epitelióides e manutenção do granuloma. Esse mecanismo é necessário para inibir a replicação do fungo na PCM⁶³.

O interferon gama (IFN- γ) é produzido por todos os linfócitos T CD8+ e por linfócitos do subtipo Th0 e Th1 (CD4+) após estimulação antigênica. Também é produzido por células NK. Estudos *in vitro* mostram que macrófagos ativados por IFN- γ têm ação fungicida para formas em levedura do *P. brasiliensis*. Os macrófagos não ativados, por outro lado, são destruídos após infecção, permitindo a liberação de leveduras⁵⁵.

Em pacientes com a forma crônica, altos níveis de IL10 e TNF- α e baixos níveis de IFN- γ foram relacionados à progressão da doença^{64,65}. Silva *et al.*⁶⁶ ao comparar os níveis de citocinas séricas em doentes com as formas crônica e disseminada verificaram que os doentes com a forma disseminada apresentam maiores níveis de IL1, IL6 e TNF- α e produção aumentada de IL2 em pacientes crônicos. Cano *et al.*⁶⁷ verificaram, por trabalho

experimental *in vivo*, que o IFN- γ tem papel crucial na contenção da infecção pelo *P. brasiliensis*. A sua depleção induziu a disseminação do fungo a partir do foco pulmonar. Entretanto, IFN- γ foi detectado em camundongos susceptíveis. De acordo com esses autores, os níveis dessa citocina no local da infecção não determinam o fenótipo de resistência. A quantidade de IL4, IL5, IL10 ou TGF- β é que poderia estar relacionada à susceptibilidade.

A expressão de TGF- β é necessária para o início da diferenciação das células Th17^{68,69}. Esse fator foi previamente relacionado ao desenvolvimento de células Treg Foxp3+. Ambas populações celulares tem sido estudadas na PCM.

As células Treg constituem uma subpopulação de linfócitos T importantes na indução e manutenção da tolerância imunológica e finalização de resposta imune. Uma deficiência ou disfunção dessas células pode levar à autoimunidade ou agravamento da inflamação induzida por patógenos.

Células Th17 constituem uma linhagem de células TCD4+ recentemente descritas. São caracterizadas pela produção de um perfil distinto de citocinas, incluindo IL17 e IL6 e parecem ter se desenvolvido para auxiliar no “*clearance*” de patógenos que não são eficientemente destruídos por linhagens Th1 ou Th2. Seu desenvolvimento necessita de TGF- β , estabelecendo uma ligação entre as Treg e Th17.

A IL17 é considerada um importante fator patogênico das doenças inflamatórias crônicas mediadas por células T. Estudos recentes têm mostrado seu papel na imunidade protetora contra infecções. A resposta imune envolve a indução de neutrófilos para os locais da infecção de patógenos extra-celulares. Essa citocina parece ter papel crucial na imunidade protetora contra fungos. Sua importância foi inicialmente descrita em infecção por *Candida albicans* em 2004⁷⁰. Embora a IL17 participe na imunidade inata contra *C. albicans*, células T de memória do tipo Th17 foram detectadas em humanos, sugerindo sua importância na imunidade adquirida⁷¹.

Uma diminuição de IL17 foi detectada experimentalmente como indicadora de susceptibilidade à infecção por *Cryptococcus neoformans* e a proteção na pneumonia por *Pneumocystis carinii* também parece estar relacionada a essa citocina^{72,73}.

A indução de células Th17 no curso das infecções fúngicas parece depender do reconhecimento de “PAMPs” (padrões moleculares associados a patógenos) fúngicos por dectinas expressas em macrófagos ou células dendríticas. Haveria um desvio para o desenvolvimento de Th17 a partir de uma produção preferencial de IL23⁷⁴.

1.2.3 Resposta tecidual e interação hospedeiro-fungos na paracoccidioidomicose

A reação granulomatosa representa resposta tecidual especializada, eficiente e imune específica contra antígenos do *P. brasiliensis*, visando destruí-lo e bloqueá-lo, impedindo a sua multiplicação e disseminação.

O granuloma paracoccidióidico é formado por células epitelióides e células gigantes multinucleadas, que são numerosas, de tamanho variável, muitas contendo fungos degenerados, em quiescência ou em reprodução ativa por brotamento múltiplo ou simples. São observados granulomas bem organizados, por vezes confluentes com formação de nódulos epitelióides maiores. Em torno dessa reação granulomatosa há fibrose e infiltrado inflamatório constituído por linfócitos, macrófagos, eosinófilos e plasmócitos. É comum também formarem-se granulomas frouxos, pouco organizados, caracterizados por pequenos grupamentos de células epitelióides distribuídas em meio a número variável de células gigantes, edema e exsudação inflamatória de polimorfonucleares e mononucleares. Além disso, pode-se ainda observar fibrose, tecido de granulação, com posterior formação de tecido fibroso cicatricial⁷⁵.

O *P. brasiliensis* é capaz de fixar e ativar o sistema complemento pela via alternativa. Essa ação provoca opsonização e facilita sua fagocitose por macrófagos que são células importantes na defesa. Componentes do sistema complemento podem se fixar e lisar os fungos. Sua presença nos macrófagos exerce função ativadora para quimiotaxia de neutrófilos. Na fase inicial da infecção, células NK têm papel defensivo, há multiplicação fúngica e lise dos macrófagos, com formação de focos de necrose. Uma vez nos alvéolos, há constituição de resposta inflamatória aguda, que leva à destruição dos fungos ou sua entrada no interstício dos septos alveolares.

O mecanismo patogênico associado à resistência, e, portanto melhor resposta do hospedeiro é mediado pela ativação de macrófagos por citocinas Th1 e poder fungicida

aumentado, caracterizando a doença menos grave. Antígenos do *P. brasiliensis* estimulam linfócitos TCD4 que se concentram no sítio inflamatório e produzem citocinas quimiotáticas para monócitos e linfócitos, ativam macrófagos que podem se fundir em células gigantes multinucleadas e favorecem o aumento de colágeno. Esses fatores originam o granuloma e em seguida ocorre o processo de cicatrização. Linfócitos TCD8 também têm função protetora, seja pela produção de citocinas ou ação citotóxica mediada por granzima. No polo de maior susceptibilidade e doença difusa, há predomínio de citocinas Th2 que proporcionam aumento no nível de anticorpos. A resposta tecidual é menos efetiva e há proliferação e disseminação de formas fúngicas^{75,76}.

1.2.4 Lesões de pele na paracoccidioidomicose

As lesões cutâneas na paracoccidioidomicose são frequentes e podem ter sua origem pela disseminação hematogênica a partir do foco primário da infecção (pulmões), a partir de lesões contíguas pré-existentes ou, raramente, da inoculação direta do fungo na pele. Considerando-se que em sua maioria essas lesões constituem foco secundário da infecção, elas seriam um reflexo da resposta imune ineficiente, que não foi capaz de conter a infecção nos pulmões.

As alterações histológicas da pele são caracterizadas por acantose, paraqueratose, hiperqueratose e hiperplasia pseudo-epiteliomatosa da epiderme. O infiltrado dérmico é caracterizado por macrófagos, linfócitos, neutrófilos e, em menor número, plasmócitos e eosinófilos. É comum a presença de microabscessos dérmicos e intraepiteliais, com ou sem a presença de formas fúngicas. A resposta inflamatória na derme é ainda caracterizada pela presença de granulomas que variam de compactos a frouxos, sendo possível a presença de ambos os tipos na mesma lesão.

São poucos os trabalhos que abordam as lesões cutâneas ou de mucosa nasal e oral na paracoccidioidomicose no contexto de resposta imune local⁷⁵⁻⁷⁸. Tem-se observado que uma falha na resposta imune específica mediada por células T pode permitir a multiplicação e disseminação do fungo, incluindo manifestações muco-cutâneas. Por exemplo, demonstrou-se baixo número de macrófagos iNOS+ em lesões orais que podem representar um mecanismo de evasão do parasita para sobreviver dentro dos macrófagos^{78,79}.

Estudos mostram que células dendríticas humanas capturam antígenos via dectina-1 e fazem a apresentação a linfócitos TCD4+ e CD8+ e a ativação celular favorece o desenvolvimento da resposta do tipo Th17⁸⁰.

A dectina-1 é um receptor da resposta imune inata⁸¹ que reconhece β -glucana e desempenha papel importante na resposta contra fungos. Glucana é um dos componentes da parede do *P. brasiliensis*. Em trabalho recente, Loures *et al.*⁶² estudaram esse receptor na paracoccidioidomicose pulmonar experimental. Nesse trabalho observaram a função na ativação da resposta TCD8+ citotóxica e envolvimento na produção de IL17. Além disso, a ausência de dectina-1 favoreceu a prevalência de macrófagos do perfil M2 que têm capacidade fungicida diminuída, assim como diminuição da produção de óxido nítrico.

1.3 CROMOBLASTOMICOSE

A Cromoblastomicose é uma infecção fúngica crônica que acomete a pele e tecido subcutâneo. Originalmente descrita na cidade de São Paulo em 1914, a maioria dos casos ocorre em regiões tropicais e subtropicais, principalmente em trabalhadores rurais⁸². A região amazônica é considerada a principal área endêmica no Brasil⁸³. Essa prevalência parece estar associada a fatores sociais e ambientais que contribuem para a manutenção dos fungos viáveis na natureza, bem como as atividades de trabalho onde indivíduos têm contato frequente com o ambiente aumentando a chance de contato com o agente.

Várias espécies de fungos dematiáceos podem causar a cromoblastomicose, dentre elas: *Fonsecaea pedrosoi*, *Phialophora verrucosa*, *Cladosporium carrionii* entre outros, sendo o primeiro o mais frequentemente identificado em casos do Brasil.

A principal forma de infecção é pela inoculação traumática do fungo na pele. Os membros inferiores são os mais frequentemente acometidos por lesões, seguidos pelos membros superiores e dorso.

Os fungos no tecido apresentam coloração pardacenta e morfologia arredondada, com dupla membrana, formato de lente côncavo-convexa e são encontrados associados ou não a células gigantes, isolados ou em grupos, no centro de microabscessos.

1.3.1 Características clínicas e histológicas das lesões

Em geral a cromoblastomicose inicia-se pela erupção de pápulas, verrugas (planas, crostosas ou papilomatosas) ou nódulos. As lesões têm evolução lenta, porém progressiva, podendo apresentar-se sob a forma de nódulos, placas verrucosas ou placas ulceradas.

A epiderme das lesões exibe hiperqueratose, paraqueratose e acantose. São comuns proliferações epiteliais atípicas. Na derme há vasos sanguíneos neoformados.

A epiderme tem papel ativo na eliminação de alguns fungos. A hiperplasia pseudoepiteliomatosa é considerada um achado importante, embora somente critérios histológicos não sejam suficientes para se concluir o diagnóstico dessa infecção fúngica⁸⁴⁻⁸⁶.

A infiltração dérmica é constituída por plasmócitos, histiócitos, neutrófilos, linfócitos e eosinófilos. O infiltrado inflamatório pode ser difuso ou nodular. Os granulomas podem ser do tipo tuberculoide ou supurativo⁸⁶. Os de tipo tuberculoide associam-se a lesões do tipo placa atrófica e os supurativos às lesões verrucosas⁸⁷.

Resposta tecidual com presença de granulomas mistos também ocorre na cromoblastomicose assim como descrito na paracoccidioidomicose, que parece ser consequência da presença de polimorfonucleares que modificam a resposta imune local⁸⁶.

As lesões verrucosas apresentam principalmente inflamação supurativa, que são reflexo de uma resposta imune celular pouco efetiva. Por isso, são mais graves, de longa duração e de difícil tratamento. Além disso, a presença de melanina na parede dos fungos é fator patogênico que lhes confere proteção contra a resposta imune⁸⁸.

1.3.2 Resposta imune na cromoblastomicose

A imunofenotipagem dos elementos inflamatórios não evidencia diferença no número de linfócitos T CD4+, CD8+, B, macrófagos ou células de Langerhans entre as formas do tipo placa atrófica e verrucosa. O perfil citocínico na forma verrucosa é caracterizado pela expressão de IL10, IL4 e TNF- α , com ausência de IFN- γ (Th2). Na forma tipo placa atrófica evidencia-se IFN- γ e TNF- α principalmente (Th1)⁸⁷.

Em ensaios com células mononucleares do sangue periférico demonstrou-se IFN- γ associado à melhor forma da doença e IL10 à forma mais grave⁸⁹. Sousa *et al.* também evidenciaram a participação da resposta imune celular mediada por linfócitos TCD4+ e produção de citocinas de perfil Th1 no controle da infecção por *F. pedrosoi*^{90,91}.

1.4 DOENÇA DE JORGE LOBO

A Doença de Jorge Lobo (DJL) é uma micose de evolução crônica, granulomatosa, causada pelo fungo *Lacazia loboi*. Manifesta-se geralmente por lesões cutâneas queloidianas, localizadas predominantemente no pavilhão auricular e membros inferiores e superiores. Embora sejam descritos casos em diferentes países na América Latina, o maior número de pacientes pertence à região Amazônica^{92,93}.

O *Lacazia loboi* possui morfologia globosa, unicelular, com frequentes ramificações em cadeia de células conectadas, semelhantes a um colar de contas^{94,95}. Esse fungo é considerado parasita intracelular obrigatório, possui características filogenéticas próximas ao *P. brasiliensis*, possuindo inclusive molécula GP43-“like”, semelhante a este⁹⁶.

Seu habitat natural é o solo, vegetais e água, em florestas tropicais e subtropicais e o acometimento cutâneo ocorre por implantação traumática⁹⁷.

A doença predomina no sexo masculino com maior possibilidade de exposição ao fungo, provavelmente pela atividade de trabalho no meio rural, como a de seringueiros, garimpeiros, caçadores e mateiros⁹⁸. Essa micose foi descrita pela primeira vez em 1931 em Recife, pelo dermatologista brasileiro Jorge Lobo.

A Doença de Jorge Lobo não é de notificação compulsória e é, portanto, considerada negligenciada e de difícil exatidão do número de casos. Estima-se cerca de 322 casos no Brasil^{99,100} e 550 casos no mundo¹⁰¹. Destaca-se a alta prevalência da micose entre a população indígena Caiabi, onde foram descritos 63 casos, sendo 39 homens e 24 mulheres¹⁰².

A infecção pelo *L. loboi* ocorre, além de nos humanos, em diferentes espécies de golfinhos, como *Tursiops truncatus* (na costa da Flórida, Brasil entre outros), *T. aduncus* (Japão) e *Sotalia guianensis* (Suriname e Brasil)¹⁰³⁻¹⁰⁵.

1.4.1 Características clínicas e histológicas das lesões

Clinicamente, as lesões possuem aspecto queloidiano, são isoladas ou confluentes, ou verrucosas, ulceradas, gomosas, esclerodermiformes, maculares e infiltrativas¹⁰⁶.

A disseminação da infecção pode ocorrer por contiguidade, por via linfática ou hematogênica¹⁰⁷.

O aspecto histológico das lesões é caracterizado por atrofia da epiderme. Nos casos verrucosos há hiperplasia epitelial e hiperqueratose. Uma faixa clara (zona grenz), de espessura variável separa a epiderme do infiltrado. A derme caracteriza-se pela presença de vasos dilatados, infiltrado inflamatório granulomatoso, nodular, composto por macrófagos (muitos xantomizados pela presença de polissacarídeos e lipídeos), linfócitos, células gigantes do tipo Langhans e do tipo corpo estranho, alguns eosinófilos e plasmócitos. Corpos asteróides foram relatados presentes no infiltrado granulomatoso nas células multinucleadas tipo Langhans, como é observado em outras doenças granulomatosas¹⁰⁸.

O diagnóstico da DJL é feito pela observação de formas fúngicas nos tecidos e não existe até o momento um tratamento efetivo, sendo importante a remoção cirúrgica com margens ampliadas, a fim de se evitar recidivas^{107,108}.

A diversidade de aspectos das lesões sugere que haja variabilidade na resposta imune desencadeada frente ao fungo. Esse polimorfismo lesional é o mais encontrado no exame clínico, principalmente nos casos de longa evolução. Pode haver pontos enegrecidos na superfície das lesões, correspondendo à eliminação transepitelial do fungo como ocorre em outras micoses profundas, a exemplo da cromoblastomicose^{106,109}.

A patogenia da DJL ainda não é bem esclarecida, não havendo cultivo do agente em laboratório e os experimentos em animais ainda não têm resultados consistentes¹⁰⁸. É muito variável a resposta individual do paciente frente às agressões causadas pelo *L. loboi*. Há

indivíduos em que, num curto período, as lesões evoluem para uma forma mais extensa e disseminada e outros que por muitos anos persistem com a forma localizada da doença¹⁰⁷.

1.4.2 Resposta imune na Doença de Jorge Lobo

Uma deficiência parcial da resposta imune local parece ser fator importante para a susceptibilidade à doença. No infiltrado inflamatório verifica-se a presença de macrófagos, células NK, linfócitos TCD4+, TCD8+, entretanto, com grande número de formas fúngicas¹¹⁰.

O perfil Th2 de citocinas parece ser o mais frequente, tendo como consequência a manutenção da lesão. Há também alto número de células com expressão de TGF- β 1, seguida por IL-10, e uma expressão mínima de TNF- α e iNOS nos pacientes¹¹¹. Com relação à expressão de TGF- β 1, Xavier *et al.*¹¹² sugerem seu papel na inibição da atividade macrofágica e indução de fibrose.

A evolução e extensão das lesões podem formar agregados multiloculares, relacionados com a expressão de TGF- β ¹⁰⁶, citocina importante que desempenha função sobre células CD8+ e diferenciação de células Treg Foxp3+ ou Th17⁵².

Consideramos importante a compreensão dos mecanismos de lesão e resposta imune do hospedeiro na paracoccidioidomicose, cromoblastomicose e Doença de Jorge Lobo, pois são doenças cuja progressão pode levar para um estado de cronicidade e dificuldade de tratamento.

Apesar dos inúmeros avanços na compreensão da interação fungo-hospedeiro, dados da literatura mostram que ainda são necessários muitos esforços para aumentar nossos conhecimentos acerca do papel da resposta imune inata e adaptativa nessas micoses de grande importância no Brasil e outras regiões.

Objetivos

2 OBJETIVOS

O objetivo deste texto sistematizado é descrever e avaliar no contexto patógeno-hospedeiro as pesquisas realizadas em lesões cutâneas de Paracoccidioidomicose, Cromoblastomicose e Doença de Jorge Lobo, fazendo correlações com formas clínicas das lesões, abordando os seguintes aspectos:

- a) Elementos da resposta imune inata: células dendríticas dérmicas, células de Langerhans, macrófagos, mastócitos, queratinócitos;
- b) Elementos da resposta imune adaptativa: linfócitos, perfis de citocinas Th1, Th2 e Th17;
- c) O fenótipo do endotélio nas lesões de Doença de Jorge Lobo.

Resultados comentados

3 RESULTADOS COMENTADOS

3.1 A interação dos Dendrócitos Dérmicos Fator XIIIa+ com formas fúngicas

(Anexo 1) Pagliari C, Sotto MN. Correlation of Factor XIIIa+ dermal dendrocytes with paracoccidioidomycosis skin lesions. Medical Mycology 2002; 40:407–410.

O macrófago é a principal célula parasitada pelo *Paracoccidioides sp.* Entretanto, vários trabalhos têm evidenciado o parasitismo de outros elementos celulares.

Em condições inflamatórias é descrito um número aumentado de dendrócitos dérmicos Fator XIIIa+, mas ausência em processos granulomatosos como sarcoidose e tuberculose¹¹³. Alguns trabalhos evidenciam sua capacidade de internalizar, processar e apresentar antígenos para linfócitos T, com subsequente indução da produção de citocinas de padrão Th1 pelos linfócitos T^{19,114,115}.

Considerando-se a escassez de estudo sobre a atividade dos dendrócitos Fator XIIIa na paracoccidioiomiose¹¹⁶, nosso trabalho de 2002 foi resultado do início dessa linha de pesquisa em nosso grupo. Demonstramos a interação de dendrócitos dérmicos FXIIIa+ com o *Paracoccidioides brasiliensis* em lesões cutâneas da paracoccidioidomiose. Essa abordagem foi feita através de técnica imuno-histoquímica de dupla-marcação, com detecção concomitante dos dendrócitos dérmicos e estruturas fúngicas. Utilizamos casuística composta de 61 biópsias provenientes dos arquivos do Laboratório de Dermatopatologia do Departamento de Dermatologia da FMUSP e para comparação foram utilizadas 10 biópsias de pele sem atividade inflamatória.

A formação do granuloma, específico ao *Paracoccidioides sp.*, reflete a resposta imune local do hospedeiro na tentativa de conter e destruir o patógeno.

A análise histológica dos espécimes nos permitiu classificar as amostras de pele em três grupos, de acordo com o tipo de granuloma presente na lesão. Desta forma, obtivemos um grupo com presença de granuloma compacto, um grupo com granuloma pouco organizado e um terceiro grupo, com a presença de ambos os tipos de granulomas. Essa

subdivisão foi importante para este e demais trabalhos, possibilitando-nos fazer inúmeras correlações do ponto de vista de resposta imune *in situ*.

Inicialmente, ao fazermos a reação de imuno-histoquímica simples, ou seja, somente utilizando o marcador anti-Fator XIIIa, verificamos alterações tróficas e numéricas nessas células, quando comparadas a pele normal. Os dendrócitos, nas lesões cutâneas de paracoccidiodomicose, estão distribuídos em região perivascular, na derme superficial e reticular e ao redor de granulomas. Sua morfologia nas lesões é de aspecto dendrítico, ao contrário do que visualizamos na pele sã onde tais células têm aspecto fusiforme.

Sugere-se que essas alterações morfológicas sejam consequência da expressão aumentada de HLA-DR e, portanto, da atividade dos dendrócitos dérmicos Fator XIIIa+ como células capazes de apresentar antígenos¹¹⁵. Ao verificarmos a presença dessas células ao redor de vasos, especulamos que esse seria um local estratégico para interação com linfócitos T e desencadeamento de resposta imune local. Alguns autores sugerem, inclusive, que essa população celular poderia assessorar as células de Langerhans na função de apresentadora de antígenos ou ainda, que seriam precursoras dessas últimas²⁵. Vale lembrar que em trabalho anterior a esse, Sandoval *et al.* ao utilizar técnica de dupla-marcação para visualização de antígenos fúngicos e células de Langerhans (marcador S100), não detectaram co-localização positiva e sugeriram que outra população celular, prováveis macrófagos, atuaria como apresentadora de antígenos na paracoccidiodomicose¹¹⁷.

A partir de uma primeira análise dos resultados imuno-histoquímicos com reação simples, nos sentimos induzidas a elaborar um protocolo de dupla-marcação imuno-histoquímica com o anticorpo policlonal anti- *P. brasiliensis* e monoclonal anti-Fator XIIIa. A primeira reação foi revelada com peroxidase (castanho) e a segunda com fosfatase alcalina (vermelho). Como resultado, demonstramos de modo inequívoco a interação dos dendrócitos dérmicos Fator XIIIa+ com formas fúngicas. Esses resultados nos permitiram sugerir um papel para esse grupo celular como apresentadoras de antígenos nas lesões cutâneas de paracoccidiodomicose.

Uma abordagem parecida foi feita por Sotto *et al.* em 18 biopsias de lesões de cromoblastomicose¹¹⁸. Entre os elementos estudados, também foi possível verificar por

metodologia semelhante a interação dos dendrócitos dérmicos Fator XIIIa+ com material antigênico do *Fonsecaea pedrosoi*. Os autores sugerem sua capacidade fagocítica e de apresentadores de antígenos nessa micose.

(Anexo 2) Barboza TC, Quaresma JAS, Brito AC, Xavier MB, Oliveira CMM, Unger DAA, Duarte MIS, Sotto MN, **Pagliari C.** Jorge Lobo's Disease: Immunohistochemical Characterization of Dendritic Cells in Cutaneous Lesions. *Mycopathologia* (1975. Print), JCR v. 179, p. 269-274, n. **2015**. FI 1.6.

O *Lacazia loboi*, agente causador da Doença de Jorge Lobo e o *Paracoccidioides sp* diferem quanto a alguns aspectos morfológicos, porém apresentam similaridades químicas e antigênicas¹¹⁹.

A imunologia da Doença de Jorge Lobo é pouco conhecida, sobretudo pelo agente etiológico ainda não ter sido cultivado *in vitro*, não havendo antígeno adequado para o teste sorológico. Muitas das hipóteses são baseadas em estudos realizados em outros tipos de micoses, como a PCM.

A caracterização histológica das lesões é representada na derme por vasos dilatados e infiltrado inflamatório granulomatoso constituído principalmente por macrófagos e linfócitos e em menor quantidade, eosinófilos e plasmócitos. Neutrófilos e necrose são raros. As estruturas nervosas estão íntegras nos interstícios do conjuntivo e no granuloma^{106,108}.

Na Doença de Jorge Lobo, micose confinada à pele, sem envolvimento sistêmico, a primeira abordagem de que temos conhecimento envolvendo os dendrócitos dérmicos Fator XIIIa+ foi a dissertação de mestrado da aluna Clivia Oliveira, orientada pelo Prof. Arival Cardoso de Brito em 2010 no Núcleo de Medicina Tropical, UFPA¹²⁰.

Nesse trabalho de Oliveira envolvendo 33 biopsias de pacientes com Doença de Jorge Lobo, a quantidade de dendrócitos Fator XIIIa+ foi maior quando comparado a grupo de amostras de pele sã e de lesões de paracoccidioidomicose. Embasados na literatura que descreve a presença de TGF- β nessas lesões e a capacidade dos dendrócitos em produzirem

TNF- α , os autores sugerem que a Doença de Jorge Lobo seja espectral e apresente padrão de resistência ou susceptibilidade¹²⁰.

Em nossa publicação de 2015, produto da dissertação de mestrado da aluna Tania Cristina Barboza sob minha orientação, evidenciamos os dendrócitos Fator XIIIa+ hipertróficos, à semelhança da morfologia observada nas lesões de paracoccidioidomicose, e frequentemente visualizados em meio ao infiltrado inflamatório das lesões. De maneira intrigante, embora tivessem alterações tróficas e aparentemente estivessem presentes em número expressivo, não houve diferença significativa com relação à pele normal.

Nessa casuística com 41 biópsias de lesões de pacientes, 14 espécimes não tiveram expressão de Fator XIIIa. Esse fato pode ter sido responsável pela ausência de diferença do grupo de lesões de DJL com o grupo de pele normal controle, por nós estudado.

Pretendemos em continuidade com essa linha de pesquisa, verificar o perfil citocínico e de outros elementos celulares da resposta imune *in situ* nas lesões de DJL com presença e ausência de dendrócitos dérmicos, com o objetivo de traçar um perfil que demonstre a característica espectral dessa doença, provavelmente relacionado a contextos imunes distintos.

3.2 Células dendríticas, macrófagos, linfócitos e citocinas: a resposta imune *in situ*

(Anexo 3) Pagliari C, Sotto MN. Dendritic cells and pattern of cytokines in paracoccidioidomycosis skin lesions. The American Journal of Dermatopathology (print), JCR Estados Unidos, v. 25, n. 2, p. 107-112, n. 2003. Citações: Web of Science (23), SCOPUS (25) FI 1.4.

Como sabemos, o primeiro estágio no reconhecimento de antígenos pelo sistema imune é o processamento pelas células apresentadoras de antígenos. No tópico anterior foi destacada a importância dos dendrócitos dérmicos Fator XIIIa+ nas três infecções fúngicas que são objeto de nossas pesquisas. Na sequência de nossos estudos sobre as lesões cutâneas na paracoccidioidomicose, investigamos outros grupos de células dendríticas, além do perfil de citocinas envolvidas na resposta imune local, bem como uma possível relação com o tipo de granuloma observado.

Enquanto os dendrócitos Fator XIIIa são classificados por dendrócitos tipo I, as células dendríticas do tipo II são marcadas pela expressão da molécula CD34. Esses dendrócitos possuem localizações e funções distintas na pele^{19,20,114,121,122}. As células dendríticas que expressam CD34 são consideradas por alguns autores como precursoras locais dos dendrócitos dérmicos Fator XIIIa+ e das células de Langerhans^{25,123,124}.

Estudos importantes estabeleceram que o perfil Th1 (IFN- γ e IL2) de citocinas representa o principal mecanismo de resistência na paracoccidiodomicose¹²⁵⁻¹²⁷, enquanto a susceptibilidade está associada ao perfil Th2 (IL4, IL5, IL6 e IL10)¹²⁷⁻¹²⁹. A progressão da doença está associada, entre outros fatores, a elevados níveis de IL10 e TNF- α e baixos níveis de IFN- γ ^{64,65}.

Nessa primeira abordagem das células de Langerhans imunomarcadas pelo anticorpo anti-CD1a e dendrócitos CD34+, verificamos a distribuição de pequeno número de células de Langerhans ao longo da epiderme, com dendritos curtos e irregulares, diferente do que se observa na pele normal, em que os dendritos são numerosos e alongados. A redução numérica e trófica das células de Langerhans poderia ser resultado de fatores produzidos pelo *P. brasiliensis* como mecanismo de escape, ou ainda, a redução numérica poderia ser explicada pela migração dessas células para a derme, resultando em alterações imunofenotípicas.

Células CD34+ apresentaram morfologia alongada e distribuição na derme, especialmente na adventícia de glândulas sebáceas e sudoríparas. Essa população celular nas lesões de paracoccidiodomicose não diferiu da pele normal em número ou morfologia.

Além de células dendríticas, a expressão de CD34 é observada nas células endoteliais e células fusiformes ao redor de anexos. Essas células não expressam CD1a e proteína S100. Somente cerca de 2% delas expressa a molécula HLA-DR¹²³.

Em nosso estudo, não verificamos correlação dos dendrócitos CD34+ com as demais células dendríticas estudadas, nem seu parasitismo ou co-localização com o *Paracoccidioides brasiliensis*. Acreditamos que mais estudos acerca dessa população celular seriam interessantes.

Por outro lado, a pesquisa dos padrões de citocinas nos revelou dados interessantes. Demonstramos que células expressando TNF- α distribuem-se na derme em meio ao infiltrado inflamatório granulomatoso e células gigantes multinucleadas. Células de morfologia dendrítica imunomarcadas pelo TNF- α distribuem-se ao redor dos granulomas, com localização semelhante à de alguns dendrócitos Fator XIIIa+ que descrevemos anteriormente. A partir desse dado, sugerimos que na paracoccidioidomicose os dendrócitos dérmicos Fator XIIIa+ poderiam ser produtores de TNF- α como células auxiliares no recrutamento de macrófagos e constituição do granuloma.

A citocina clássica de perfil Th1 (IFN- γ) foi visualizada em maior quantidade nas lesões com granulomas compactos, enquanto as citocinas de perfil Th2 (IL5 e IL10) predominaram nas lesões com granulomas pouco organizados. De maneira interessante, as lesões com ambos os tipos de granulomas apresentaram ambos os tipos de padrão citocínico, sem predomínio de um ou outro.

Outra observação que derivou um tópico de pesquisa que será discutido adiante, foi a imunomarcagem de mastócitos com o anticorpo anti-IL10, predominando nos grupos de granulomas pouco organizados e de resposta mista.

Com esses dados corroboramos a idéia de que experimentalmente é possível estabelecer-se dois polos bem definidos de resposta imune, porém, em humanos essa doença parece ser mais dinâmica e polimórfica¹³⁰.

Há vários fatores que regulam a expressão do perfil Th1 ou Th2 de citocinas, como o conteúdo genético, o microambiente de citocinas e o tipo de células apresentadoras de antígenos que interagem com os linfócitos T^{131,132}. Nesse último aspecto, Calich *et al.* verificaram haver diferenças na resposta ao *P. brasiliensis* diante de diferentes células apresentadoras de antígenos¹³³.

Desta forma, acreditamos que a presença de diferentes populações de células dendríticas na pele e sua capacidade de apresentar antígenos constitua fator importante na resposta imune local na paracoccidioidomicose. A compreensão da correlação entre essas células e citocinas pode contribuir para o conhecimento de possíveis alvos de tratamento.

Essas células podem ser alvo do fungo ou desempenhar papel de apresentadoras de antígenos, com indução da produção de citocinas que modulam a resposta imune contra o *P.brasiliensis*.

(Anexo 4) D'Avila SP, **Pagliari C**, Duarte MIS. *The cell-mediated immune reaction in the cutaneous lesion of Chromoblastomycosis and their correlation with different clinical forms of the disease. Mycopathologia, JCR Netherlands, v. 156, p. 51-60, n. 2003. Citações: Web of Science (21), SCOPUS (23) FI 1.6.*

Os mecanismos de defesa nas infecções fúngicas vêm sendo cada vez mais explorados, no sentido de melhor compreender a interação hospedeiro–agente e contribuir, principalmente, para o conhecimento de possíveis alvos terapêuticos. No ano de 2003 iniciamos os estudos envolvendo a resposta imune *in situ* em lesões de cromoblastomicose, ao colaborar com o trabalho de tese de doutorado da Dra. Solange Correia Garcia Pires DÁvila, sob orientação da Profa. Maria Irma Seixas Duarte⁸⁷.

A resposta imune mediada por células e caracterização histológica foram correlacionadas a diferentes formas clínicas da doença, em particular as lesões verrucosas e do tipo placa atrófica.

Nesse trabalho estudamos a resposta imune *in situ* considerando linfócitos T totais (CD45RO), TCD4+, TCD8+, macrófagos e linfócitos B (CD20+), células de Langerhans (CD1a) e células CD34+. As citocinas estudadas foram TNF- α , IFN- γ , IL4 e IL10 (perfil Th1 e Th2).

As lesões do tipo verrucoso exibiram hiperplasia pseudoepiteliomatosa da epiderme, granulomas supurativos intraepidérmicos, infiltrado dérmico composto por macrófagos, neutrófilos, linfócitos, plasmócitos, mastócitos, eosinófilos, células epitelioides e células gigantes multinucleadas. Nessas lesões houve predomínio de expressão de IL10. Além desses elementos celulares, verificamos fibrose e intensa proliferação vascular.

As lesões do tipo placa atrófica apresentaram epiderme atrófica e resposta tecidual melhor organizada com granulomas bem formados, infiltrado inflamatório principalmente na derme superficial, pequeno número de mastócitos e poucos fungos. A proliferação vascular e fibrose eram, por outro lado, moderadas. Houve predomínio de células com

expressão de IFN- γ . Em estudo com casuística de 65 pacientes provenientes da região amazônica, os dados referentes a caracterização histológica foram semelhantes aos nossos⁸⁶.

Os resultados obtidos permitiram caracterizar dois perfis distintos de resposta imune local, que nessa infecção fúngica se correlacionam com duas diferentes formas clínicas. Em pacientes que respondem melhor à infecção há uma melhor resposta tecidual, com granulomas bem formados, fenótipo celular e expressão de citocinas compatíveis com o perfil Th1. Por outro lado, as lesões consideradas mais agressivas, nos pacientes que não respondem bem à infecção, o fenótipo foi constituído por granulomas pouco organizados e perfil de citocinas do tipo Th2.

Os estudos que focam a atividade macrofágica na cromoblastomicose relatam que, embora ocorra a fagocitose, são visualizados muitos fungos íntegros nas lesões, o que sugere uma deficiência no processo de destruição do agente pelos macrófagos. A manutenção de fungos viáveis nos sítios de lesão seria o principal fator para a morbidade dessa doença^{134,135}. É interessante ressaltar que diferentes células com capacidade de apresentar antígenos podem ser visualizadas nas lesões interagindo com formas fúngicas, que Sotto *et al.* caracterizaram como sendo os dendrócitos dérmicos Fator XIIIa+, descritos anteriormente, e as células de Langerhans¹¹⁸.

Acreditamos que haja vários fatores implicados nas diferenças de resposta tecidual e conseqüente reflexo na apresentação clínica da cromoblastomicose, especificamente, fatores que influenciam na constituição da resposta do tipo Th1 ou Th2, como aqueles derivados das células apresentadoras de antígenos, imunidade inata e citocinas produzidas por outras células^{136,137}. Os mastócitos, por exemplo, contribuem para o direcionamento da resposta para o polo Th2¹³⁸.

A partir desses resultados, nos sentimos estimulados a dar continuidade aos estudos sobre a cromoblastomicose. Novas abordagens serão discutidas mais à frente.

(Anexo 5) Carneiro FRO, Fischer TRC, Brandão CM, **Pagliari C**, Duarte MIS, Quaresma JAS. *Disseminated infection with *Lacazia loboi* and immunopathology of the lesional spectrum. Human Pathology (Print)*, v. 46, p. 334-338, n. **2015**. Citações: Web of Science (1), SCOPUS (1) FI 2.7.

A Doença de Jorge Lobo, embora confinada à pele, se manifesta sob diferentes aspectos lesionais, cuja variabilidade parece estar diretamente relacionada ao tipo de resposta imune do hospedeiro frente ao *L. loboi*, de maneira semelhante ao que ocorre por exemplo, na hanseníase.

Alguns pacientes desenvolvem a forma disseminada, enquanto outros apresentam lesões localizadas. Ao que parece, uma deficiência parcial da resposta imune celular leva a maior susceptibilidade a essa doença. O infiltrado inflamatório nas lesões é composto por macrófagos, células NK, plasmócitos, linfócitos B, linfócitos T CD3+, linfócitos T CD4+, linfócitos T CD8+¹¹⁰.

O perfil Th2 de citocinas favorece o estabelecimento da infecção e reduz a capacidade dos mecanismos responsáveis pela contenção do patógeno. Além disso, o aumento de TGF- β e expressão reduzida de TNF- α e iNOS contribuem para a patogênese dessa infecção induzindo fibrose e inibição da capacidade microbicida dos macrófagos^{108,111,112,139}.

Estudamos um caso muito interessante de paciente da região amazônica submetido a tratamento contra a Doença de Jorge Lobo por cerca de quatro meses, sem sucesso. As lesões persistentes apresentavam cinco aspectos clínicos diferentes: queloidiforme, infiltrativa, nodular, papular e verrucosa. Investigamos o perfil celular e citocínico em cada uma delas.

Os principais achados histológicos foram presença de ectasias vasculares, infiltrado granulomatoso nodular ou difuso rico em macrófagos, linfócitos e células gigantes do tipo Langhans e do tipo corpo estranho, contendo fungos.

Demonstramos diferenças no padrão de resposta imune entre os diferentes tipos de lesão, semelhante a estudos prévios^{108,111}. O tipo de lesão se correlaciona com a resposta

imune. Lesões nodulares têm perfil do tipo Th1, as lesões infiltrativas são do tipo Th2 de citocinas. As demais são intermediárias e, no conjunto, a Doença de Jorge Lobo compreende um espectro clínico e imunológico de manifestações de lesões.

3.3 O papel dos mastócitos como produtores de IL10 na paracoccidiodomicose

(Anexo 6) Pagliari C, Fernandes ER, Guedes F, Alves C, Sotto MN. Role of mast cells as IL10 producing cells in paracoccidiodomycosis skin lesions. *Mycopathologia* (1975. Print), JCR v. 162, p. 331-335, n. 2006. Citações: Web of Science (10), SCOPUS (8) FI 1.6.

Os mastócitos pertencem ao sistema imune inato e alguns estudos destacam seu papel importante na resposta imune, sugerindo-se inclusive que possam ter função de células apresentadoras de antígenos, já que expressam moléculas de MHC classe II^{140,141}. Entretanto, a capacidade de apresentar antígenos é inibida por IFN- γ , sugerindo-se que essa função seja, portanto, inibida mediante o desenvolvimento de resposta imune mediada pelo padrão Th1 de citocinas. Por outro lado, os mastócitos estão envolvidos na resposta imune mediada por citocinas de padrão Th2, caracterizada pela secreção de IL4 e IL10.

Sabe-se que os mastócitos sintetizam e secretam muitas citocinas incluindo IL4, IL5, IL6 e TNF- α . Este último é estocado nos grânulos citoplasmáticos de mastócitos dérmicos humanos e liberado no processo de degranulação¹⁴². Em 1999, ao estudar a resposta tecidual cutânea experimental, Streilein *et al.* sugeriram que mastócitos poderiam ser fonte de IL10¹.

Uma vez que tínhamos caracterizado três grupos de lesões de paracoccidiodomicose, baseados na expressão de citocinas Th1, Th2 ou ambas, e tendo verificado à imuno-histoquímica número considerável de células com aspecto de mastócitos imunomarcados com o anticorpo anti-IL10, decidimos estudar em detalhes essas células nos grupos de lesões. Em uma primeira análise, os mastócitos foram visualizados pela coloração histoquímica de azul de toluidina, sendo detectados em maior número nas lesões caracterizadas por granulomas mal formados e perfil Th2 de citocinas. A seguir, realizamos reação imuno-histoquímica de dupla-marcação com anticorpo específico para IL10 e subsequente imunomarcção de mastócitos. O encontro frequente da co-localização de

mastócitos e IL10, permite-nos afirmar que na paracoccidioidomicose essas células são produtoras de IL10 e contribuem para a resposta menos efetiva do hospedeiro frente aos antígenos fúngicos.

Além de serem produtoras de IL10, os mastócitos também são produtores de TNF- α , o que os torna células importantes na formação e manutenção da resposta granulomatosa¹⁴².

As citocinas IL3 e IL4 podem influenciar a proliferação de mastócitos. Uma vez nos tecidos, terminam seu processo de maturação influenciados pelo microambiente tecidual^{143,144}. Em doenças alérgicas essas células funcionam como efetoras na defesa do organismo pela ativação de *Toll-like receptor* (TLR) 2 e 4.

A ativação mediada por TLR2 induz a degranulação e produção de IL4 e IL5, citocinas de perfil Th2^{145,146}. O TLR2 reconhece partículas fúngicas¹⁴⁷. Desta forma, ao reconhecer antígenos do *Paracoccidioides sp*, além de ativar macrófagos, esse receptor também parece poder atuar na degranulação de mastócitos e consequente produção de citocinas Th2, que caracterizam o grupo de lesões com resposta imune menos efetiva.

3.4 A resposta imune citotóxica: avaliação de sua importância nas infecções fúngicas

(Anexo 7) Pagliari C, Pereira NV, Kanashiro L, Stegun FW, Duarte MIS, Sotto MN. *Characterization of cytotoxic immune response in skin and mucosal lesions of paracoccidioidomycosis. Journal of Cutaneous Pathology, JCR v. 37, p. 565-570, n. 2010.*
Citações: Web of Science (3), SCOPUS (3) FI 1.4.

A resposta imune mediada por linfócitos TCD8+ representa importante mecanismo de defesa contra patógenos intracelulares pois, além da ação citotóxica com produção de perforina e granzima, são células produtoras de IFN- γ , importante citocina na indução da resposta do tipo Th1.

A função protetora da resposta imune citotóxica na paracoccidioidomicose tem sido descrita em diversos trabalhos. Em 2000, Cano *et al.* evidenciaram experimentalmente que a

depleção de linfócitos TCD8+ dificulta a defesa do hospedeiro na paracoccidioidomicose pulmonar¹⁴⁸.

Por meio da análise do lavado broncoalveolar de pacientes com paracoccidioidomicose pulmonar, sugere-se que além dos macrófagos, os linfócitos TCD8+ podem ter papel importante em sua patogenia¹⁴⁹.

O primeiro estudo que temos conhecimento sobre a atividade citotóxica das células NK nessa doença foi experimental, em hamsters infectados. A atividade prejudicada das células NK ao longo da infecção e aumento das lesões pulmonares permitiu uma correlação entre a dificuldade dessas células em controlar a infecção e os estágios mais avançados da doença fúngica¹⁵⁰.

Em nosso trabalho, realizamos um estudo nas lesões cutâneas e de mucosa oral de paracoccidioidomicose abordando a função citotóxica dos linfócitos TCD8+ e células NK, ambas populações capazes de produzir perforina e granzima. As lesões, como nos trabalhos anteriores foram divididas em três grupos de acordo com o tipo de granuloma presente.

Ambas as populações celulares estão presentes nos três grupos de lesões cutâneas e de mucosa oral, sem diferenças numéricas estatisticamente significantes entre os grupos, distribuídas no infiltrado inflamatório e ao redor dos granulomas. Linfócitos TCD8+ predominam sobre células NK. A expressão de granzima B é visualizada em todos os grupos, em número maior associada aos granulomas mal formados nas lesões cutâneas e, de maneira interessante, no citoplasma de células gigantes multinucleadas. As lesões de mucosa oral têm expressão semelhante de granzima B nos três grupos de resposta tecidual.

A expressão de perforina foi raramente detectada tanto em pele como em mucosa.

Já é bem estabelecido que linfócitos TCD4+ têm papel chave no controle da infecção pelo *Paracoccidioides brasiliensis*. As lesões orais têm predomínio de linfócitos TCD4+ associados a granulomas bem formados e um balanço entre CD4 e CD8 em áreas não granulomatosas¹⁵⁰.

Sugerimos que, embora linfócitos TCD4+ sejam essenciais na defesa do hospedeiro contra o *P. brasiliensis*, os linfócitos TCD8+ e células NK podem ter papel na resposta imune *in situ* tanto nas lesões cutâneas como de mucosa oral, pela ação citotóxica com liberação de perforina e granzima, semelhante ao que foi descrito no lavado broncoalveolar de pacientes, independentemente do tipo de reação granulomatosa¹⁵¹.

Um estudo aprofundado sobre as células NK evidenciou que reconhecem e eliminam formas fúngicas (leveduras) independente de perforina, porém dependente de granzima. Por outro lado, o mecanismo de citotoxicidade sobre monócitos infectados é mediado por perforina, conferindo, portanto a capacidade de agir diretamente sobre o fungo ou sobre células infectadas com mecanismos distintos¹⁵².

Na cromoblastomicose ao estudarmos a resposta imune nas lesões, demos prioridade à caracterização do perfil de citocinas e observamos predomínio de linfócitos TCD4+, embora tenhamos verificado a presença de linfócitos TCD8+. Acreditamos que essa abordagem mereça um olhar mais detalhado em trabalhos futuros.

Estudos experimentais evidenciam em linfonodos regionais que há predomínio de linfócitos TCD4+ e o aumento de células TCD8+ só ocorre em fases mais tardias da infecção, entretanto, sem representar resposta protetora contra o *F. pedrosoi*⁹⁰.

(Anexo 8) Alexandre AF, Quaresma JA, Barboza TC, de Brito AC, Xavier MB, de Oliveira CM, Unger DA, Kanashiro-Galo L, Sotto MN, Duarte MI, **Pagliari C.** The cytotoxic T cells may contribute to the *in situ* immune response in Jorge Lobo's Disease human lesions. *Medical Mycology*. v. 55(2), p. 145-149, **2017**. FI 2.1.

A pesquisa da participação de mecanismos de citotoxicidade *in situ* nas lesões fúngicas estendeu-se à Doença de Jorge Lobo, onde estudamos células NK, linfócitos TCD8+ e expressão de granzima B.

Alguns trabalhos, além da nossa e outras abordagens sobre a paracoccidioidomicose, têm mostrado a importância da atividade citotóxica em outras infecções fúngicas importantes¹⁵³⁻¹⁵⁵.

Por meio da aplicação de técnicas de imuno-histoquímica pudemos visualizar linfócitos TCD8+, células NK e expressão de granzima B em todos as lesões de Doença de Jorge Lobo estudadas. Demonstramos maior número de linfócitos TCD8+ que células CD4+ (quantificadas em trabalho prévio que analisou as mesma casuística) e de células NK.

Nos questionamos porque, havendo número expressivo de linfócitos TCD4+ e ainda maior população de células TCD8+, as lesões cutâneas na DJL são extensas e persistentes. Xavier *et al.* demonstraram a elevada expressão de TGF- β por macrófagos nessas lesões. Esse fator é um potente imunossupressor, embora promova a proliferação de linfócitos TCD8+. O TGF- β apesar de favorecer o aumento da população de linfócitos TCD8+ nas lesões, poderia também favorecer um déficit na atividade citotóxica^{156,157}.

Pelo conjunto dos resultados de nossos trabalhos podemos sugerir que a resposta imune citotóxica tem papel importante na Doença de Jorge Lobo. Os nossos resultados aliados a conhecimentos prévios da literatura poderiam auxiliar ou embasar o desenvolvimento de novas estratégias para o controle e remissão das lesões.

3.5 Novas vias de resposta imune: o perfil Th17 e T regulador em lesões cutâneas e de mucosa oral

(Anexo 9) Pagliari C, Fernandes ER, Stegun FW, Silva WLF, Duarte MIS, Sotto MN. Paracoccidioidomycosis: Cells expressing IL17 and Foxp3 in cutaneous and mucosal lesions. Microbial Pathogenesis, JCR v. 50, p. 263-267, n. 2011. Citações: Web of Science (12), SCOPUS (12) FI 1.8.

A citocina IL17 tem recebido atenção considerável em vários contextos desde sua descoberta em 1993. Sua secreção é promovida por IL23 produzida por linfócitos TCD4+^{158,159}. O perfil Th17 inclui ainda a secreção de IL6, IL22 e TNF- α ¹⁶⁰.

O perfil Th17 de citocinas apresenta funções distintas dos grupos Th1 e Th2^{161,162}. O desenvolvimento dessas células depende da presença de TGF- β e determinadas citocinas inflamatórias.

A função protetora dos linfócitos do tipo Th17 contra infecções fúngicas tem sido proposta, como por exemplo, na candidíase, criptococose e pneumocistose^{71,72,74,163}.

Os linfócitos T reguladores constituem um importante subpopulação de células na indução e manutenção da tolerância, assim como na finalização da resposta imune, considerando-se que uma deficiência ou disfunção nestas, pode exacerbar a inflamação induzida por determinados patógenos. Sua ativação também é dependente de TGF- β ⁶⁹.

Na paracoccidiodomicose, foi demonstrado o número elevado de linfócitos T reguladores no sangue e em lesões orais de pacientes⁷⁷. Essas células acumulam-se nos locais de infecção limitando a resposta imune efetora e permitindo a persistência do agente e indução da cronicidade.

Em nosso estudo tivemos por objetivo verificar a participação de ambos os grupos de células, Th17 e T reguladores Foxp3+, em lesões cutâneas e de mucosa oral, fazendo novamente a correlação com o tipo de resposta tecidual granulomatosa nos subgrupos já descritos anteriormente.

Observamos que linfócitos com expressão de IL17 distribuem-se no infiltrado inflamatório e na região dos granulomas, próximos a células gigantes multinucleadas, tanto nas lesões de pele como de mucosa oral. Alguns neutrófilos também têm imunomarcagem positiva para a IL17, principalmente aqueles nos microabscessos, bem como algumas células na parede vascular. Essa localização poderia em parte contribuir para a formação da lesão, ao induzir a maior expressão de moléculas de adesão. Essas considerações são baseadas em conhecimentos prévios de que a IL17 induz os macrófagos a liberarem IL1- β e TNF- α , que são citocinas estimuladoras de moléculas de adesão. Em modelos experimentais, a expressão de tais moléculas se correlaciona à intensidade da resposta inflamatória¹⁶⁴⁻¹⁶⁶.

A expressão de IL17 em neutrófilos já havia sido descrita em modelo pulmonar da paracoccidiodomicose¹⁶⁷. Nesse modelo verificou-se que a ativação de células Th17 é prevalente e associada a alto número de polimorfonucleares e redução de células Tregs nas lesões.

Por meio de uma análise por dupla-marcação imuno-histoquímica observamos que nas lesões de paracoccidiodomicose, tanto linfócitos TCD4⁺ como TCD8⁺ expressam IL17, sendo esta maior em células TCD4⁺.

Células Foxp3 estão presentes no infiltrado inflamatório das lesões de pele e de mucosa oral na paracoccidiodomicose. O número dessas células é semelhante nos diferentes tipos de resposta tecidual na pele, entretanto são mais numerosas nas lesões mucosas que exibem predomínio de granulomas compactos.

As células Treg podem interferir na eficácia da imunidade do hospedeiro ou representar um mecanismo que o beneficia, uma vez que diminuem os danos que geralmente se seguem a uma resposta imune¹⁶⁸.

Como já destacamos previamente, os granulomas compactos estão associados ao padrão Th1 de citocinas. As células Treg estariam envolvidas na modulação da resposta imune nas lesões mucosas ao contribuir localmente para diminuir o dano tecidual. Por outro lado, foi inesperada a semelhança numérica dessas células nos grupos de lesões cutâneas.

A função das células Treg parece depender de alguns fatores. Em infecções crônicas estão envolvidas no controle da infecção e manutenção da imunidade contra reinfecção devido a um balanço entre elas e células T efectoras¹⁶⁹⁻¹⁷¹.

Vale ressaltar mais uma interessante função das células T reguladoras, que é sua capacidade de aumentar a expressão de granzima B, função, portanto, citotóxica à semelhança dos linfócitos TCD8⁺¹⁷². Essa função vai diretamente de encontro aos nossos achados descritos em tópico anterior, sobre o papel da resposta imune citotóxica na paracoccidiodomicose. Uma de nossas intenções futuras é realizar reação imuno-histoquímica de dupla-marcação para averiguar a possível co-localização de Foxp3 (marcador de Treg) e granzima B (grânulo citotóxico) nos diferentes grupos de resposta tecidual das lesões de pele e mucosa da paracoccidiodomicose.

Concluindo, nossos achados nesse estudo foram de uma expressão importante de linfócitos Treg nas lesões de pele e mucosa na paracoccidiodomicose, o que poderia representar um mecanismo imunorregulador na patogenia dessa infecção. Até o momento

dessa publicação não tínhamos conhecimento da abordagem das células Th17 em tais lesões. Considerando as dúvidas que permaneceram e nosso interesse no assunto, demos prosseguimento a essa linha de pesquisa, com mais resultados descritos abaixo.

(Anexo 10) Silva AAL, Sotto MN, Duarte MIS, **Pagliari C.** *Regulatory T cells in cutaneous lesions of patients with Paracoccidioidomycosis. Microbial Pathogenesis, JCR v. 65, p. 36-40, n. 2013. Citações: : Web of Science (1), SCOPUS (1) FI 1.8.*

O presente estudo teve como intuito contribuir com a caracterização da resposta tecidual cutânea na paracoccidioidomicose, abordando a resposta T reguladora nos diferentes grupos de lesão. Trata-se dos resultados da monografia de conclusão de curso da aluna Aline Alves de Lima Silva, sob minha orientação. Especificamente, o objetivo foi detectar a presença dos marcadores Foxp3, CD25, TGF- β e IL10, quantificar sua expressão e associar os resultados aos diferentes tipos de granulomas paracoccidióidicos.

Além da positividade para Foxp3 no infiltrado inflamatório nas lesões, visualizamos a presença de células CD25+ em todos os espécimes, bem como TGF- β e IL10.

As células Treg possuem fenótipo CD4+CD25+¹⁷³, porém a molécula CD25 é expressa por outras populações celulares como linfócitos B, T e macrófagos ativados, de modo que a presença apenas de CD25 não indica necessariamente a presença de células Treg. Por este motivo, avaliamos as células Foxp3+ e CD25+, uma vez que a expressão nuclear do gene Foxp3 em células T reguladoras naturais é marcador fidedigno desta linhagem celular¹⁷⁴.

A população de linfócitos Treg parece fazer uso de citocinas inibitórias, como a IL10, que inibe a ação das células apresentadoras de antígenos e é antagonista de IFN- γ , assumindo dessa forma um papel supressor de resposta imune, com ação anti-inflamatória¹⁷⁵.

A expressão de TGF- β relaciona-se a ativação da expressão de Foxp3. Além disso, em conjunto com IL10 constituem perfil inibidor dos mecanismos da imunidade.

(Anexo 11) Silva AAL, Criado PR, Nunes RS, Silva WLF, Kanashiro-Galo L, Duarte MIS, Sotto MN, **Pagliari C.** *In Situ Immune Response in Human Chromoblastomycosis - A Possible Role for Regulatory and Th17 T Cells. PLoS Neglected Tropical Diseases (Online), JCR v. 8, p. e3162-, n. 2014. Citações: Web of Science (2), SCOPUS (2) FI 4.4.*

Prosseguimos a análise desses mecanismos reguladores na cromoblastomicose. Esse trabalho foi o resultado da dissertação de Mestrado da aluna Aline Alves L. Silva, sob minha orientação.

A casuística desse estudo foi constituída apenas por lesões do tipo verrucoso da cromoblastomicose, uma vez que na pesquisa de prontuários clínicos e do arquivo do laboratório de Dermatopatologia do Departamento/Divisão de Clínica Dermatológica do HCFMUSP, não encontramos casos de lesões do tipo placa atrófica, que seriam muito interessantes para comparação. Talvez esse fato seja relacionado ao tipo de atendimento terciário de nossa instituição, com seleção de doentes com curso clínico prolongado e de difícil abordagem terapêutica.

Nesse estudo, além da abordagem dos marcadores para Foxp3, IL17, CD25 e TGF- β realizados na paracoccidioidomicose, incluímos a IL6 e IL23.

O método imuno-histoquímico nos permitiu observar presença de células Foxp3 e CD25+ em meio ao infiltrado inflamatório e, em particular, ao redor de granulomas. A expressão de TGF- β foi visualizada em células mononucleares do infiltrado inflamatório. Nessas lesões há discreta expressão de IL6. Células mononucleares e polimorfonucleares IL17+ estão presentes nos focos granulomatosos, bem como células IL23+.

Ao compararmos as amostras de cromoblastomicose com a de lesões de paracoccidioidomicose, observamos que a expressão de Foxp3 é maior nessas últimas. Já a expressão de CD25 ocorre de maneira inversa. Células que expressam TGF- β , IL6 e IL23 não diferiram nas lesões das duas micoses estudadas. Por outro lado, a expressão de IL17 foi superior na cromoblastomicose.

As lesões verrucosas são as mais comumente observadas na cromoblastomicose. A sua histopatologia caracteriza-se por infiltrado inflamatório com granulomas mal formados,

neutrófilos, macrófagos e extensiva fibrose e fungos numerosos. As citocinas mais frequentes nos sítios de lesão são as que caracterizam o perfil Th2⁸⁷.

Pudemos mostrar nesse trabalho o predomínio de células IL17+ sobre células Foxp3+, bem como a presença de TGF- β e IL23. A IL23 é responsável por induzir a expressão de IL17. Uma vez que a resposta Th17 está estabelecida, a expressão de IL23 parece diminuir. Considerando-se que nas lesões verrucosas há predomínio de citocinas de perfil Th2, poderíamos supor que a resposta Th17 que verificamos possa ser desencadeada numa tentativa do organismo em conter a infecção.

O TGF- β media, dependendo do contexto de outras citocinas presentes, o direcionamento para resposta do tipo T regulador ou Th17. Na presença de citocinas pró-inflamatórias há promoção da síntese de IL23 com subsequente desenvolvimento da resposta do tipo Th17^{68,176}.

A interação equilibrada entre linfócitos Treg e os produtores de IL17 é importante na homeostase, já verificada em mucosa intestinal. A IL17 tem atividade pró-inflamatória e supõe-se que uma interação desregulada entre essa citocina e patógenos possa levar a um viés no equilíbrio imune predispondo o indivíduo à cronicidade¹⁷⁷.

Desta forma, acreditamos que nossos resultados evidenciando uma resposta Th17 exacerbada possam contribuir para a compreensão da imunopatogenia da cromoblastomicose e a possibilidade de focar em terapias que possam modular a resposta imune do hospedeiro para um padrão mais efetivo capaz de conter a infecção com remissão das lesões.

(Anexo 12) Kanashiro-Galo L, **Pagliari C**, Barboza TC, Brito ACD, Xavier MB, Oliveira CMM, Uger, DAA, Sotto MN, Quaresma JAS, Duarte MIS. Th17 and regulatory T cells contribute to the in situ immune response in skin lesions of Jorge Lobo's disease. *Medical Mycology* (Oxford. Online), JCRv. 54, p. 23-28, n. **2016**. Citações: SCOPUS (1) FI 2.1.

Uma vez que já havíamos estudado o perfil Th17 e T regulador de citocinas na paracoccidiodomicose e cromoblastomicose e, estando desenvolvendo diferentes

abordagens em lesões na Doença de Jorge Lobo, ficamos curiosos quanto à participação de Th17 também nesta infecção fúngica e seu papel na patogenia da doença.

Verificamos que linfócitos Foxp3+ estavam presentes em todos os casos estudados. A expressão de IL17 foi visualizada tanto em linfócitos como em neutrófilos. A expressão de IL1, CD25, IL6 e IL23 foi observada no infiltrado inflamatório e participando da formação granulomatosa. A citocina mais frequente foi a IL17, seguida por IL6. Na análise comparativa, o número de células com a expressão de IL17 foi maior que de células Treg Foxp3+.

Xavier *et al.*¹¹² relataram que as lesões da Doença de Jorge Lobo têm expressão acentuada de TGF- β , uma citocina reguladora. Em nossas observações, ao estudar a mesma micose, detectamos elevado número de células expressando IL6, uma citocina pró-inflamatória.

Nossos resultados evidenciam a participação da resposta Th17 nessa infecção fúngica. A presença de citocinas pró-inflamatórias IL1 e IL6, aliada aos achados prévios da presença expressiva de TGF- β seriam fatores estimuladores dessa resposta. É importante considerar, entretanto, que a elevada expressão do padrão Th17 pode ter papel deletério e contribuir para a patogenia da doença, o que ainda é um assunto controverso, à semelhança do que evidenciamos na cromoblastomicose.

3.6 Revisitando as células de Langerhans nas lesões cutâneas

(Anexo 13) Pagliari C, Fernandes ER, Silva WLF, Silva AAL, Stegun FW, Duarte MIS, Sotto MN. *Revisiting Langerhans cells in paracoccidioidomycosis: expression of CD207/langerin in human cutaneous and mucosal lesions. Microbes and Infection, JCR v. 13, p. 1012-1017, n. 2011. Citações: Web of Science (1). SCOPUS (1) FI 2.2.*

Como já mencionado anteriormente, as células de Langerhans são células dendríticas constitutivas da epiderme na pele normal. Além de seus marcadores conhecidos, como a expressão da proteína S100 e da molécula CD1a, marcador específico ao lado dos grânulos de Birbeck, foi descrito mais recentemente outro marcador - a Langerina, classificada como CD207. Esse marcador está relacionado com os grânulos de Birbeck de células de Langerhans maduras²⁹.

Alguns artigos da literatura discutem a possibilidade de uma possível nova população de células dendríticas langerina+ na derme, ou se as células CD207+ seriam células de Langerhans dérmicas em processo de migração. Entretanto, não há muitas evidências de tratar-se de uma nova subpopulação celular dendrítica na pele humana^{15,31}.

Como já relatado, realizamos estudo de paracoccidioidomicose focando as células de Langerhans, através de sua imunomarcção com o anticorpo anti-CD1a. Revisitamos agora as células de Langerhans nessa e outras infecções, pelo estudo com o marcador específico langerina/CD207.

Ao imunomarcar as células de Langerhans com o anticorpo CD207 demonstramos, como em trabalho anterior com o anticorpo CD1a que as células de Langerhans são escassas, distribuídas ao longo da epiderme e apresentam dendritos curtos e algumas vezes ausentes. Além disso, descrevemos pela primeira vez células langerina+ nos espécimes de mucosa oral da paracoccidioidomicose. As células CD207+ mostraram-se localizadas em meio ao infiltrado inflamatório e nos granulomas da derme e lâmina própria da mucosa oral. Na análise prévia com o marcador CD1a não visualizamos células positivas na derme ou lâmina própria das lesões da paracoccidioidomicose. Embasados nesses resultados, sugerimos que as células de Langerhans possam sofrer alterações fenotípicas e não expressar mais a molécula CD1a quando na derme, por isso não terem sido identificadas. Entretanto, nesse trabalho com o anticorpo CD207, observamos que essas células mantêm a expressão de langerina, que persiste no processo de maturação e migração, como o descrito em outros processos patológicos da pele¹⁷⁸.

Na cromoblastomicose, o trabalho de Sotto *et al.*¹¹⁸ evidenciou as células de Langerhans com o marcador para proteína S100 na epiderme dos casos estudados e na derme de parte da casuística. Estas últimas apresentavam raramente antígenos fúngicos no seu citoplasma.

(Anexo 14) Quaresma JAS, Unger D, **Pagliari C**, Sotto MN, Duarte MIS, Xavier MB, Britto AC. *Immunohistochemical study of Langerhans cells in cutaneous lesions of the Jorge Lobo's disease. Acta Tropica*, JCR v. 114, p. 59-62, n. **2010**. Citações: Web of Science (8), SCOPUS (9) FI 2.3.

Neste trabalho tivemos por objetivo investigar o possível papel das células de Langerhans na patogenia da Doença de Jorge Lobo. Demonstramos que essas células estão presentes na epiderme, com morfologia e análise numérica similares a pele normal, visualizadas com o anticorpo anti-CD1a.

Tendo como parâmetro a expressão de CD1a e considerando as pesquisas prévias quanto à expressão exacerbada do fator imunossupressor TGF- β , sugerimos haver na Doença de Jorge Lobo um possível distúrbio imunorregulador. Esse distúrbio poderia resultar na inibição da atividade das células de Langerhans enquanto apresentadoras de antígenos e por isso, teriam aspectos morfológicos e densidade semelhante ao tecido cutâneo na normalidade.

(Anexo 2) Barboza TC, Quaresma JAS, Brito AC, Xavier MB, Oliveira CMM, Unger DAA, Duarte MIS, Sotto MN, **Pagliari C**. *Jorge Lobo's Disease: Immunohistochemical Characterization of Dendritic Cells in Cutaneous Lesions. Mycopathologia (1975. Print)*, JCR v. 179, p. 269-274, n. **2015**. FI 1.6.

De maneira intrigante, obtivemos resultados controversos cinco anos depois, ao empregarmos a mesma metodologia com anticorpo anti-langerina. O grupo de lesões de Doença de Jorge Lobo evidenciou número escasso de células langerina positivas na epiderme, mas as mesmas mostraram-se na derme em meio ao infiltrado inflamatório. Esta foi a primeira documentação da expressão de langerina nessa infecção fúngica.

A langerina liga-se à manose e β -glucana da parede celular de alguns fungos e parece ser um importante receptor nas células de Langerhans humanas¹⁷⁹. A langerina é atualmente considerada o “estado da arte” para identificar essa população celular. Sua identificação na derme parece indicar que as células de Langerhans mantêm a expressão de langerina ao

migrar ou poderia ser evidência da presença de outra população de células dendríticas que também sejam langerina+ no microambiente dérmico^{15,180}.

3.7 Células dendríticas plasmocitoides nas micoses cutâneas

(Anexo 15) Pagliari C, Kanashiro-Galo L, Silva AADL, Barboza TC, Criado PR, Duarte MIS, Brito ACD, Xavier MB, Unger D, Oliveira CMM, Sotto MN. Plasmacytoid dendritic cells in cutaneous lesions of patients with chromoblastomycosis, lacaziosis, and paracoccidioidomycosis: a comparative analysis. Medical Mycology (Oxford. Print), JCR v. 52, p. 397-402, n. 2014. FI 2.1.

As células dendríticas plasmocitoides (pDC), caracterizadas pela expressão do receptor da IL3 (CD123) e da molécula CD303, estão envolvidas na resposta imune aos vírus, por produzirem IFN- α .

Essa população de células dendríticas foi estudada em processos infecciosos granulomatosos como a hanseníase e leishmaniose tegumentar^{181,182}.

Fizemos nesse estudo a pesquisa das células plasmocitoides com ambos marcadores (CD123 e CD303), por técnica imuno-histoquímica e sua análise comparativa em lesões de paracoccidioidomicose, cromoblastomicose e Doença de Jorge Lobo.

Células CD123+ e CD303+ foram detectadas em 16/43 (37%) biopsias de cromoblastomicose. Na paracoccidioidomicose, a expressão de CD303 foi rara e de CD123 foi visualizada em 24/46 casos (cerca de 50%). Na Doença de Jorge Lobo as células dendríticas plasmocitoides não foram observadas à análise com os anticorpos CD123 e CD303.

As células dendríticas plasmocitoides têm origem mieloide, atuam como apresentadoras de antígenos e sua participação tem sido estudada principalmente em neoplasias como o melanoma, alguns tipos de carcinoma e tumor de ovário. Em condições inflamatórias há relatos em lúpus eritematoso sistêmico, psoríase e dermatite de contato, onde foram relacionadas à produção de determinadas citocinas e ativação de linfócitos¹⁸³⁻¹⁸⁶.

Os nossos resultados focando as lesões de cromoblastomicose permitem sugerir que as pDC, ao expressar a molécula CD303 (um receptor de lectina-c) podem interagir com as formas fúngicas, à semelhança da interação de fungos com CD205, CD207 e CD209, que são também receptores de lectina-c. Ademais, as pDC poderiam atuar nessa infecção assim como na paracoccidioidomicose como colaboradoras na produção de citocinas. As pDC, por serem produtoras de IFN- α/β , seriam capazes de participar da imunomodulação da resposta imune como na diferenciação de células dendríticas mieloides, ativação da resposta Th1, ativação de células NK e indução da resposta humoral.

Na Doença de Jorge Lobo, a ausência de pDC pode ser mais um dos fatores que caracterizam os mecanismos imunes deficientes no combate e eliminação dos fungos.

3.8 Interação dos queratinócitos com formas fúngicas na paracoccidioidomicose

(Anexo 16) Silva WLF, **Pagliari C**, Duarte MIS, Sotto MN. *Paracoccidioides brasiliensis interacts with dermal dendritic cells and keratinocytes in human skin and oral mucosa lesions. Medical Mycology (Oxford. Print), JCR v. 54, p. myv112-, n. 2016. FI 2.1.*

Neste trabalho abordamos a interação do *Paracoccidioides brasiliensis* com dendrócitos dérmicos Fator XIIIa+ em lesões de mucosa oral, células langerina+ e queratinócitos, usando protocolos de dupla-marcação imuno-histoquímica. Para comprovar a interação do fungo com os queratinócitos também utilizamos a técnica de microscopia confocal a laser. Este trabalho é resultado da dissertação de mestrado do aluno Wellington Luiz Ferreira da Silva, sob orientação da Profa. Mirian Nacagami Sotto.

À semelhança do que havíamos descrito em biopsias de pele em 2002, as biopsias de mucosa também exibiram a interação de formas fúngicas com dendrócitos Fator XIIIa. Essa interação pode ser resultado da capacidade desses dendrócitos em apresentar antígenos ou somente serem células alvo para o fungo na paracoccidioidomicose humana. Na mucosa oral, o número maior de dendrócitos com presença de leveduras do fungo no seu citoplasma foi observado no grupo de granulomas pouco organizados, que apresentam, como já descrito em 2003, o perfil Th2 de citocinas.

Não verificamos co-localização de células langerina+ e formas fúngicas, corroborando os achados prévios nas lesões cutâneas. Por outro lado, as técnicas de dupla-marcação por imuno-histoquímica e por imunofluorescência ao microscópio confocal evidenciaram a frequente interação das leveduras com os queratinócitos, tanto das lesões de pele como de mucosa. Brito *et al.*¹⁸⁷ já haviam demonstrado, por microscopia eletrônica de transmissão, o parasitismo de queratinócitos pelo *Paracoccidioides brasiliensis*. Relacionaram seus resultados como demonstração da capacidade de apresentar antígenos que os queratinócitos possuem. Os queratinócitos expressam muitos receptores para uma variedade de patógenos e produzem citocinas e quimiocinas em resposta a estímulos patogênicos¹⁸⁸. Com base nos nossos resultados, podemos dizer que na paracoccidioidomicose humana, os queratinócitos parecem ter papel no reconhecimento imune de antígenos fúngicos.

3.9 O fenótipo do endotélio nas lesões da Doença de Jorge Lobo

(Anexo 17) Quaresma JAS, Brito MV, Sousa JR, Silva LM, Hirai KE, Araujo RS, Brito AC, Carneiro FRO, Fuzii HT, **Pagliari C**, Sotto MN, Duarte MIS. Analysis of microvasculature phenotype and endothelial activation markers in skin lesions of lacaziosis (Lobomycosis). *Microbial Pathogenesis*, JCR v. 78, p. 29-36, n. **2015**. FI 1.8.

Nesse trabalho enfocamos o fenótipo dos elementos vasculares e marcadores de ativação endotelial foram estudados em lesões da Doença de Jorge Lobo. Este trabalho é resultado da tese de doutorado da aluna Maysa Brito, orientada pelo Prof Juarez Antonio Simões Quaresma na Universidade Federal do Pará, na qual nosso laboratório teve participação na metodologia e discussão dos resultados.

Como descrito anteriormente, as lesões nessa doença exibem intensa fibrose, ausência de granulomas bem formados e grande número de formas fúngicas, indicando uma resposta imune celular deficiente.

Os vasos têm papel importante nas doenças inflamatórias crônicas e agudas. Na pele inflamada o remodelamento vascular consiste de aumento de permeabilidade, expansão da rede de vasos acompanhada por aumento no fluxo sanguíneo e influxo celular. Em

inflamações crônicas o endotélio ativado expressa quimiocinas, citocinas e moléculas de adesão que induzem a aderência e entrada de leucócitos para a pele¹⁸⁹.

Nesta análise, verificamos alterações microvasculares nas lesões. Vasos linfáticos e sanguíneos estão reduzidos em número (imunomarcação com anticorpos anti-D2-40 (podoplanina) e CD34) quando comparados a pele normal. Essa regressão vascular já foi descrita em casos de aspergilose pulmonar¹⁹⁰.

Discutimos a possibilidade de haver dois fatores para essa redução vascular. Do ponto de vista imunológico, estudos mostram que TGF- β pode inibir a proliferação de células endoteliais, atenuando o estímulo angiogênico¹⁹¹, além de inibir a expressão de E-selectina. Outra possibilidade é a perda de matriz extracelular e degradação de fibras elásticas com consequente rompimento do endotélio¹⁹².

A densidade reduzida de vasos pode também explicar porque a disseminação da infecção para outros órgãos é rara, apesar do alto número de fungos nas lesões.

Com relação as moléculas de adesão, houve um maior número de vasos expressando ICAM-1 e VCAM-1, contrário a baixa expressão de E-selectina. Esta última atua no contato inicial de células Th1 e Th17 com o endotélio ativado¹⁹³.

A expressão de ICAM-1 é importante componente para o recrutamento de leucócitos e formação do infiltrado inflamatório¹⁹⁴.

Novamente, a expressão importante de TGF- β merece atenção, pela capacidade de interferir na expressão de E-selectina.

Resumindo, há disfunções na microvasculatura cutânea na Doença de Jorge Lobo com redução na densidade de vasos, o que parece limitar os fungos nesse local de infecção e interferir na adequada recirculação de células e no estabelecimento de uma resposta adaptativa adequada. Por outro lado, a expressão de ICAM-1 permite a manutenção do processo infeccioso e afluxo de macrófagos.

3.10 A expressão de IL17 em células de Langerhans na cromoblastomicose.

(Anexo 18) Silva AAL, Criado PR, Nunes RS, Kanashiro-Galo L, Duarte MIS, Sotto MN, **Pagliari C.** *Langerhans cells express IL17-A in the epidermis of chromoblastomycosis lesions. Biomedicine Hub, v.2, 477954, n. 2017.*

Nesse trabalho apresentamos resultados de reação imuno-histoquímica de dupla marcação por microscopia óptica convencional e confocal a laser, onde foi possível visualizar a co-expressão de langerina (CD207) e IL17 em células da epiderme, sugerindo, portanto, a expressão dessa citocina por células de Langerhans.

Essa expressão foi previamente descrita em casos de histiocitose. Acreditamos que esse dado, descrito pela primeira vez em lesões de cromoblastomicose, possa trazer alguma contribuição na compreensão da imunopatogenia dessa importante micose.

As células de Langerhans poderiam estar ainda atuando como sentinelas imunes locais ao expressarem IL17.

3.11 Macrófagos M1 e M2 – as pesquisas em desenvolvimento

A população de macrófagos exibe forte plasticidade que varia de acordo com os estímulos no microambiente de lesões e pode ser classificada como do tipo M1 ou M2.

Os macrófagos do tipo M1, considerados como pró-inflamatórios, expressam citocinas pró-inflamatórias como IFN- γ e TNF- α , óxido nítrico sintetase indutível e moléculas MHC classe II que são importantes para a morte de patógenos intracelulares.

Os macrófagos do tipo M2 apresentam um perfil imunomodulador com a diminuição na expressão das moléculas citadas acima e são identificados pela expressão de outras citocinas (IL4 e IL13) e uma ampla variedade de marcadores, tais como a arginase-1 e o receptor de manose (CD206), sendo importante na resposta imune contra parasitas³⁹. A população M2 parece estar relacionada à evolução para a cronicidade das doenças infecciosas⁴⁰.

Na resposta imune frente a diversos vírus, bactérias, fungos e protozoários sabe-se que macrófagos ativados consomem L-arginina e a expressão de enzimas que degradam essa proteína é regulada por citocinas. A expressão de arginase I é induzida por IL4, IL10 e IL13⁴².

Nos últimos anos, a expressão de arginase e consequente depleção de L-arginina tem sido demonstrada como importante fator de imunossupressão⁴⁵.

STAT-1 em sua forma fosforilada (pSTAT-1) liga-se a regiões promotoras de genes estimulados por IFN e aumenta em resposta aos IFN dos tipos I, II e III. cMAF é fator de transcrição essencial para a expressão gênica de IL10 pelos macrófagos.

A caracterização da polarização de macrófagos *in situ* foi realizada em trabalho recente. Foram analisados os fatores de transcrição relacionados a essa polarização pelo método imuno-histoquímico de dupla-marcação. Utilizou-se os marcadores CD68 e CD163 em associação ao pSTAT-1, RBP-J (M1) e cMAF (M2)⁴⁶.

Estamos desenvolvendo simultaneamente três pesquisas, cada uma com um dos fungos abordados nesse texto sistematizado.

Na cromoblastomicose, sob minha orientação no doutorado, a aluna Aline Alves de Lima Silva está em fase final de análise dos dados. Na Doença de Jorge Lobo, também sob minha orientação no doutorado, a aluna Tânia Cristina Barboza está realizando os protocolos de imuno-histoquímica. Na paracoccidiodomicose, tive recém aprovado no Edital Universal do CNPq um projeto para compra de insumos e desenvolvimento dessa pesquisa, que será em parte, tema de dissertação de uma aluna.

4 DISCUSSÃO GERAL

Ao longo do tópico anterior, foram discutidos os achados referentes a cada um dos artigos publicados da linha de pesquisa que versa sobre a resposta imune compartimentalizada em lesões tegumentares da Paracoccidioidomicose, Cromoblastomicose e Doença de Jorge Lobo. Nesta seção será feita uma discussão geral, considerando-se não só uma análise comparativa entre as três infecções abordadas, mas também a colaboração de nossos resultados ao melhor conhecimento da resposta imune *in situ* em lesões cutâneas e mucosas dessas infecções humanas.

4.1 Paracoccidioidomicose

A paracoccidioidomicose é uma doença crônica granulomatosa causada pelo *Paracoccidioides sp*, sendo atualmente conhecidas duas espécies, o *P. brasiliensis* e o *P. lutzii*.

Clinicamente, as lesões cutâneas na paracoccidioidomicose variam em número e aspecto morfológico, podendo ser múltiplas, devido à disseminação hematogênica do fungo, ou únicas. Seu aspecto mais frequente é o de lesões ulceradas, e sua localização no segmento cefálico¹⁹⁵.

A resposta tecidual na paracoccidioidomicose caracteriza-se pela formação do granuloma, que é o reflexo da resposta imune específica aos antígenos fúngicos^{195,196}.

Nas lesões de paracoccidioidomicose são descritos dois tipos diferentes de granulomas: os compactos e bem organizados e os frouxos, ou pouco organizados. Seus constituintes macrofágicos expressam HLA-DR e junto com os linfócitos T apresentam papel importante na constituição do processo inflamatório, através da expressão de citocinas e outras moléculas^{197,198}.

O padrão de citocinas que se estabelece se relaciona com a configuração de dois polos opostos de resposta imune. No polo de melhor resposta são observados granulomas epitelioides bem organizados com poucos fungos enquanto que no polo de resposta imune

menos eficiente os granulomas são pouco organizados, frouxos, com número maior de fungo e exudação supurativa frequente¹⁹⁷.

Considerando-se os estudos acerca dos dendrócitos dérmicos Fator XIIIa+ como células fagocíticas e produtoras de citocinas, verificamos nas lesões cutâneas e de mucosa oral na paracoccidiodomicose a presença de número elevado dessa população celular distribuída no infiltrado dérmico superior e ao redor dos granulomas. A observação de formas fúngicas no citoplasma desses dendrócitos foi uma descoberta inédita e corroborou dados prévios da literatura que consideram essas células como atuantes nos processos inflamatórios tegumentares.

Outra contribuição que fizemos sobre a interação do fungo com as células do hospedeiro na paracoccidiodomicose foi a demonstração da interação de leveduras com os queratinócitos. Os resultados obtidos com a reação imuno-histoquímica de dupla marcação foram validados pela técnica de microscopia confocal a laser. Pudemos verificar na epiderme e no epitélio de mucosa a co-localização de formas fúngicas no citoplasma dessas células, sugerindo que os queratinócitos também possam ser alvo dos fungos, como mecanismo de escape dos eventos imunes dérmicos, uma vez que ao serem capazes de internaliza-los, não desencadeariam resposta fagocítica efetiva^{195,199}.

Em uma etapa seguinte a esse achado, fizemos nas lesões a caracterização imuno-histoquímica de citocinas de perfil Th1 e Th2 nas lesões cutâneas da paracoccidiodomicose. Foi possível relacionar os granulomas compactos ao perfil Th1 e os granulomas frouxos ao perfil Th2. Havia ainda um terceiro grupo, caracterizado por ambos os tipos de perfis citocínicos e ambos os tipos de granulomas na mesma lesão.

Entre as citocinas estudadas, chamou-nos a atenção células com expressão de TNF- α e morfologia dendrítica ao redor dos granulomas, disposição semelhante aos dendrócitos dérmicos ali localizados e também células com expressão de IL10 com morfologia e localização de mastócitos.

Com base em nossos resultados pudemos inferir que os dendrócitos Fator XIIIa+, além de interagirem com o *Paracoccidioides brasiliensis* como células fagocíticas ou células-alvo, poderiam ainda ser produtores de TNF- α (propriedade descrita em outras doenças) e,

desse modo, colaborar na ativação e recrutamento de macrófagos para a constituição e manutenção do granuloma.

Quanto aos mastócitos, a co-localização de IL10 e marcador específico para tais células sugere que os mastócitos representam mais uma possível fonte desta citocina nas lesões de perfil Th2, colaborando para a resposta imune menos efetiva frente aos fungos.

A resposta imune reguladora associada à resposta Th1 é importante para a resolução das infecções. Ao contrário, uma ineficiência desse padrão imune colabora para a constituição da resposta do tipo Th2, com aumento das citocinas IL4, IL5 e IL10.

O perfil regulador na resposta imune da paracoccidiodomicose foi verificado em nossos trabalhos ao estudarmos a expressão de Foxp3, CD25, TGF- β e IL-10. As células Treg podem interferir na eficácia da imunidade do hospedeiro ou representar um mecanismo que o beneficia, uma vez que diminuem os danos que geralmente se seguem a uma resposta imune que pode ser exagerada¹⁶⁸. Células Foxp3+ foram visualizadas no infiltrado inflamatório de ambos os tipos de lesão na paracoccidiodomicose, cutânea e mucosa, sem diferenças quanto ao tipo de granuloma na pele, mas em maior número nos granulomas compactos de mucosa. Por outro lado, a ativação excessiva pode estar associada a susceptibilidade. Níveis elevados de células T reguladoras no sangue e nas lesões da paracoccidiodomicose foram também descritos²⁰⁰.

Além desses perfis citocínicos também detectamos, de modo pioneiro, a expressão de IL17 nas lesões tegumentares da paracoccidiodomicose. Essa citocina faz parte de um perfil descrito mais recentemente e acredita-se que seja acionado numa tentativa de dar suporte ao padrão Th1 quando esse não é suficiente para a resolução dos processos infecciosos.

Como já destacamos previamente nas lesões de paracoccidiodomicose, os granulomas compactos (melhor organizados) estão associados ao padrão Th1 de citocinas. As células Treg poderiam contribuir localmente para diminuir o dano tecidual e a manutenção da reação granulomatosa, e desse modo, estarem envolvidas na modulação da resposta imune nas lesões mucosas. Por outro lado, foi inesperada a semelhança numérica das células Treg nos três nos grupos de resposta tecidual observada nas lesões cutâneas. Por

outro lado, o número de células com expressão de Foxp3 é maior que os de células com expressão de IL17.

Na relação patógeno-hospedeiro da paracoccidioidomicose, deve haver o desencadeamento de mecanismo eficaz para eliminação do fungo com equilíbrio na intensidade da resposta granulomatosa a fim de evitar lesão tecidual, ou seja, equilíbrio entre a atividade pro e anti-inflamatória¹⁹⁵.

Outro aspecto interessante descrito na literatura é que células T reguladoras têm capacidade de aumentar a expressão de granzima B, função, portanto citotóxica à semelhança dos linfócitos TCD8+¹⁷². Essa função corrobora nossos achados sobre o papel da resposta imune citotóxica na paracoccidioidomicose. Verificamos um número elevado de células com expressão de granzima B nas lesões de paracoccidioidomicose, além de número elevado de linfócitos TCD8+. Originalmente a importância dos linfócitos TCD4+ é bem descrita e com nossos achados acreditamos que nas lesões de pele e de mucosa oral, a resposta citotóxica também desempenha papel importante contra os antígenos fúngicos.

Finalizando, no contexto de células dendríticas, além do envolvimento dos dendrócitos dérmicos Fator XIIIa+ descritos acima, também estudamos as células de Langerhans através da expressão de CD1a e de CD207 (langerina). Destacamos que as células langerina positivas estão presentes tanto na epiderme quanto na derme, bem como no epitélio e na lâmina própria de lesões de mucosa. A redução numérica e alteração trófica dessas células poderia ser resultado de fatores inibidores do fungo, como mecanismo de escape. Vale salientar que foram visualizadas formas fúngicas nesses sítios quando estudamos sua interação com queratinócitos. Por outro lado, há que se considerar o encontro de células langerina+ na derme e na lâmina própria das mucosas, o que poderia ser a evidência da migração dessas células ou ainda, evidência de uma nova população de células dérmicas langerina+, como sugerido em trabalhos de Romani *et al.*¹⁵.

Outra população de células dendríticas estudadas foram as pDC, evidenciadas pela expressão de CD123 e CD303. Por serem produtoras de IFN- α/β , as pDC seriam capazes de participar da imunomodulação da resposta imune como na diferenciação de células dendríticas mieloides, ativação da resposta Th1, ativação de células NK e indução da

resposta humoral^{201,202}. A Figura 1 demonstra esquematicamente a interação entre os achados discutidos nas lesões tegumentares na paracoccidioidomicose.

Figura 1 – Contribuições ao estudo da resposta imune *in situ* em lesões tegumentares na paracoccidioidomicose. Desenho esquemático dos achados discutidos nos trabalhos apresentados.

4.2 Cromoblastomicose

A cromoblastomicose é uma doença crônica confinada à pele e tecido subcutâneo. Ocorre por implantação traumática de fungos da família *Dematiaceae*, provenientes do solo e plantas. Acomete em sua maioria pessoas do sexo masculino, provavelmente pela maior exposição em atividades rurais^{203,204}.

A resposta tecidual é caracterizada pelos granulomas epitelioides, em geral de centro supurado.

As lesões verrucosas são as mais frequentes, de modo que a nossa casuística obtida da Divisão de Clínica de Dermatologia do HCFMUSP, foi constituída somente de casos do tipo verrucoso.

O polimorfismo lesional parece estar relacionado ao tipo de resposta imune tecidual. Nossos resultados evidenciam que citocinas de perfil Th1 predominam nas lesões do tipo placa atrófica, de melhor controle e prognóstico, enquanto que as citocinas de perfil Th2 estão presentes em maior número nas lesões verrucosas, que são de difícil abordagem terapêutica.

Na paracoccidioidomicose, a resposta imune menos efetiva contra os antígenos dos fungos é caracterizada pela ativação ineficiente dos macrófagos e de células dendríticas com consequente formação de granulomas pouco organizados e maior número de fungos nas lesões. Da mesma forma, na cromoblastomicose, a persistência dos fungos nas lesões e o infiltrado inflamatório exuberante, supurativo visto nas lesões do tipo verrucoso refletem em maior gravidade e cronicidade das mesmas.

Embora sejam vistos muitos macrófagos, dendrócitos dérmicos Fator XIIIa e células de Langerhans nas lesões da cromoblastomicose¹¹⁸, a fagocitose das formas fúngicas pode estar prejudicada, uma vez que os fungos persistem viáveis nas lesões. Certamente, um dos mecanismos de escape desse agente é a presença de melanina em sua parede, o que pode dificultar o seu reconhecimento como antígeno pelo sistema imune do hospedeiro.

Estamos atualmente realizando uma pesquisa mais detalhada acerca das populações macrofágicas presentes nas lesões do tipo verrucoso na cromoblastomicose. Estamos empregando o método imuno-histoquímico com anticorpos específicos para as populações de macrófagos do tipo M1 e M2. Em resultados preliminares, observamos que a população de macrófagos M2 parece prevalecer.

Ao estudar as lesões verrucosas, chamou-nos a atenção o número expressivo de células IL17+, incluindo células de Langerhans epidérmicas que expressavam essa citocina. Embora, como mencionado na discussão sobre paracoccidioidomicose, esse perfil citocínico possa auxiliar a resposta imune local quando a resposta Th1 não é eficiente, acreditamos que da mesma forma, um desequilíbrio com relação aos mecanismos reguladores possa ser deletério ao paciente, no sentido de contribuir para a patogenia e dano tecidual. Na psoríase a melhora expressiva das lesões dos pacientes tratados com drogas anti-IL17, corrobora a possibilidade aventada sobre o papel da IL17 na patogenia da cromoblastomicose.

Demonstramos também a presença de células dendríticas plasmocitoides nas lesões de cromoblastomicose. Além da frequente expressão de CD123, também evidenciamos a expressão de CD303. Sugerimos pelos nossos resultados que pelo menos na cromoblastomicose, considerando-se a expressão de CD303 (um receptor de lectina-c), as células dendríticas plasmocitoides podem interagir com as formas fúngicas, à semelhança da interação de fungos com as moléculas CD205, CD207 e CD209, que representam outros receptores deste tipo. Ademais, as pDC poderiam atuar nessa infecção como colaboradoras na produção de citocinas Th1, uma vez que são indutoras de interferons^{201,202}.

Na Figura 2 demonstramos esquematicamente a interação entre os achados discutidos nas lesões cutâneas de cromoblastomicose.

Figura 2 – Contribuições ao estudo da resposta imune *in situ* em lesões cutâneas na cromoblastomicose. Esquema dos principais achados discutidos anteriormente.

4.3 Doença de Jorge Lobo

A Doença de Jorge Lobo é uma infecção crônica que acomete a pele e tecido subcutâneo, endêmica na região amazônica.

De maneira semelhante à cromoblastomicose, a variedade de formas clínicas das lesões parece estar relacionada ao tipo de resposta imune do hospedeiro¹⁰⁶. De acordo com nossos resultados ao estudar um paciente que apresentava lesões múltiplas com aspecto clínico diverso, demonstramos que as lesões nodulares evidenciaram perfil do tipo Th1 e as lesões infiltrativas o tipo Th2 de citocinas. As demais são intermediárias quanto às citocinas presentes. Desse modo, podemos considerar que a Doença de Jorge Lobo compreende um espectro clínico de manifestações, como observado em outras doenças infecciosas.

O estudo imunofenotípico das lesões evidencia a presença de infiltrado inflamatório composto por macrófagos, linfócitos, células NK, plasmócitos, linfócitos T (CD4 e CD8) e B¹¹⁰. De acordo com nossos resultados, apesar de haver número expressivo de linfócitos TCD4+, há importante expressão de células TCD8+ e as lesões cutâneas nos pacientes são agressivas e persistentes. Xavier *et al.*¹¹² demonstraram a elevada expressão de TGF- β por macrófagos nessas lesões. Esse fator é um potente imunossupressor, embora promova a proliferação de linfócitos TCD8+. O TGF- β apesar de favorecer o aumento numérico, poderia prejudicar a atividade citotóxica dessas células^{156,157}.

Ainda nos estudos sobre células dendríticas, evidenciamos dendrócitos dérmicos Fator XIIIa+ hipertróficos e de localização à semelhança do observado nas lesões de paracoccidiodomicose. Além disso, na Doença de Jorge Lobo, apesar do número reduzido de células de Langerhans epidérmicas, foi frequente o encontro de células langerina+ na derme. Sugerimos que essas populações celulares parecem ter papel importante na resposta imune frente ao *L. loboi*. Por outro lado, verificamos que as pDC não participam da resposta imune nas lesões dessa micose.

Na Doença de Jorge Lobo o perfil citocínico evidencia predomínio de citocinas de padrão Th2, que parece modificar a regulação dos mecanismos de contenção do patógeno¹¹⁰. No trabalho de Xavier *et al.*¹¹² o número elevado de células com expressão de

TGF- β 1 sugere seu papel na patogênese da infecção por *L. loboi*, seja inibindo a resposta imune celular mediada por macrófagos ou por induzir fibrose.

A contribuição da IL17 nos mecanismos imunes eficientes em infecções fúngicas ou mesmo de outros agentes infecciosos, ainda é assunto controverso. Como discutimos em casos de paracoccidioidomicose e cromoblastomicose, apesar da IL17 representar uma tentativa do organismo em suprimir uma resposta Th1 ineficaz, o excesso dessa resposta pode vir a ser deletério ao tecido, favorecendo, inclusive, a multiplicação de organismos²⁰⁵. O equilíbrio com mecanismos imunorreguladores é fundamental para o controle das infecções e do dano tecidual resultante.

Uma vez que já havíamos estudado o perfil Th17 e T regulador de citocinas na paracoccidioidomicose e cromoblastomicose e, estando desenvolvendo diferentes abordagens em lesões na Doença de Jorge Lobo, ficamos curiosos quanto à participação desse perfil também nesta infecção fúngica e seu papel na patogenia da doença.

Nossos resultados evidenciam a participação da resposta Th17 nessa infecção fúngica. A presença de citocinas pró-inflamatórias IL1 e IL6, aliada aos achados prévios da presença expressiva de TGF- β , seriam fatores estimuladores dessa resposta. É importante considerar, entretanto, que a elevada expressão do padrão Th17 pode ter papel deletério e contribuir para a patogenia da doença, o que ainda é um assunto controverso, à semelhança do que evidenciamos na cromoblastomicose.

Verificamos a presença de linfócitos Foxp3+ em todos os casos estudados. A expressão de IL17 foi observada tanto em linfócitos como em neutrófilos presentes na resposta tecidual. A expressão de IL1, CD25, IL6 e IL23 foi também verificada no infiltrado inflamatório e junto dos focos granulomatosos. A citocina mais frequente foi a IL17, seguida por IL6. Entretanto, o número de Treg (Foxp3+) foi maior que o de células com expressão de IL17. Esse resultado sugere a predominância dos mecanismos reguladores, o que poderia ser fator relacionado à persistência das lesões na Doença de Jorge Lobo.

De acordo com Azevedo (2015) as células Th17 não estariam associadas à proteção na Doença de Jorge Lobo, por haver expressão elevada de IL17 nos pacientes com possível papel deletério e envolvido na patogenia da doença²⁰⁶.

Demonstramos que a densidade microvascular sanguínea e linfática está reduzida nas lesões da Doença de Jorge Lobo. Aspecto semelhante foi descrito na aspergilose pulmonar¹⁹⁰. A redução da densidade microvascular pode explicar porque a disseminação da infecção para outros órgãos é muito rara, apesar do alto número de fungos nas lesões.

A redução da rede vascular sanguínea e linfática na Doença de Jorge Lobo pode estar relacionada ao fato de que o TGF- β , expressivo nos sítios de lesão, pode inibir a proliferação de células endoteliais, atenuando o estímulo angiogênico¹⁹¹, além de inibir a expressão de E-selectina. Outra possibilidade é a alteração da matriz extracelular e degradação de fibras elásticas com consequente dano vascular¹⁹².

Verificamos também maior número de vasos expressando as moléculas ICAM-1 e VCAM-1 e, por outro lado, baixa expressão de E-selectina. A expressão de ICAM-1 é importante componente para o recrutamento de leucócitos e formação do infiltrado inflamatório¹⁹⁴. A expressão elevada de TGF- β nessa doença merece atenção, pela capacidade de interferir na expressão de E-selectina.

Resumindo, na Doença de Jorge Lobo ocorre disfunção da microvascularização das lesões. Ao mesmo tempo em que a redução na densidade vascular seria fator limitante local para a proliferação dos fungos, representaria fator de interferência na adequada recirculação de células inflamatórias necessárias para o estabelecimento de uma resposta adaptativa adequada. Por outro lado, a expressão de ICAM-1 compensaria a manutenção do processo infeccioso e afluxo de monócitos para prover os macrófagos da resposta tecidual.

Na Figura 3 demonstramos a interação dos principais achados discutidos nos trabalhos realizados.

Figura 3 – Contribuições ao estudo da resposta imune *in situ* em lesões cutâneas na doença de Jorge Lobo. Desenho esquemático da relação entre os achados discutidos.

5 CONSIDERAÇÕES FINAIS

Nossos estudos que enfocam diversos aspectos da relação parasita-hospedeiro em ambiente tegumentar das três micoses endêmicas em nosso país, demonstram aspectos semelhantes e comuns às três doenças e aspectos particulares a cada uma delas.

Devemos ressaltar que, embora na Paracoccidioidomicose o acometimento cutâneo e mucoso são frequentes e expressivos, a doença é sistêmica. O fungo tem a capacidade de se disseminar quer seja por via linfática, hematogênica e por contiguidade a diferentes órgãos. Além disso, a pele e a mucosa podem ser acometidas nas diferentes formas clínicas da doença, que se manifestam com quadro clínico e características imunológicas diferentes. As lesões tegumentares, por outro lado, mostram perfis imunológicos que acompanham, de certo modo, o padrão de resposta tecidual e a capacidade de resposta do hospedeiro.

A cromoblastomicose, apesar de sua evolução prolongada, rebelde a diferentes manobras terapêuticas, permanece confinada ao ambiente cutâneo e subcutâneo. A sua resposta tecidual básica granulomatosa é semelhante à da paracoccidioidomicose, assim como o comportamento de alguns elementos da resposta imune nas lesões. Nessa doença observam-se diferenças no perfil imunológico local, de acordo com a apresentação clínica das lesões, mas apesar dessas diferenças a doença permanece confinada à pele e, raramente se dissemina para outros órgãos.

A Doença de Jorge Lobo, apesar de também permanecer confinada à pele e tecido subcutâneo, tem curso prolongado e rebelde ao tratamento, como a cromoblastomicose. Demonstramos que essa micose difere da paracoccidioidomicose e cromoblastomicose quanto ao comportamento das células dendríticas, em particular as células de Langerhans e pDC. Além disso, os aspectos da densidade microvascular e do endotélio vascular estariam envolvidos na permanência dos fungos nos sítios de lesão e, por outro lado, na incapacidade de sua eliminação.

A fim de concluir o painel de pesquisas que desenvolvemos até o momento: participação de células dendríticas, subpopulações de linfócitos, perfil de citocinas, participação de queratinócitos, do endotélio, da microcirculação e subpopulações de macrófagos, de maneira a comparar todos esses aspectos nas três infecções fúngicas de

acometimento cutâneo, pretendemos avaliar a participação dos mastócitos e queratinócitos nas lesões de cromoblastomicose e Doença de Jorge Lobo e estudar os marcadores de endotélio e expressão de moléculas de adesão na paracoccidioidomicose e cromoblastomicose.

Acreditamos que essas pesquisas poderão contribuir para o melhor entendimento dos mecanismos imunopatológicos dessas doenças endêmicas no Brasil e também naqueles de outras doenças infecciosas com acometimento tegumentar, uma vez que a pele é um órgão de fácil acesso para a observação e análise dos processos patológicos que a envolvem.

Conclusões

6 CONCLUSÕES

Ao avaliarmos nossas pesquisas nas infecções fúngicas de acometimento tegumentar Paracoccidioidomicose, Cromoblastomicose e Doença de Jorge Lobo, acreditamos ter obtido dados interessantes que contribuíram para a compreensão da interação patógeno-hospedeiro.

Na Paracoccidioidomicose, cujo foco primário de infecção é o pulmão, as lesões cutâneas apresentam mecanismos patogênicos semelhantes aos que ocorrem em outros órgãos, porém apresentam elementos característicos de seu sistema imune local.

Na Cromoblastomicose e Doença de Jorge Lobo, esses elementos do sistema imune da pele também desempenham papel importante frente aos antígenos fúngicos.

Especificamente, podemos concluir que:

6.1 Na Paracoccidioidomicose, Cromoblastomicose e Doença de Jorge Lobo ocorre a participação das **células dendríticas cutâneas**:

a) Os **dendrócitos dérmicos Fator XIIIa+** participam da resposta imune tegumentar na paracoccidioidomicose: a sua interação com o *Paracoccidioides sp.* sugere sua atuação como apresentadora de antígenos e/ou célula-alvo para a infecção fúngica; relacionam-se com a produção e indução de TNF- α que modula a resposta imune, atuam na ativação de macrófagos e na organização e manutenção dos granulomas.

b) Na Doença de Jorge Lobo, os **dendrócitos dérmicos Fator XIIIa+** embora exibam alterações tróficas, não diferem em número do observado na pele normal.

c) Na Paracoccidioidomicose as **células de Langerhans** têm alterações tróficas e estão diminuídas em número. Essas alterações resultariam de ação lesiva do fungo.

d) Demonstramos pela primeira vez a participação de **células CD207+** nas lesões tegumentares da Paracoccidioidomicose. Essas células comportaram-se como as células de Langerhans nos epitélios das lesões, entretanto a sua presença na derme/lâmina própria dos tegumentos pode ser interpretada como células de Langerhans no processo de migração ou uma nova população de células dendríticas dos tegumentos.

e) Na Doença de Jorge Lobo a população de **células de Langerhans** epidérmicas é semelhante à da pele normal. As células **dendríticas CD207+**, comportam-se de modo semelhante às da Paracoccidioidomicose.

f) As células dendríticas plasmocitoides participam da resposta tecidual da Paracoccidioidomicose e Cromoblastomicose, entretanto são ausentes nas lesões da Doença de Jorge Lobo. Essas células podem interagir com o agente fúngico da Cromoblastomicose, uma vez que expressam a molécula CD303 (lectina ligante do agente infeccioso).

g) A ausência de **células dendríticas plasmocitoides** nas lesões da Doença de Jorge Lobo pode representar um dos prováveis mecanismos imunes deficientes para a completa eliminação do *Lacazia loboi* e manutenção das lesões.

6.2 O perfil de citocinas Th1 e Th2 da Paracoccidioidomicose, Cromoblastomicose e Doença de Jorge Lobo, de acordo com a organização da resposta tecidual e tipos clínicos das lesões cutâneas:

a) O **perfil de citocinas** na Paracoccidioidomicose é concordante com a melhor/pior organização da resposta tecidual: resposta tecidual com granulomas organizados e perfil Th1; resposta tecidual pouco organizada e perfil Th2.

b) Na cromoblastomicose o **perfil de citocinas** tem relação com o tipo de lesão clínica observada: lesões em placa atrófica e perfil Th1; lesões verrucosas e perfil Th2.

c) Na doença de Jorge Lobo o **perfil de citocinas** reflete a natureza espectral das lesões: lesões nodulares e perfil Th1; lesões infiltrativas e perfil Th2. Nessa doença ocorre a compartimentalização da resposta imune de acordo com as diferentes expressões clínicas num mesmo paciente.

6.3 O perfil de citocinas Th17 da Paracoccidioidomicose, Cromoblastomicose e Doença de Jorge Lobo, de acordo com a organização da resposta tecidual e tipos clínicos das lesões cutâneas:

a) Há participação de células com expressão de **IL17** na paracoccidioidomicose, inclusive em localização perivascular, o que poderia ser fator ativador do endotélio.

b) A constituição de uma resposta imune local com elevada expressão de **IL17** na cromoblastomicose poderia representar uma tentativa do sistema imune em eliminar a infecção fúngica. Por outro lado, esses valores elevados quando comparados com a expressão de células T reguladoras Foxp3+ poderia ter como consequência um desequilíbrio imune e consequente resposta menos efetiva.

c) Na Doença de Jorge Lobo, o perfil **Th17** está bem caracterizado, com expressão de citocina inflamatórias IL1 β e IL6, bem como TGF- β , além da IL17. O perfil Th17 poderia em parte ser benéfico a eliminação fúngica. Tais células se sobrepõem numericamente à expressão de Foxp3, o que pode significar um desequilíbrio imune e consequente resposta imune ineficaz.

6.4 Mecanismos de citotoxicidade nas lesões tegumentares da Paracoccidioidomicose, Cromoblastomicose e Doenças de Jorge Lobo:

a) **Linfócitos TCD8+** possuem função citotóxica na paracoccidioidomicose, expressando perforina e granzima. A localização desta última em células gigantes permite inferir que a ação citotóxica é importante na eliminação de formas fúngicas.

b) **Linfócitos TCD8+** estão presentes nas lesões de cromoblastomicose

c) Na Doença de Jorge Lobo a resposta imune citotóxica parece também representar um importante mecanismo no controle da infecção, evidenciada pela elevada expressão de **linfócitos TCD8+** e granzima B.

6.5 Mecanismos reguladores nas lesões tegumentares da Paracoccidioidomicose, Cromoblastomicose e Doenças de Jorge Lobo:

a) **Linfócitos T reguladores Foxp3+** parecem participar de mecanismos moduladores na Paracoccidioidomicose, em lesões cutâneas e de mucosa oral. Sua expressão se correlaciona às diferentes respostas granulomatosas verificadas, sendo maior nos granulomas compactos.

b) Na Cromoblastomicose, bem como na Doença de Jorge Lobo, os mecanismos reguladores mediados por **linfócitos T Foxp3+** estão diminuídos, podendo significar um desequilíbrio na resposta imune, com exacerbação da resposta inflamatória evidenciada.

6.6 Participação dos mastócitos nas lesões tegumentares da Paracoccidioidomicose:

a) Os **mastócitos**, avaliados na Paracoccidioidomicose, participam da resposta imune local nas lesões com granulomas pouco organizados e perfil Th2 de citocinas. Essa participação é evidenciada pela expressão de IL10, a qual poderia contribuir para a resposta menos efetiva frente aos antígenos fúngicos.

6.7 Interação do queratinócito com o *Paracoccidioides sp.* nas lesões tegumentares da Paracoccidioidomicose:

a) Os **queratinócitos**, avaliados nas lesões de pele e mucosa oral na paracoccidioidomicose, são parasitados pelo *Paracoccidioides sp.*, o que pode representar um possível processo de evasão do fungo dos mecanismos imunes locais. Além disso, por suas características fenotípicas, tais células poderiam atuar como apresentadoras de antígenos, na tentativa de suprir as funções reduzidas das células de Langerhans.

6.8 A densidade microvascular cutânea e **moléculas de ativação/adesão endotelial** nas lesões da Doença de Jorge Lobo:

a) Na Doença de Jorge Lobo, há **disfunção microvascular** que reflete na progressão clínica da doença. A expressão de ICAM-1 e inibição de E-selectina permite a manutenção do processo infeccioso, com conseqüente microambiente com resposta imune deficiente mediada por células.

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Correlation of factor XIIIa+ dermal dendrocytes with paracoccidioidomycosis skin lesions

C. PAGLIARI* & M. N. SOTTO*†

*Department of Pathology and †Dermatology, University of São Paulo School of Medicine, São Paulo, Brazil

We demonstrated and quantified by immunohistochemistry the factor XIIIa+ dermal dendrocytes (FXIIIa+ DD) in paracoccidioidomycosis skin lesions. Sixty-one biopsies were classified according to the tissue response in well-organized granulomas (group 1), poorly organized granulomas (group 2) and samples showing both kinds of granuloma (group 3). Ten biopsies from normal skin were used as controls. In order to verify the internalization of *Paracoccidioides brasiliensis* antigens by FXIIIa+ DD, we performed a double immunostaining technique. FXIIIa+ DD were hypertrophied with prominent dendrites and their number in the test groups was higher than in the control group, especially in the dermal papillae. *P. brasiliensis* yeasts were seen within the cytoplasm of FXIIIa+ DD in 40% of the immunostained biopsies. We could correlate these findings with the probable role of FXIIIa+ DD as antigen-presenting cells in the pathogenesis of skin lesions in paracoccidioidomycosis.

Keywords dermal dendrocytes, factor XIIIa, immunohistochemistry, paracoccidioidomycosis

Introduction

Paracoccidioidomycosis (PCM) is a chronic, systemic granulomatous disease caused by the dimorphic fungus *Paracoccidioides brasiliensis* and is endemic in Brazil and other Latin America countries [1]. Clinical manifestations are mainly characterized by involvement of the lungs, lymph nodes, adrenal glands, respiratory tract mucous membranes and skin [2]. Cell-mediated immunity (CMI) plays an important role in host defense against the PCM agent and the intensity and severity of the disease is related to CMI depression [3].

In skin lesions, the pathogen may be found in the centers of granulomas that contain it and prevent its multiplication [4,5]. These lesions are characterized by a reduced number of Langerhans cells (LC), reflecting the depressed CMI response secondary to infection with *P. brasiliensis*. It has been suggested that low numbers of

LC may result in an impairment in the local processing of foreign antigens [6,7].

There are diverse populations of immunocompetent dendritic cells in skin [810]. A recently described group of such cells is the factor XIIIa+ dermal dendrocytes (FXIIIa+ DD), distinguished by the expression of the coagulation factor XIIIa (which is a transglutaminase) in their cytoplasm [11,12]. In normal skin, these dendritic cells are particularly numerous in the dermal papillae, although they are also present throughout the reticular and deep dermis [13].

In inflammatory conditions, increased numbers of FXIIIa+ DD have been described, but not in granulomatous diseases such as sarcoidosis and tuberculosis [14]. Some authors have shown that they are as potent as LC in the internalization, processing and presentation of soluble protein antigens to T cells [15–17]. In psoriasis, it was demonstrated *in vitro* that FXIIIa+ DD induced T lymphocytes to produce interferon- γ and interleukin 2 [18].

In view of these considerations, the objectives of the present paper were to demonstrate and quantify the FXIIIa+ DD in PCM skin lesions and to verify their possible role in the pathogenesis of the disease.

Correspondence: Mírian N. Sotto, Av. Dr. Arnaldo, 455 sala 1118, cep 01246-903, São Paulo, SP, Brazil. Tel./Fax: +55 11 3066-7238; E-mail: mnsotto@ajato.com.br

Material and methods

Skin biopsies

Sixty-one biopsies from PCM patients were selected from the files of the Dermatopathology Laboratory of the Department of Dermatology of the University of São Paulo Medical School. All specimens had been fixed in neutral-buffered formalin and embedded in paraffin. They were classified according to whether the tissue response [19,20] showed well-organized granulomas (group 1), poorly organized granulomas (group 2) or both kinds of granulomas (group 3).

Ten biopsies of normal skin obtained from orthopedic knee surgery were utilized as controls (group 4).

Antibody purification

A positive immunoreactivity of fungal walls for Factor XIIIa has been described, suggesting a relationship between this biological marker and the pathogenicity of fungi [21]. In order to block this immunostaining we performed a purification of the anti-FXIIIa antibody. *P. brasiliensis* strain Pb 339 was cultured in the yeast phase at 37 °C for 10 days in Sabouraud medium with chloramphenicol (100 mg l⁻¹). These cultures were formalin-killed for 3 days at 4 °C. After centrifugation, the supernatant was removed and the resulting sediment of yeast cells (80 mg) was incubated for 3 days in 1000 µl of the anti-FXIIIa antibody at the dilution of 1:50.

Immunohistochemistry

Tissue sections were cut 4 µm thick and were transferred to glass slides coated with amino-propyltriethoxy-silane (Sigma Chemical Co, St. Louis, MO, USA). They were dewaxed in xylene and hydrated through a graded series of ethanol dilutions. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 30 min at room temperature. Pre-incubation with trypsin (Sigma) was performed for 15 min at a concentration of 0.015% in 0.134% CaCl₂ (E. Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS; 0.01 M, pH 7.4) at 37 °C.

The rabbit-derived polyclonal antibody anti-human coagulation factor XIIIa-subunit (Calbiochem-Novabiochem Co., La Jolla, CA, USA) was applied to the sections at a dilution of 1:200 overnight at 4 °C. Biotinylated anti-rabbit antibody was then used at a dilution of 1:1000 for 30 min at 37 °C (Dako Corporation, Carpinteria, CA, USA). The peroxidase-labeled streptavidin-biotin complex (SABC, Dako Corporation, Carpinteria, CA, USA) was applied for 30 min at 37 °C at a dilution of 1:1000. 3,3-Diaminobenzidine tetrahy-

droxychloride (Sigma) was used as chromogen and the slides were counterstained with hematoxylin.

Immunohistochemical double staining

In order to verify the internalization of *P. brasiliensis* antigens by FXIIIa+ DD, a double immunostaining was performed using a Doublestain Envision System (Dako) [22]. After deparaffinization with xylene and hydration of the tissue, endogenous peroxidase activity was blocked with 0.03% hydrogen peroxide for 5 min. Slides were incubated for 30 min at 37 °C with the polyclonal rabbit antibody anti-*P. brasiliensis* (kindly supplied by Prof. Dr Carlos da Silva Lacaz—Tropical Medicine Institute of São Paulo Medical School) diluted 1:2000. Peroxidase-labeled polymer conjugated to affinity purified goat anti-rabbit antibody was applied for 30 min at 37 °C and the reaction was visualized with diaminobenzidine. The first reaction was blocked with doublestain blocking solution and incubated with trypsin (Sigma) at a concentration of 0.015% in 0.134% CaCl₂ (E. Merck) in PBS buffer at 37 °C for 15 min. Slides were incubated for 30 min at 37 °C with a 1:200 dilution of polyclonal antibody anti-FXIIIa (Calbiochem-Novabiochem). Alkaline phosphatase-labeled polymer conjugated to affinity-purified anti-rabbit antibody was applied for 30 min at 37 °C and the reaction was visualized with fast red and naphthol.

Factor XIIIa+ DD quantification

FXIIIa+ DD were quantified by counting the number of immunolabelled cells in 15 randomized high power fields (HPF) for each specimen with a × 10 ocular with a square grid and a × 40 objective [23].

Statistical analysis

The number of FXIIIa+ DD was statistically analyzed by using Graph Pad Prism version 3.00 for Windows (GraphPad software, San Diego, CA, USA) to perform one-way ANOVA via the Kruskal–Wallis test with the level for significance set at 95% ($P \leq 0.05$).

Results

FXIIIa+ DD were hypertrophied with prominent dendrites in the three PCM groups lesions, mainly in the dermal papillae and around the superficial blood vessels (Fig. 1a). In the control group, these cells exhibited a spindle morphology (Fig. 1b). FXIIIa+ DD were not part of the granuloma, although they were found around them (Fig. 1c) and throughout the dermis.

The double immunostaining technique showed *P. brasiliensis* yeasts within the cytoplasm of FXIIIa+ DD in

Fig. 1 a, PCM skin lesion: hypertrophic dendrocytes in the dermal papillae labeled in brown (arrowheads) [anti-factor XIIIa (FXIIIa) antibody, peroxidase-labeled SABC (SABC/HRP) method] (bar = 25 μ m). b, Control skin: FXIIIa+ spindle cells labeled in brown (arrowhead) in papillary dermis [anti-FXIIIa antibody SABC/HRP method] (bar = 25 μ m). c, PCM skin lesion: FXIIIa+ DD labeled in brown around granulomatous lesion [anti-FXIIIa antibody SABC/HRP method] (bar = 50 μ m). d, PCM skin lesion: Doublestain Envision System with anti-*P. brasiliensis* and anti-FXIIIa antibodies showing yeast-form of *P. brasiliensis* labeled in brown (arrow) within the cytoplasm of FXIIIa+ DD labeled in red (arrowhead). There are hypertrophic FXIIIa+ DD strongly stained in red (arrowhead) (bar = 25 μ m).

40% of the biopsies studied (Fig. 1d). The polyclonal anti-*P. brasiliensis* antibody showed the fungi in brown, whereas FXIIIa+ DD were stained red. The latter cells were seen to be hypertrophied just as the positively labeled cells had been when the anti-factor XIIIa antibody alone was used. Yeast cells were found in the cytoplasm of both FXIIIa+ DD and other phagocytic cells that were FXIIIa negative.

There was a statistically significant difference in the number of FXIIIa+ DD in the reticular dermis of group 3 as compared with the control group ($P < 0.01$; Fig. 2).

Discussion

PCM is thought to be initiated in the lungs following inhalation of mycelial particles. Dissemination to other organs then occurs. Cutaneous lesions are very frequent and may originate from hematogenous dissemination,

Fig. 2 Distribution of FXIIIa+ DD/mm² in Paracoccidioidomycosis skin lesions and control group: ■ control group; □ well-organized granuloma; ● poorly-organized granuloma; ○ both kinds of granuloma.

pre-existing contiguous lesions and, rarely, from the direct inoculation of the fungus into the skin. Skin lesions that have arisen as a result of hematogenous dissemination from pre-existing lung lesions may be considered a sign of an inefficient cellular immune response [4].

The granuloma formation caused by *P. brasiliensis* reflects the host immune system's attempt to contain and destroy the pathogen. The first stage in the recognition of antigen by the immune system is its processing by antigen presenting cells (APC). In a previous report, Sandoval *et al.* [24] could not demonstrate *P. brasiliensis* antigen in the cytoplasm of Langerhans cells in cutaneous PCM lesions when they used a double immune staining technique (monoclonal antibody anti-S100 protein and polyclonal antibody anti *P. brasiliensis*). They suggested that macrophages may act as APC in PCM skin lesions. Although LC are well characterized as APC, another group of dendritic cells with similar characteristics have been described, namely, the FXIIIa+ DD [15–17,25,26]. We demonstrated that in PCM skin lesions the FXIIIa+ DD appeared hypertrophic and hyperplastic, and were found localized with unusual frequency in the superficial and reticular dermis around the granuloma. These morphological alterations have been suggested to derive from human leukocyte antigen system (HLA-DR) overexpression and from the degree of FXIIIa+ DD activity in antigen presentation [17]. The perivascular location of FXIIIa+ DD is ideal for interaction with T lymphocytes and for initiation of an immune response. Some authors have speculated that FXIIIa+ DD can replace LC in this function [26], whereas others have proposed that these cells may be precursors of LC [27].

The results of the present study suggest a role for FXIIIa+ DD as antigen-presenting or target cells in PCM skin lesions.

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Jorge Lobo's Disease: Immunohistochemical Characterization of Dendritic Cells in Cutaneous Lesions

Tania Cristina Barboza · Juarez Antonio Simões Quaresma · Arival Cardoso de Brito ·
Marilia Brasil Xavier · Clivia Maria Moraes de Oliveira · Deborah Aben Athar Unger ·
Maria Irma Seixas Duarte · Mirian Nacagami Sotto · Carla Pagliari

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Abstract

Background Jorge Lobo's disease (JLD) is a cutaneous chronic mycosis caused by *Lacazia loboi*. We studied Factor XIIIa + dermal dendrocytes (FXIIIa + DD), Langerhans cells (LC) through the expression of langerin and the expression of S100 protein.

Methods A total of 41 biopsies and 10 normal skins (control) were developed with a polymer-based immunohistochemical method.

Results Lesions presented infiltrate comprising macrophages, some asteroid corpuscles, lymphocytes, multinucleated giant cells and a large number of

fungi. LCs presented short dendrites and were scarcely distributed. Dermal langerin + cells were detected in nine JLD lesions. FXIIIa + DD were hypertrophic, visualized in the inflammatory infiltrate of JLD lesions. Cells S100+ were present in JLD and control group with a similar number of cells. A total of 14 specimens did not express FXIIIa, and this considerable number probably contributed to the statistical similarity with the control group.

Conclusions The results indicate that LCs are present in the immune response against *Lacazia loboi*. Some dermal langerin + cells could be another subset of dendritic cells. Our data indicate changes of LCs in JLD cutaneous lesions and present, for the first time, results that show langerin + cells in the dermis and corroborate previous observations on the participation of FXIIIa + DD in the in situ immune response in JLD.

Keywords Jorge Lobo's disease · Dendritic cells · Immunohistochemistry

T. C. Barboza · C. Pagliari
Instituto de Assistência Médica ao Servidor Público
Estadual, Programa de pós-graduação em Ciências da
Saúde, São Paulo, SP, Brazil

T. C. Barboza · M. I. S. Duarte · M. N. Sotto ·
C. Pagliari (✉)
Laboratório da Disciplina de Patologia de Moléstias
Transmissíveis, Departamento de Patologia, Faculdade de
Medicina, Universidade de São Paulo, Av. Dr. Arnaldo,
455, sala 1118, Cerqueira Cesar, São Paulo, SP, Brazil
e-mail: cpagliari@usp.br

J. A. S. Quaresma · A. C. de Brito ·
M. B. Xavier · C. M. M. de Oliveira · D. A. A. Unger
Núcleo de Medicina Tropical, Universidade Federal do
Pará, Belém, PA, Brazil

J. A. S. Quaresma · M. B. Xavier
Centro de Ciências Biológicas e da Saúde, Universidade
do Estado do Pará, Belém, PA, Brazil

Introduction

Jorge Lobo's disease (JLD) is a cutaneous chronic granulomatous disease caused by the pathogenic fungus *Lacazia loboi*. It is usually characterized by lesions predominantly on the ears and upper and lower limbs. The disease is described in several Latin

American countries, but the largest number of cases is in Brazil's Amazon region [1–5].

The fungus *Lacazia loboi* is globular, unicellular, yeast type and may be presented either alone or in chains of cells connected resembling a “pearl necklace”.

The disease predominates in males with rural activity [6].

Clinically, there are differences related to the time of evolution, as well as extension of the lesions [7]. The most frequent aspect is the keloid-like type, with isolated or confluent lesions. This aspect seems to be related to high expression of TGF- β in the lesions. There may still be verrucous, ulcerated, gummy, scleroderma or macular infiltrative ones [8–10].

The skin dendritic cells have been studied in many pathologic processes once they are sentinels of the local immune surveillance and play a role to present antigens and induce a specific immune response.

Langerhans cells (LC) are well studied, although, over the past decades, other populations of cutaneous dendritic cells have been investigated, revealing its peculiarities in the in situ immune response in different lesions. LCs are constitutive of the epidermis and epithelium and characterized by the specific expression of CD207 (langerin) and CD1a. Furthermore, as antigen-presenting cells, they also express S100 protein [11, 12].

In more recent years Factor XIIIa + dermal dendrocytes (FXIIIa + DD) have been identified and characterized. In normal skin, those cells are preferably located in the papillary dermis around vessels. They express MHC II, indicating their ability to present antigen, and the immunophenotypic characteristics are distinct from LC and fibroblasts [13, 14]. The localization of these cells around vessels seems to confer strategic position to interact with several antigens.

Little is known about the characterization of the inflammatory cellular population in cutaneous lesions of JLD, and some speculations are based on paracoccidioidomycosis (PCM), a better studied disease that shares some similarities with JLD. Regarding the in situ immune response in PCM skin lesions concerning dendritic cells, there is an increase in the number of factor XIIIa + dermal dendrocytes and decrease in the number of LC when compared to normal skin [15].

By studying the population of LC using the CD1a marker, Quaresma et al. [16] observed similarities between JLD lesions and a group of normal skin and suggested a mechanism of immune escape of the fungus that inhibits phagocyte activity and antigen presentation of this population cell. On the other hand, macrophages seem to be ineffective in destroying the fungus. It is suggested that the significant presence of TGF- β can also play an important role in inhibiting the cellular immune response in these lesions [16]. FXIIIa + DDs were also frequently observed in such lesions (Oliveira C, unpublished data).

We intended to evaluate the in situ immune response in cutaneous lesions in JLD, focusing on dendritic cells. Considering the recent development of a sensible and more specific anti-langerin (CD207) antibody, we revisited the LC in such lesions and tried to identify dermal langerin + cells in this mycosis. Also, we investigated the population of FXIIIa + DD and cells expressing S100 protein.

Materials and Methods

Biopsies

Forty-one skin biopsies from patients with clinical and histopathologic diagnosis of JLD were selected from the files of the Núcleo de Medicina Tropical, Universidade Federal do Pará, Belém, PA, Brazil. Ten biopsies from normal skin were used as control.

Immunohistochemistry

It was performed a polymer-based immunohistochemical method. Following deparaffinization in xylene and hydration in ethanol, antigen recovery was performed in a TRIS/EDTA solution pH 9.0 for 20 min at 95 °C. The primary antibody anti-langerin (Leica CD207) was applied at a 1:400 dilution; anti-Factor XIIIa (Biogenex PU337-P) was applied at a 1:3,000 and anti-S100 protein (Dako Z311), at a 1:1,000 dilution. Following an overnight incubation at 4 °C, it was applied the second antibody and a polymer system. 3, 3'-diaminobenzidine tetrahydroxychloride was used as chromogen, and the slides were counterstained with hematoxylin. All reactions were performed with positive and negative controls. The

second ones were constituted by the use of isotype controls and the omission of the primary antibody.

Quantitative Analysis

Epidermal LC were quantified by counting the epidermal area fraction with positivity to langerin using a 10× ocular lens with a square grid and an 40× objective.

Dermal cells expressing Factor XIIIa and S100 protein were quantified by counting the number of positive cells in nine randomized fields using the same square grid described above.

Statistical Analysis

The number of positive cells was expressed as cells/mm² and statistically analysed using the Mann–Whitney test with the level for significance set at 95 % ($p \leq 0.05$).

Results

Lesions were characterized by granulomatous inflammatory infiltrate, nodular and diffuse, comprising macrophages, some of them with asteroid corpuscles, lymphocytes, epithelioid cells, numerous multinucleated cells of Langhans and foreign body types, with a large number of fungi. Neutrophils, eosinophils and plasma cells were rarely visualized (Fig. 1).

Langerhans cells, detected by the expression of langerin, were distributed along the epidermis of control group and presented either long or short dendrites. The group of JLD lesions presented cells with short dendrites scarcely distributed or, in some cases (15 %), absent. Cells expressing langerin in the dermis were detected in nine skin lesions of JLD group (22 %) distributed in the inflammatory infiltrate, five of them negative in the epidermis (Fig. 2). The comparison between groups presented a decreased number of positive cells in the JLD group ($p = 0.02$).

Factor XIIIa + dermal dendrocytes were hypertrophic and frequently visualized in the inflammatory infiltrate of 66 % of specimens of JLD lesions ($n = 27$). In the control group of normal skin, those cells presented a fusiform aspect (Fig. 3a, b). Although this cell population seemed to be increased in number in JLD group, the comparison to control group did not presented significant difference ($p > 0.05$).

Fig. 1 Histopathology aspect of lesion, characterized by inflammatory infiltrate with macrophages (M), lymphocytes, multinucleated giant cells (GC) and high number of fungal cells (F). (Hematoxylin-Eosin, magnification ×400)

Cells expressing S100 protein were also present in JLD specimens and control group. In JLD, cells were visualized in epidermis and in the dermal inflammatory infiltrate and were absent in 31 % of lesions (Fig. 3c, d). Some cells presented prominent dendrites. The numerical comparative analysis evidenced a similar number of cells in both groups ($p > 0.05$).

The comparative analysis of dendritic cells between JLD lesions and control group of normal skin is presented in Fig. 4.

Discussion

It is speculated that the genesis of symptoms of JLD is related to the host defective cellular immune response [17, 18].

In the present study, we have characterized the dendritic cell population present in the cutaneous lesions of patients with JLD, focusing on the expression of Factor XIIIa, langerin and S100 protein, to better understand the host–parasite relationship in this mycosis and the importance of such group of cells.

The histopathologic characterization of the inflammatory infiltrate was a predominance of macrophages and multinucleate giant cells, lymphocytes and large numbers of fungi. Also, we could observe the participation of Factor XIIIa + dermal dendrocytes, LC in epidermis and dermis, and cells expressing S100 protein.

Previously, FXIIIa + DDs were observed in high quantities in JLD lesions (Oliveira C, unpublished

Fig. 2 Immunohistochemistry expression of langerin. **a, b** Langerhans cells with short dendrites scarcely distributed in the epidermis of JLD lesion ($\times 200$ and $\times 400$). **c, d** Expression of

langerin in the dermis intermingled with fungal cells and dermis–epidermis junction ($\times 200$ and $\times 400$)

Fig. 3 Immunohistochemistry characterization of Factor XIIIa + dermal dendrocytes (**a, b**) and S100 protein (**c, d**). **a** Cells distributed in the inflammatory infiltrate in the lesions and **b** around vessels in the dermis (magnification $\times 400$).

c Immunohistochemistry expression of S100 protein in cells distributed in epidermis (magnification $\times 200$) and **d** in the dermal inflammatory infiltrate (magnification $\times 400$)

Fig. 4 Comparative analysis of cells expressing Factor XIIIa, S100 protein and langerin in Jorge Lobo's disease and control groups. FXIIIa: 6.22 ± 29.4 and 15.45 ± 15.45 ($p > 0.05$)/S100 protein: 3.55 ± 20.31 and 8.88 ± 11.14 ($p > 0.05$)/

langerin: 0.023 ± 0.021 and 0.047 ± 0.031 ($p = 0.02$) (values are given as median $\pm$ SD; JLD and control group, respectively)

data). In the present work, we detected such cells in the inflammatory infiltrate of 66 % of specimens of JLD lesions ($n = 27$). In those cases, the cells were hypertrophic and some of them presented high numbers. On the other hand, 14 specimens did not express FXIIIa and this considerable number of cases without expression probably contributed to the statistical similarity with the control group, in which there is naturally low number of these cells.

Compared with PCM, which shares some similarities with JLD, some studies have shown that FXIIIa + DD are hypertrophied and hyperplastic and are found with unusual frequency in the superficial and reticular dermis and around granulomas, probably related to their ability to present antigens. Also, it is known that FXIIIa + DD are able to produce TNF- α , and it was speculated their role in activation of macrophages and demonstrated the interaction with fungal cells [19].

Cells expressing S100 protein were visualized both in cells of epidermis and dermis, with similar number between lesions and control group. The used marker to recognize this protein is a subunit B of the S100 family. It is normally expressed in dendritic cells, melanocytes, macrophages and some other cutaneous elements. This marker is also used to detect LC [20].

A similar result addressing LC in JLD lesions was performed using a CD1a marker, in which the authors verified numerical similarities with normal skin [16]. The authors suggested a possible immunoregulatory disturbance in the patients, probably as a result of escape mechanisms of the fungus.

In order to revisit the LC in JLD, in the present work we tried to identify this cell population using an anti-langerin antibody.

Langerin binds mannose and beta-glucan in the wall of some fungal cells, and it is suggested as the major fungal receptor on human LCs [21].

When the expression of CD1a was investigated in JLD, the authors did not find differences from normal skin [16]. In the present study, however, we could observe a reduced number of langerin + cells in epidermis and the presence of positive cells in the dermis. Langerin expression seems to be a highly sensitive and specific marker of Langerhans cell. This molecule is considered the “state-of-the-art marker” for identifying LCs in mouse and human and persists during the maturation and migration of LCs [11]. The relatively low number of cells detected in epidermis and the positive cells found in dermis could be migrating LCs following their maturation process.

These data suggest that LCs play a role in the immune response against *Lacazia loboi* and keep the expression of langerin when migrate from supra-basal layer of epidermis. However, it is noteworthy that a number of researches have suggested that some dermal langerin + cells could be a novel subset of dendritic cells [22–24]. Bearing in mind that in JLD the dermal positive cells could be in fact this new group of cells, that is such an interesting result.

Taken together, our data indicate the numerical and morphological changes of LCs in JLD cutaneous lesions when analysed the expression of CD207 and present, to the best of our knowledge for the first time,

results that show langerin + dendritic cells in dermis of such lesions. Also, the results corroborate previous observations on the participation of Factor XIIIa + dermal dendrocytes in the in situ immune response in JLD.

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Conflict of Interest None of the authors have any potential conflict of interest or financial support in the subject matter.

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Dendritic Cells and Pattern of Cytokines in Paracoccidioidomycosis Skin Lesions

Carla Pagliari, Ph.D., and Mírian N. Sotto, M.D., Ph.D.

We demonstrated and quantified by immunohistochemistry epidermal Langerhans cells, CD34+ dermal dendrocytes (DDs), and cells expressing TNF α , interferon- γ (IFN γ), IL-5, and IL-10 in skin lesions of paracoccidioidomycosis (PCM). Sixty-one biopsies were classified in three groups according to the pattern of tissue response: Group 1, well-organized granuloma; Group 2, poorly organized granuloma; and Group 3, both kinds of granuloma. Langerhans cells had short and irregular dendrites in all groups and were decreased in number in Groups 1 and 2. CD34+ DDs did not differ in number from the control group. Group 1 was characterized by many cells expressing IFN γ . Groups 2 and 3 exhibited large numbers of cells expressing IL-5 and IL-10. The data obtained suggest that well-organized granulomas reflect a better cellular immune response, and the large number of cells expressing IL-5 and IL-10 in Group 2 indicate an ineffective response in PCM skin lesions. Both kinds of granuloma in the same cutaneous lesion probably represent an intermediate response between the anergic and hyperergic poles. Group 3 also showed higher numbers of cells expressing TNF α when compared with the control group. Some cells expressing TNF α were dendritic and localized around the granuloma similar to the factor XIIIa+ DD localization that we previously described.

Key Words: Skin—Dendritic cells—Cytokines—Paracoccidioidomycosis.

Paracoccidioidomycosis (PCM) is a human systemic mycosis caused by the dimorphic fungus *Paracoccidioides brasiliensis*. It is confined to Latin America, with endemic areas in Brazil, Argentina, Colombia, and Venezuela. Primary lesions are thought to occur in the lungs. From these lesions, the fungi can disseminate via the bloodstream or lymphatics to any organ of the host, and the skin is involved in approximately 50% of cases (1). Cutaneous lesions constitute an important clinical element of the diagnosis because they represent an exteriorization of the disease.

The lesions of PCM are characterized by a granulomatous inflammatory response with formation of epithelioid granulomas with giant cells. The hyperergic pole of PCM is characterized by compact epithelioid granulomas, and the anergic pole is represented by a loose and parasite-rich granulomatous response. The skin lesions reflect a reduction in the number of Langerhans cells (LCs), resulting in an impairment in the local processing of antigens (2).

Dermal dendrocytes (DDs) constitute a group of dendritic cells with an antigen-presenting cell phenotype (3). These cells were identified by the expression of factor XIIIa (FXIIIa), a protransglutaminase involved in hemostasis, and classified as type I dendrocytes. Another dendritic cell described within the human dermis and identified by the expression of the human progenitor cell antigen CD34 was classified as type II (4–7).

Brandão et al. (8) detected more FXIIIa+ DDs in PCM oral lesions, suggesting that these cells might be related to granuloma formation. We recently described the presence of fungi inside FXIIIa+ DDs (9). These cells were hypertrophic and increased in number and were localized in the superficial and reticular dermis. The precise role of CD34+ DDs remains to be established. It seems that these cells might differentiate toward more differentiated cells such as CD1a+ LCs or FXIIIa+ DDs (10–12).

The cellular immune response mediated by CD4+ T cells producing a T helper (Th) 1 pattern of cytokines (interferon [IFN] and IL-2) represents the main resis-

From the Departamento de Patologia (C.P.) e Dermatologia (M.N.S.), Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil.

Address correspondence and reprint requests to Mírian N. Sotto, M.D., Ph.D., Av. Dr. Arnaldo, 455 sala 1118, Cep 01246-903, Faculdade de Medicina da Universidade de São Paulo, São Paulo, SP, Brazil. E-mail: mnsotto@ajato.com.br.

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tance mechanism against *P. brasiliensis*. Susceptibility is associated with the Th2 pattern of cytokine production (IL-4, IL-5, IL-6, and IL-10) (13–17). Tumor necrosis factor is a potent stimulant of leukocyte function and granuloma formation. In patients with chronic PCM, high serum levels of IL-10 and TNF α and low levels of IFN γ were related to progression of the disease (18,19).

In the current study, we investigated the correlation between skin dendritic cells (LCs and CD34+ DDs), the morphologic characteristics of the granulomatous response, and the expression of cytokines of the Th1 and Th2 patterns in the skin lesions of PCM.

MATERIALS AND METHODS

Sixty-one skin biopsies from patients with a clinical diagnosis of PCM confirmed by direct examination or fungal culture were selected from the files of the Dermatopathology Laboratory of the Department of Dermatology of the University of São Paulo Medical School according to the amount of tissue available to perform all the proposed immunohistochemical techniques. All specimens had been fixed in neutral buffered formalin and embedded in paraffin. They were classified according to tissue response (20,21) into well-organized granuloma (Group 1), poorly organized granuloma (Group 2), and both kinds of granuloma (Group 3). Ten biopsies from normal skin were used as controls.

Immunohistochemistry

Four-micrometer sections were dewaxed in xylene and hydrated through a graded series of ethanol. Endogenous peroxidase was blocked with 3% hydrogen peroxide, and preincubation with trypsin (Sigma Chemical Company, St. Louis, MO) in 0.01 M of PBS, pH 7.4, was performed to enhance antigen exposure when necessary. The following primary antibodies were applied, and sections were incubated overnight at 4°C: rabbit anti-*P. brasiliensis* (kindly supplied by Prof. Carlos Lacaz, Tropical Medicine Institute, São Paulo Medical School) diluted 1:1,000, mouse anti-CD1a (Immunotech, Marseille, France), mouse anti-CD34 (Novocastra, Newcastle, UK) diluted 1:50, rabbit anti-TNF α diluted 1:100, goat anti-IFN γ diluted 1:250, and rabbit anti-IL-5 (Genzyme, Cambridge, MA) and mouse anti-IL-10 (R&D Systems, Minneapolis, MN) used at 1:50 and 1:10 dilutions, respectively. Biotinylated antirabbit and antimouse antibody was used at a dilution of 1:1,000 for 30 minutes at 37°C (DAKO Corporation, Carpinteria, CA). For the detection of IFN γ , biotinylated antigoat antibody was used at a dilution of 1:800 (DAKO Corporation). Peroxidase-labeled streptavidin-biotin complex (SABC; DAKO Corporation) was applied for 30 minutes at 37°C at a dilution of 1:1,000. Langerhans cells and cells expressing IL-5

were better visualized by using the catalyzed signal amplification (CSA) system (DAKO Corporation) based on the peroxidase-catalyzed deposition of a biotinylated phenolic compound followed by a secondary reaction with streptavidin peroxidase (22). As a chromogen, we used 3,3 diamino-benzidine tetrahydrochloride (Sigma Chemical Company), and the slides were counterstained with Harris hematoxylin.

Specimens of PCM lesions, normal skin, Kaposi sarcoma, and reactive lymph node were used as positive controls for the immunohistochemical detection of *P. brasiliensis* antigens, LCs, CD34+ DDs, and cells expressing cytokines, respectively. The negative controls were obtained by omitting the primary antibodies for each reaction, which were replaced by PBS.

Quantitative Analysis

Cells were quantified by counting the number of immunolabeled cells in 15 randomized high-power fields for each specimen with a $\times 10$ ocular lens with a square grid and a $\times 40$ objective. Epidermal LCs were evaluated by determining the epidermal area fraction with positivity for CD1a (23).

Statistical Analysis

The number of positive cells was statistically analyzed by the Graph Pad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA) using a Kruskal-Wallis test with the level of significance set at 95%.

RESULTS

Histopathologic Analysis

The histopathologic study revealed 22 biopsies characterized by well-organized granuloma (Group 1) (Fig. 1A), 20 characterized by poorly organized granuloma (Group 2) (see Fig. 1B), and 19 with both kinds of granuloma in the same biopsy (Group 3). The most frequent epidermal changes were hyperkeratosis, parakeratosis, acanthosis, and pseudocarcinomatous hyperplasia. Epidermal microabscesses were observed in 60% of the biopsies, some of them with fungi among the polymorphonuclear cells. Besides the granulomatous reaction, the dermis presented a perivascular inflammatory infiltrate composed predominantly of lymphocytes, plasma cells, and macrophages. Neutrophils were frequently observed, and eosinophils were present in almost 20% of the biopsies. Dermal necrosis and fibrosis were rarely seen. Vascular alterations were characterized by swelling of endothelial cells. Fungi were seen in the cytoplasm of macrophages and giant cells in all sections and were better demonstrated by anti-*P. brasiliensis* antibody.

FIG. 1. Patterns of granulomatous tissue reaction in paracoccidioidomycosis. (A) Well-organized granuloma characterized by compact clusters of epithelioid cells, giant cells, and mononuclear cells in a peripheral halo around the granulomas. (B) Poorly organized granuloma characterized by loose aggregates of epithelioid cells intermingled with mononuclear cells (hematoxylin–eosin).

Distribution of Dendritic Cells

Langerhans cells were distributed throughout the epidermis with short and irregular dendrites in Groups 1 and 2 (Fig. 2A). Dendrites were long and numerous in the control group (Fig. 2B). Quantitative analysis showed a statistically significant decrease in the number of cells immunolabeled with the anti-CD1a antibody in Groups 1 and 2 when compared with the control group ($P < 0.001$) (Fig. 3). CD34+ DDs were spindle shaped and distributed in the dermis, especially in the adventitia of sebaceous and sudoriparous glands. In the control group, these cells had the same characteristics as in PCM. Statistical analysis did not show differences between the groups in the number of CD34+ DDs.

Distribution of the Cytokines in the Skin Lesions

Cells expressing TNF α were distributed throughout the dermis in the inflammatory infiltrate. Interestingly,

some positive dendritic cells were localized around the granulomas similar to the distribution of FXIIIa+ DDs that we previously described (9). The quantitative analysis revealed a statistically significant increase in the number of cells expressing TNF α in Group 3 when compared with the control group (Fig. 4A). Cells expressing IFN γ were detected in the dermal inflammatory infiltrate of the three PCM groups. Group 1 was characterized by more of these cells when compared with Group 2 ($P < 0.001$) and the control group (Fig. 4B). Interleukin-5–stained lymphocytes were distributed in the dermal inflammatory infiltrate. A statistically significant increased number of positive cells were detected in Groups 2 and 3 when compared with the control group (Fig. 4C). Lymphocytes and some mast cells immunolabeled with the anti-IL-10 antibody were distributed in the dermis among the inflammatory infiltrate. More of these cells were present in Groups 2 and 3 when compared with the control group ($P < 0.01$) (Fig. 4D). On the whole, the

FIG. 2. Epidermal Langerhans cells immunostained by anti-CD1a antibody. (A) Paracoccidioidomycosis lesion with well-organized granuloma showing epidermal Langerhans cells with short and irregular dendrites. (B) Langerhans cells distributed throughout the epidermis with long and numerous dendrites in the control group (streptavidin-biotin complex immunohistochemical technique).

FIG. 3. Distribution of CD1a+ epidermal area fraction (Langerhans cells) in paracoccidioidomycosis lesions according to the type of granulomatous reaction and in the control group. Horizontal lines represent the median values, boxes represent the 25th to 75th percentiles, and whiskers show the highest and lowest values. *Statistically different from the control group (Kruskal-Wallis test).

morphology of cells expressing TNF α , IFN γ , IL-5, and IL-10 present in the control group was similar to that found in the PCM groups.

DISCUSSION

Cutaneous involvement in PCM is frequent. The infection begins in the lungs and reaches the skin via bloodstream dissemination of *P. brasiliensis*.

The specific cell populations that are involved in disease control in different organs have been identified so as to better understand the organ-specific immune response (24). The skin has many different immunologically active cells, and the most important are probably the dendritic cells (25).

Although it is possible to identify two well-defined poles in PCM in experimental animal models, there are a number of immune and histopathologic responses in human beings. The clinical form reflects a particular phase in a dynamic and polymorphic disease (26).

The reduction of LCs in diverse pathologic processes has been described (27–29). In our work, epidermal LCs (CD1a+) showed degenerative alterations and a reduced number in Groups 1 and 2 when compared with the control group ($P < 0.01$). This numeric reduction could be a result of factors produced by *P. brasiliensis*, or these cells may have migrated to the dermis, resulting in an immunophenotypic alteration in membrane expression of CD1a. Sandoval et al. (30) did not detect *P. brasiliensis* antigens in the cytoplasm of LCs, suggesting that they would not be capable of presenting fungal antigens efficiently. On the other hand, we could detect some yeast cells in the cytoplasm of FXIIIa+ DDs by double immunostaining (9). It was suggested that DDs might represent a possible dermal precursor of epidermal LCs (11). We

observed that Group 3 did not present numeric depletion of LCs and previously observed that FXIIIa+ DDs appeared hypertrophied and hyperplastic in this group (9). We suppose that FXIIIa+ DDs could be a reservoir for the repopulation of epidermal LCs in this PCM group.

CD34 antigen is present in human hematopoietic progenitor cells from bone marrow. Within the dermis, CD34 is expressed by endothelial cells, dendritic cells, and spindle-shaped cells around adnexal structures (31). Few works demonstrate the function of CD34+ DDs as well as their relation with other cutaneous dendritic cells. They do not express CD1a or S100 protein, are more frequent in the reticular and deep dermis, and only 2% express HLA-DR under normal conditions (10). It is suggested that these cells can differentiate into more specialized dendritic cells such as FXIIIa+ DDs or LCs after the stimulatory effects of TNF α and granulocyte macrophage colony-stimulating factor (GM-CSF) (10–12). We observed few CD34+ DDs in the superficial and reticular dermis, without differences between the groups. It was not possible to correlate this cell population with the other dendritic cells. Their role in skin tissue reaction to *P. brasiliensis* warrants further investigation.

Products from *P. brasiliensis* may stimulate macrophages to secrete TNF α , which is involved in granuloma development (16,21). We demonstrated the presence of dermal cells that expressed TNF α in the three groups of PCM. Some of these cells had a dendritic morphology and distribution in the periphery of granulomas similar to the distribution of FXIIIa+ DDs that we previously described (9). Group 3 showed the highest numbers of cells expressing TNF α and FXIIIa+ DDs, suggesting their participation as TNF α -producing cells following fungal stimulation.

Interferon- γ plays a pivotal role in host resistance against *P. brasiliensis* (17,32,33). Although we detected cells expressing IFN γ in the three PCM groups, Group 1 had more of these cells, correlating with a well-organized tissue response against *P. brasiliensis* with this cytokine.

Interleukin-5 is a Th2 type cytokine related to the maturation and differentiation of eosinophils (34,35). In experimental PCM, high levels of IL-5 and IL-10, eosinophilia, and low levels of IFN γ characterize the progressive disease in susceptible animals (32,36). We demonstrated more cells expressing IL-5 in Groups 2 and 3 similar to the results of cells expressing IL-10. These results suggested that poorly organized granulomas, which characterize Group 2, represent a worse response against *P. brasiliensis*.

It was interesting to observe that Group 3, the one with both kinds of granuloma formation, did not differ from Group 1 in the number of IFN γ -expressing cells. We could demonstrate that the number of FXIIIa+ DDs was increased in Group 3 of skin lesions (9) and so was the number of TNF α -expressing cells, some of them with

FIG 4. Distribution of the number of cytokine-expressing cells per square millimeter in paracoccidioidomycosis skin lesions according to the type of granulomatous reaction and control group. (A) Tumor necrosis factor- α -expressing cells. (B) Interferon- γ -expressing cells. (C) Interleukin-5-expressing cells. (D) Interleukin-10-expressing cells. Horizontal lines represent the median values, boxes represent the 25th to 75th percentiles, and whiskers show the highest and lowest values. *Statistically different from the control group (Kruskal-Wallis test). **Group 1 greater than Group 2 (Kruskal-Wallis test).

dendritic morphology. FXIIIa⁺ DDs have been related to Th1 type cytokine cutaneous processes (37). We suppose that these cells expressing TNF α in PCM skin lesions might be activated to improve the immune response against *P. brasiliensis*.

There are many factors that can regulate Th1/Th2 cell expression such as genetic background, the type of antigen-presenting cell interacting with T cells, and the cytokine microenvironment at the time of activation. Calich et al. (36) related the difference in the immune response to *P. brasiliensis* antigens to the interaction between the antigens and different populations of anti-

gen-presenting cells. Almeida and Lopes (38) studied dendritic cells in resistant and susceptible mice following inoculation with GP43 and demonstrated that the preferential activation of Th1 and Th2 responses, respectively, by those cells seems to be determined by the costimulatory microenvironment of cytokines in the early phases of infection. The presence of IFN γ would stimulate these cells to produce IL-12, inducing a Th1 response. On the other hand, IL-10, prostaglandin E, or IL-4 stimulates a Th2 response (39).

The presence of different populations of dendritic cells in the skin and their antigen-presenting capacity consti-

tute important factors in the immune response against *P. brasiliensis*. Understanding the correlation between these cells and cytokines could contribute to possible treatment targeting. The immunologic mechanisms that confer resistance to PCM are dependent on various factors, and dendritic cells seem to play a role.

Our results suggest the participation of LCs and FXIIIa+ DDs in the pathogenesis of the PCM cutaneous tissue response. These cells could act as antigen-presenting cells and/or fungal target cells. They would be related to the production and induction of the cytokines that modulate the immune response against the parasite.

The cutaneous tissue response in PCM correlates with the mechanisms that occur in other organs but with characteristic mechanisms that are related to its own immune system. □

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The cell-mediated immune reaction in the cutaneous lesion of Chromoblastomycosis and their correlation with different clinical forms of the disease

Solange Corrêa Garcia Pires d'Ávila, Carla Pagliari & Maria Irma Seixas Duarte
University of São Paulo School of Medicine, Department of Pathology, São Paulo, Brazil

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Abstract

Chromoblastomycosis is a fungal infection caused by dematiaceous fungi inducing skin lesions of difficult treatment and of frequent recurrence. The objective of the present investigation was to characterize cell-mediated tissue reactions in the skin in cases of Chromoblastomycosis using histopathology and immunocytochemistry methods and to correlate them with different clinical forms of Chromoblastomycosis. Biopsies from 19 patients were stained with HE and Giemsa, and serial sections were immunohistochemically stained using CD45RO, CD20, CD4, CD8, CD68, CD1a, CD34, IL4, IL10, TNF- α and IFN- γ antibodies. A quantitative and semiquantitative analysis of the cell subsets and cytokines in the inflammatory infiltrates was performed by counting ten high-power fields (400 $\times$). The cutaneous lesion presented as verrucous plaque ($n = 15$) or erythematous atrophic plaque ($n = 4$). We observed two types of tissue reaction: A) a granulomatous reaction with a suppurative granuloma with several fungi cells in the cutaneous lesion presenting as verrucous plaque; B) a granulomatous reaction with a tuberculoid granuloma with few fungi cells in the cutaneous lesion presenting as atrophic plaque. The data obtained suggest that patients with lesion presented as verrucous plaque have a type Th2 immunological response, while patients with lesion presented as erythematous atrophic plaque have a type Th1 response.

Key words: Chromoblastomycosis, immunohistochemistry, immunity, granuloma, skin

Introduction

Chromoblastomycosis is a chronic skin infection caused by several dematiaceous fungi occurring in tropical and subtropical regions. These fungi are found in the soil and on decomposing plant matter. The infections are caused by traumatic implantation of hyphae or conidia into the skin. Five species of fungi are usually more associated with the disease: *Fonsecaea pedrosoi* (*Phialophora pedrosoi*), *Phialophora compacta* (*Fonsecaea compacta*), *Phialophora verrucosa*, *Cladosporium carrionii*, and *Rhinocladiella aquaspersa* (*Acrotheca aquaspersa*), with the species *Fonsecaea pedrosoi* being more frequently isolated from cultivated environments [1, 2, 3].

Clinically, during the initial stage, the disease presents erythematous papules that develop into vari-

ous verrucous papules, or plaques. These lesions mainly attack the lower limbs of male individuals, and rarely of females. The disease is usually insidious and the lesions increase slowly but progressively, challenging the usual treatments and quite often reappearing. In most cases, the disease causes great discomfort to the patients due to esthetical and functional mutilations, mainly on hands and feet, preventing even the use of shoes. The patients' general condition is not usually compromised [1, 4, 5, 6]. Carrión (1950) described five different clinical forms: nodules, tumors, plaques, verrucous plaques and atrophic plaques lesions [7].

The diagnosis is made by fungus identification in culture or by anatomopathologic studies when fungi are found in tissues [1]. Histologic examinations reveal hyperkeratosis, pseudoepitheliomatous hyperplasia of epidermis, and granulomatous suppurative

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dermatitis. The fungal spores can be inside giant multi-nucleated cells or free in the tissue. They are round, brown colored, ranging in size from 6.0 to 13 μm , and generally having central septation. A chronic granulomatous inflammatory reaction occurs accompanied by extensive and progressive dermal fibrosis. These characteristics are associated with persistent antigenic stimuli and, together with the pseudoepitheliomatous hyperplasia, give a verrucous aspect to the lesions [8, 9, 10].

Analysis of 19 cases of Chromoblastomycosis diagnosed at the University Hospital of the Faculty of Medicine of São José do Rio Preto, Brazil, showed that the lesions manifested in two different forms, i.e., they were verrucous or atrophic plaque lesions. This fact points to the possibility that different lesion forms are correlated with different host tissue reactions.

We are aware of only one report describing the phenotype of the cells of the inflammatory infiltrate and the cytokines present in the lesions of patients with a chronic granulomatous suppurative inflammatory process [9]. On the other hand, we are unaware of any studies correlating different clinical forms of Chromoblastomycosis with different histological features.

Thus, the objectives of the present study on patients with Chromoblastomycosis were:

1. To evaluate the cell-mediated immune tissue response using standard staining techniques with (HE) and Giemsa and immunohistochemical methods using antibodies to label macrophages, lymphocyte subtypes, endothelial cells, and Langerhans cells.
2. To search by immunohistochemistry for the following cytokines in tissue: $\text{TNF-}\alpha$, $\text{IFN-}\gamma$, interleukin-4 and interleukin-10, in order to characterize the lesions in terms of Th1 or Th2 pattern.
3. To correlate the clinical forms of Chromoblastomycosis presentation with the type of host tissue inflammatory response.

Material and methods

Nineteen patients with Chromoblastomycosis were studied in the Department of Dermatology of the University Hospital, Faculty of Medicine of São José do Rio Preto, from January 6, 1988 to January 6, 2000. The diagnosis was made on the basis of skin lesion biopsies and visualization of yeast- fungal cells with

the characteristics of dematiaceous fungi by histological examination. In vitro cultures of pathogenic fungi were performed in seven cases.

Mean patient age was 34 years (range: 23 to 63 years), 68% ($n = 13$) were men and 32% ($n = 6$) were women, and 42.2% ($n = 8$) were farmers. 52.6% ($n = 10$) of the lesions were located on the upper limbs and 47.4% ($n = 9$) in the lower limbs.

The verrucous type of lesion (Picture 1) occurred in 78% of cases (group A) and the atrophic plaque type (Picture 3) in 22% (group B) (Table 1).

Fonsecaea pedrosoi was identified in *in vitro* cultures in seven cases (5 of verrucous type lesion and 2 of atrophic plaque type lesion).

Skin biopsies were taken from each patient. The specimen was fixed in formalin and embedded in paraffin for histological examination. We used five biopsies of normal skin as controls. A section (5.0 μm) from each biopsy was stained with HE and with Giemsa for mast cell visualization. The following parameters were evaluated: epidermis hyperplasia (acanthosis), distribution of inflammatory infiltrate cells, types of granulomas, presence of microabscesses, proliferation of blood vessels, fibrosis intensity. Serial sections were immunohistochemically stained with the streptavidin-biotin complex (11). The panel of antibodies included: CD45RO, CD20, CD4, CD8, CD68, CD34, CD1a, anti- $\text{TNF-}\alpha$, anti- $\text{IFN-}\gamma$, anti-IL-4, anti-IL-10 (Table 2).

A quantitative estimate of the different cell subsets in each biopsy was obtained by counting the number of positive cells with the respective antibodies in ten high power fields ($\times 400$) in the area of highest density of the inflammatory infiltrate [10]. In respect to CD34 cells, only endothelial cells were estimated. Cells expressing cytokines were analyzed semi-quantitatively. According to the following scale: 0 = Absence of detection in the examined fields; + = few positive cells; ++ = moderate number of positive cells; +++ = large number of positive cells.

Comparison between groups was made by the Student t test, after homogeneity of variance was determined by the Levene test. Frequency data among groups were analyzed by the Fisher exact test. In all analyses, the level of significance was set at 0.05% or less.

Results

The histopathological exam demonstrated the following pattern of inflammatory reaction:

Figure 1. (Picture 1) Chromoblastomycosis. Verrucous plaque form. (Picture 2) Chromoblastomycosis. Verrucous plaque form. Epidermal hyperplasia. H&E $\times 100$. (Picture 3) Chromoblastomycosis. Atrophic plaque form. (Picture 4) Chromoblastomycosis. Atrophic plaque form. Epidermal atrophy. Typical tuberculoid granuloma with only one fungal element H&E $\times 200$.

Table 1. Chromoblastomycosis: social, and clinical data of the patients

Case	Sex	Age	Profession	Lesion placement	Evolution time	Reccurrence	Clinical forms
1	M	52	farmer	left thigh	3 years	N.I	Atrophic plaque
2	M	55	guard	left hand	16 years	N.I.	Verrucous lesion
3	M	38	bricklayer	left knee	1 year	Yes	Verrucous lesion
4	F	45	hw	left knee	2 yeras	N.I.	Verrucous lesion
5	F	54	hw	right hand	8 years	No	Atrophic plaque
6	M	62	farmer	left thigh	2 years	No	Verrucous lesion
7	M	35	farmer	right hand	6 months	Yes	Verrucous lesion
8	M	54	carpenter	left hand	1 year	Yes	Verrucous lesion
9	M	25	farmer	left foot	4 years	Yes	Verrucous lesion
10	M	29	driver	elbow	8 months	N. I.	Verrucous lesion
11	M	56	mechanic	left leg	5 years	No	Atrophic plaque
12	F	50	hw	left leg	3 years	Yes	Verrucous lesion
13	M	59	farmer	forearm	8 years	No	Atrophic plaque
14	M	23	farmer	right leg	10 years	N.I.	Verrucous lesion
15	F	62	hw	left forearm	10 years	Yes	Verrucous lesion
16	F	62	hw	left forearm	1 year	N.I.	Verrucous lesion
17	F	59	hw	left elbow	1 year	Yes	Verrucous lesion
18	M	63	farmer	right elbow	26 years	Yes	Verrucous lesion
19	M	56	farmer	left foot	30 years	Yes	Verrucous lesion

N.I. = no information. hw = house wife.

Table 2. Primary antibodies used

Antibody	Code	Dilution	Antigenic recovery
Monoclonal mouse anti-T cells (CD45RO)	DAKO/M742	1:100	–
Monoclonal mouse anti-B cells(CD20)	DAKO/M755	1:50	–
Monoclonal mouse Anti-macrophage (CD68)	DAKO/M876	1:50	Damp heat
Monoclonal mouse Anti-endothelial cell (CD34)	NOVACAstra	1:50	Tripsina 0.015% +CaCl2 0,134 mg em PBS
Monoclonal mouse Anti-CD4(OPD4)	DAKO M834	1:1000	CSA
Monoclonal mouse Anti-CD8	DAKO m707	1:50	CSA
Monoclonal mouse Anti-CD1a	Immunotech/1590	Puro	CSA
Polyclonal rabbit Anti-TNF- α	Genzime/IP300	1:400	Trypsin 0,1% in PBS
Monoclonal mouse Anti-IL-10	R&D Systems/MAB217	1:10	Trypsin 0,1% in PBS
Polyclonal goat Anti-IFN-	Genzime/41285	1:300	Trypsin 0,1% in PBS
Monoclonal mouse Anti-IL-4	Genzime/1842-01	1:40	Trypsin 0,1% in PBS

Figure 2. (Picture 5) Chromoblastomycosis. Verrucous plaque form. Dense infiltrates in the dermis, rich in neutrophils, eosinophils and plasma cells. H&E $\times 400$. (Picture 6) Chromoblastomycosis. Verrucous plaque form. Polymorphonuclear neutrophils surround several fungal elements. H&E $\times 400$. (Picture 7) Chromoblastomycosis. Verrucous plaque form. Several mast cells. Giemsa $\times 400$. (Picture 8) Chromoblastomycosis. Verrucous plaque form. The cell immunostained with anti-IL 10 mAb. Immunohistochemistry $\times 400$. (Picture 9) Chromoblastomycosis. Atrophic plaque form. TCD4+ lymphocytes in tuberculoid granuloma. Immunohistochemistry $\times 200$. (Picture 10) Chromoblastomycosis. Atrophic plaque form. The macrophages in tuberculoid granuloma. Immunohistochemistry $\times 200$. (Picture 11) Chromoblastomycosis. Atrophic plaque form. The cell immunostained with anti-IFN-gamma mAb. Immunohistochemistry $\times 400$.

A group ($n = 15$) showed superficial and deep granulomatous dermatitis with suppurative granulomas. The dermis showed a diffuse mixed cellular infiltrate composed of macrophages, neutrophils, lymphocytes, plasma cells, eosinophils, epithelioid cells and giant multinucleated isolated cells (Picture

5). True granulomas were not found. Several microabscesses with a great quantity of fungus usually in division were observed (Picture 6). Intense proliferation of dermal capillary vessels, dermal fibrosis, and numerous mast cells were also observed (Picture 7). The epidermis exhibited significant pseudoepithelio-

matous hyperplasia (Picture 2). LTCD4+ cells were diffusely distributed. Some cells expressing IL-10 were observed (Picture 8).

Another group ($n = 04$) showed superficial granulomatous dermatitis with well formed tuberculoid granulomas, presenting epithelioid cells with compact arrangement, Langerhans cells and lymphocytes. In addition, the epidermis was found to be atrophic (Picture 4). The inflammatory infiltrate was mainly superficial. CD4+ cells were identified at the periphery of the granuloma (Picture 9) and macrophages in the center (Picture 10). Mast cells were present in small numbers and microabscesses were absent. The fungi were scarce and usually located within giant cells. (Picture 4). There was a moderate proliferation of dermal capillary vessels and fibrosis. Cells expressing IFN- γ were also observed (Picture 11).

The results of the immunohistochemical exams are demonstrated in Tables 2, 3 and 4 and illustrated in Pictures 8, 9, 10 and 11.

Statistical analysis of numerical data showed significance for the following parameters: acanthosis intensity, presence of microabscesses, proliferation of blood vessels, fibrosis intensity, number of mast cells, and CD34+ cells, fungal cells, cells expressing IFN- γ , and cells expressing IL-10.

Discussion

We were intrigued by the fact that some patients with Chromoblastomycosis treated in our Service exhibit a type of clinical lesion different from that usually observed.

We noticed two types of clinical presentation of the lesions. In 15 cases the lesions were of the verrucous type (group A) and in four cases they were of the atrophic plaque type (group B). In group A, the lesions were larger, raised and poorly delimited and many of them returned after antimycotic treatment and even after lesion excision.

The lesions of group B were smaller, flat, well delimited and did not recur after surgical excision. The differences observed between the two types of lesions were not related to the time of evolution (Table 1). The data concerning patient gender, age and occupation coincided with those reported in the literature [4, 5, 6, 12, 13, 14]. In our series, the lesions predominated in the upper limbs (52.6%), in contrast to most of the series described in the literature, in which the lesions were most often located in the lower limbs.

A systemic mycosis usually presents as a granulomatous response, with the presence of significant numbers of neutrophils, conferring a suppurative aspects to the lesions [1, 15, 16, 17]. Literature data about Chromoblastomycosis point out granulomatous inflammatory reactions with suppurative granulomas, while true tuberculoid granulomas are rarely found. [2, 8, 9, 16].

In our series, we observed two distinct types of inflammatory response: amorphous suppurative granulomas and true tuberculoid granulomas.

The patients with suppurative granulomas showed at the same time pseudopitheliomatous hyperplasia, microabscesses with large numbers of fungi, significantly higher numbers of mast cells, and CD34+ cells, higher proliferation of dermal capillary vessels, and fibrosis. Some cells expressing IL-10 were part of this profile.

The cases with true tuberculoid granulomas showed atrophy of the epidermis or light acanthosis, well formed granulomas with epithelioid cells, Langerhans cells, lymphocytes, and absence of microabscesses. Few fungi, generally fragmented, were observed in the cytoplasm of giant multinucleated cells. We even noted less proliferation of dermal capillary vessels and fibrosis, and fewer numbers of mast cells and CD34+ cells. Some cells expressing IFN- γ were found.

It is important that the distinct histopathological alterations were correlated with the clinical aspects of the lesions. This permits to characterize Chromoblastomycosis as a spectral bipolar tendency disease. In good responder patients, a better response was shown by the development of well- formed granulomas and cellular phenotype and expression of cytokines compatible with the profile of Th1 type immune response. At the other pole, in poor responders true granulomas were not found, with the phenotype of inflammatory cells and the local expressions of cytokines reflecting the Th2 pattern of T cell response.

The host defense mechanism in fungal lesions is still not well understood, although during the last decade many studies have been done to evaluate the cell-mediated immune response [17–21], the cytokines related to this response [22–25], and the fungus-host interaction [26–30].

Specifically in Chromoblastomycosis, some studies have focused on fungus- host interaction, showing a predominantly cellular immune response, with the activation of macrophages involved in fungus phagocytosis. Although phagocytosis did occur, it was dam-

Table 3. Cromoblastomycosis: Immunohistochemistry (mean cell numbers counted in 10 high-power fields (400×))

Cases	CD45RO	CD20	CD68	CD4	CD8	CD34	CD1a	Mast cell
Cell type	T	B	Macr.	T	T	Endot.	Langh.	
1.	63.7	57.6	31.5	23.2	42.2	9.4	3.9	1.7
2.	78.8	72.3	26.3	29.9	48.5	10.7	3.2	5.1
3.	63.2	22.6	20.9	22.9	43.2	8.7	5.4	8.2
4.	27.2	5.6	27.3	13.3	15.1	12.4	4.1	5.1
5.	30.8	4.4	30.3	12.7	20.2	8.3	4.6	2.0
6.	17.0	8.3	30.6	4.5	13.7	12.0	3.7	1.8
7.	23.0	88.3	24.6	8.9	16.2	11.5	3.9	2.2
8.	49.8	16.8	15.4	23.7	29.6	13.2	5.3	3.0
9.	13.6	19.6	26.6	4.7	11.6	20.2	5.2	4.4
10.	23.4	15.4	34.9	3.7	23.2	14.9	3.1	3.6
11.	8.4	7.0	13.3	3.8	3.4	7.6	4.7	2.4
12.	24.4	7.1	23.1	7.5	18.5	14.0	*	2.6
13.	33.7	7.4	12.8	18.6	12.6	6.9	6.9	2.0
14.	24.2	7.2	17.2	12.8	10.7	14.9	3.5	4.7
15.	23.0	21.3	11.4	4.9	15.1	17.3	*	5.6
16.	10.9	4.8	24.6	4.4	3.4	13.3	4.1	2.9
17.	16.6	*	27.2	4.1	*	11.6	*	11.5
18.	32.2	*	18.1	*	*	11.4	*	5.9
19.	23.8	*	17.5	*	*	*	*	4.0
C1	1.2	0.0	0.0	0.5	0.3	5.4	3.7	0.6
C2	1.1	0.0	0.0	0.7	0.2	5.4	2.8	1.0
C3	1.2	0.0	0.0	1.2	0.2	6.5	2.9	1.3
C4	1.4	0.0	0.0	0.7	0.4	4.6	4.8	1.6
C5	1.2	0.0	0.0	0.6	0.4	5.8	2.3	1.7

* Damaged count. C1 = control case 1; C2 = control case 2; C3 = control case 3; C4 = control case 4; C5 = control case 5.

Table 4. Chromoblastomycosis: Mean positive cell count in groups A , B and C (control cases)

Group	CD45RO	CD20	CD4	CD8	CD68	CD1a	CD34	Mast cells
Cell type	T	B	T	T	Macr.	Langh.	Endot.	
A	30.1	25.2	11.1	20.7	22.9	4.1	12.4	4.4
B	33.5	18.8	14.5	19.6	27.0	5.0	8.5	2.0
C	1.2	0	0.7	0.3	0	3.2	5.5	1.2

aged and death of the fungal cells was rarely observed. It is believed that the persistence of fungi *in-situ* is the principal factor responsible for the morbidity of this disease [8, 10, 31].

The present data agree with the above claims, since the patients with the best response and with no post-treatment recurrence were those showing scarce, fragmented fungi phagocytized by giant cells. In contrast, in patient with large numbers of fungi the inflammat-

ory process, the fibrosis and the epidermal reaction were much more exuberant.

We believe that fungal persistence and the capacity of fungal multiplication as an exuberant inflammatory response are related to the absence of development of a true granulomatous reaction. This is probably a consequence of factors that inhibited the Th1 response, with a predominance of the Th2 response.

It is known that the development of Th cells into subtypes Th1 and Th2 depends on different factors

Figure 3. Chromoblastomycosis pathogenesis.

Table 5. Chromoblastomycosis: Cytokines

Cases	IL4	IL10	IFN- γ	TNF- α
1.	+	0	+	+
2.	+	+	0	+
3.	+	+	0	+
4.	+	+	0	+
5.	+	0	+	+
6.	+	+	0	+
7.	+	+	0	+
8.	+	+	0	+
9.	+	+	0	+
10.	+	+	0	+
11.	+	0	+	+
12.	+	+	0	+
13.	+	0	+	+
14.	+	+	0	+
15.	+	+	0	+
16.	+	+	0	+
17.	+	+	0	+
18.	+	+	0	+
19.	+	+	0	+
C1	0	0	0	0
C2	0	0	0	0
C3	0	0	0	0
C4	0	0	0	0
C5	0	0	0	0

0 = absent; + = few positive cells; C1 = control case 1; C2 = control case 2; C3 = control case 3; C4 = control case 4; C5 = control case 5.

such as the pathogen, T cells, factors derived from antigen-presenting cells (APC), different categories of dendritic cells, stromal factors, innate immunity, and cytokines produced by other cells such as the mast cells [32–34].

The bipolar spectral aspect verified in the present study allows us to infer that when the host's response tends towards the Th2 pole the lesion is more exuberant and has a significantly larger number of mast cells compared to the atrophic form. It is likely that mast cells contribute to directing the response towards the Th2 pole [35, 36].

Both polar types of response could be better understood according to the model proposed in Figure 3

We believe that further studies are needed to search for the intrinsic and extrinsic determinants of the polarization of this disease in order to develop novel therapeutic strategies that will direct the reaction to-

wards the Th1 pole and will permit to control the infection or to limit it for effective surgical exeresis.

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Address for correspondence: Maria Irma Seixas Duarte, Av. Dr. Arnaldo, 455 sala 1118, CEP. 01246-903, São Paulo, SP, Brazil
Phone/Fax: +55 11 3066-7238; e-mail: miduarte@usp.br; e-mail: spdpatho@famerp.br

Case study

Disseminated infection with *Lacazia loboi* and immunopathology of the lesional spectrum ☆, ☆ ☆

Francisca Regina Oliveira Carneiro MD, PhD^{a,*},
Thais Rodrigues da Cunha Fischer MD^a, Caroline Martins Brandão MD^a,
Carla Pagliari MD, PhD^b, Maria Irma Seixas Duarte MD, PhD^b,
Juarez Antonio Simões Quaresma MD, PhD^{a,c}

^aCentro de Ciencias Biologicas e da Saude, Universidade do Estado do Pará, 66087-670, Belem, PA, Brazil

^bDepartamento de Patologia, Universidade de São Paulo, 01246903, Sao Paulo, SP, Brazil

^cNucleo de Medicina Tropical, Universidade Federal do Pará, 66015-070, Belem, PA, Brazil

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Summary The pathogenesis of lacaziosis continues to be obscure, and works have investigated the blood systemic immune response or the dermal immune response in restricted lesions in different body regions. Some authors describe that the inflammatory infiltrate in lacaziosis lesions showed a predominance of macrophages followed by CD45RO⁺, CD4⁺, and CD8⁺ T cells; CD57⁺ natural killer cells; S-100⁺ cells; and CD20⁺ B lymphocytes. A 54-year-old man and living in the State of Para, Amazon region, Brazil, was seen with a lesion on the left lower limb, which had started as a small nodular area 18 years ago. The lesion showed progressive growth and disseminated to other parts of the body. Our findings showed that dermal immune response differs depending on the type of lesions and clinical presentation, with presence of CD1a⁺, FXIIIa⁺, CD45⁺, CD4⁺, CD8⁺, and S-100⁺ cells and cytokine profile with expression of interleukin 1 β , tumor necrosis factor α , transforming growth factor β , IL-10, and interferon γ .

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1. Introduction

Jorge Lobo's disease or lacaziosis is a chronic mycosis caused by fungal pathogen *Lacazia loboi*, an agent

phylogenetically closely related to the dimorphic pathogen *Paracoccidioides brasiliensis* [1,2]. Cases of lacaziosis have been described in various South American countries, but it assumes great importance in the Brazilian Amazon region, where the largest numbers of these cases are found [3–5]. Data regarding the immune response in *L. loboi* infection are still scarce in the literature. Vilani-Moreno et al [6] characterized the inflammatory infiltrate in lacaziosis lesions and showed a predominance of macrophages followed by, in decreasing order of frequency, CD3⁺, CD4⁺, and CD8⁺ T cells; CD57⁺ natural killer cells; CD79⁺ plasma cells; and CD20⁺ B lymphocytes. Xavier et al [7] characterized the presence of CD68 cells and intense staining for transforming growth factor β (TGF- β) that is a strong inhibitor of the microbicidal activity of macrophages and induces fibrosis in

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* Corresponding author. Ambulatório de Dermatologia, Centro de Ciências Biológicas e da Saúde, Universidade do Estado do Pará, 66087-670, Trav. Perebeui 2623, Marco, Belem, PA, Brazil.

E-mail address: reginacarneiro@globo.com (F. R. Oliveira Carneiro).

keloid-like lesions [8]. The pathogenesis of the disease has been investigated in cases of lesions limited and localized in different regions of the body. This article investigates the immune response in lesions of different clinical features in the same region of the body and in a case of disseminated disease from using immunohistochemistry technique.

2. The case history

A 54-year-old, single, black man, a farm worker, born in the town of Timbira, State of Maranhão, and living in the State of Para, Amazon region, Brazil, was seen with a lesion on the left lower limb, which had started as a small nodular area 18 years ago. The lesion showed progressive growth, and similar lesions appeared on other parts of the body. The patient sought medical assistance, and 100 mg/d clofazimine was prescribed, in addition to surgical excision of several lesions. Lacaziosis treatment was administered for approximately 4 months, but

no response was obtained. The patient currently reported the occurrence of new lesions and progressive growth of older ones.

On physical examination, the patient was afebrile and had a normal skin color. In addition, multiple keloid-like, infiltrative, nodular, papular, and verrucous skin lesions were observed on the trunk, upper and lower limbs, auricle, and abdomen (Fig. 1). These lesions were single or formed plaques that varied in shape and size. For direct mycological examination, material was scraped from the surface of the lesions. The smear demonstrated the presence of single or clustered yeast-like cells with a double membrane in a chain-like arrangement. Serology for human immunodeficiency virus, human T-lymphotrophic virus, hepatitis B, and hepatitis C was negative. A complete blood count showed 4×10^6 red blood cells/mm³, 13 g/100 mL hemoglobin, 40% hematocrit, 3800 leukocytes/mm³, and 300 000 platelets/ μ L. Mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and red cell distribution width as well as monocyte, lymphocyte, eosinophil, neutrophil,

Fig. 1 Clinical aspect of skin lesions in Jorge Lobo's disease. A, Disseminated disease with multiple lesions on the trunk and limbs. B, Verrucous lesions on the lower limbs. C, Papular and nodular lesions in the leg. D, Keloidal and infiltrated lesions in the abdomen.

and basophil counts were normal. CD4 levels in peripheral blood were 800 cells/mm³, and the CD4/CD8 ratio was 2:4. Before treatment, an excision biopsy was performed of each type of skin lesion with multiple years of evolution and stain with hematoxylin-eosin for histopathologic analysis, which revealed a normal or atrophied epidermis adjacent to the lesions, accompanied by flattening of the interpapillary ridges and basement membrane thickening. Epithelial hyperplasia and hyperkeratosis were observed in vegetative- verrucous lesions, often accompanied by basement membrane thickening. The dermis presented dilated vessels and a nodular or diffuse

granulomatous inflammatory infiltrate rich in histiocytes, which were often foamy macrophages due to the presence of intracellular polysaccharides and/or lipids. These histopathologic findings were accompanied by the presence of lymphocytes, epithelioid cells and Langerhans and foreign body giant cells, whose cytoplasm was filled with fungus. Eosinophils and plasma cells were present in smaller numbers (Fig. 2). Treatment with itraconazole (100 mg/d) was initiated, and the patient has been followed-up since June 2007 during monthly medical visits, showing no clinical improvement of the lesions or other symptoms.

Fig. 2 A, Histopathology of skin lesions showing granulomatous infiltrate rich in histiocytes with transepidermal elimination of fungal structures. B, Multiple fungal structures marked by silver staining. Pattern of immunostaining for CD1a (C), CD8 (D), CD4 (E), CD68 (F), TGF- β (G), and IL1- β (H).

3. Materials and methods

Tissue samples were collected before the treatment from different lesions in the same region by surgical excision, after consent was obtained from the patient. The samples were classified according to clinical features into keloid-like, infiltrative, nodular, papular, and verrucous. Next, the specimens were embedded in paraffin, and the blocks were cut into histologic sections. The sections were then submitted to immunohistochemistry using monoclonal antibodies for evaluation of the expression of Langerhans cells (CD1a), CD4 T cells, CD8 T cells, pan-T (CD45RO) cells, dermal dendrocytes (Factor XIIIa [FXIIIa]), and antigen-presenting cells (S-100) and expression of the cytokines tumor necrosis factor α (TNF- α), interferon γ , TGF- β , interleukin 1 β (IL-1 β), and IL-10 according to the protocol of Quaresma et al [9] (Fig. 2). For quantitative analysis of immunostained cells and cytokine expression, we use a grid scale with 10×10 subdivisions in an area of 0.0625 mm^2 in the different types of lesions. The number of positive cells was statistically analyzed by the Graph Pad Prism version 3.0 for Windows using a Mann-Whitney test with the level of significance set at 95%. The project was approved by the Ethics and Research Boards of the Tropical Medicine Center of Federal do Para University.

4. Results

Evaluation of the spectrum of clinical features showed that nodular lesions were characterized by an inflammatory infiltrate consisting of CD1a⁺ Langerhans cells; FXIIIa⁺ cells; CD45⁺, CD4⁺, and CD8⁺ T lymphocytes; and S-100⁺ antigen-presenting cells. These cells were more frequent than in the other lesions examined. The cytokine profile of nodular lesions was characterized by the significant expression of IL-1 β and TNF- α and, to a lesser extent, TGF- β and IL-10. Infiltrative lesions were characterized by moderate numbers of Langerhans cells and no expressive increase in the number of dermal dendrocytes. In addition, low expression of CD4⁺ and CD8⁺ T lymphocytes was observed despite the expressive presence of T lymphocytes in general. The expression of classic Th1 cytokines such as interferon γ and TNF- α was low in infiltrative lesions, whereas expression of IL-10 was higher than in the other lesions examined. In infiltrative lesions, the expression of TGF- β was not related to the immune response pattern but rather to the features of the lesions, with the observation of higher expression in lesions in which the characteristic connective tissue/fibrotic component was more exuberant (eg, keloid-like, papular, and verrucous lesions). These findings show infiltrative lesions with predominance of CD1a, CD45, and IL-10 and nodular lesions with predominance of CD1a, FXIIIa, CD45, CD4, CD8, S-100, IL-1 β , TNF- α , and TGF- β forms as well keloid-like, verrucous, and papular lesions with predominance of TGF- β .

5. Discussion

Clinically, lacaziosis lesions initially manifest as papules that converge into plaques or nodules. The classic clinical presentation is single or confluent brown hemispherical keloid lesions of hard consistency and with a smooth surface. These lesions form multilobular masses covered with intact, smooth, shiny skin, which are clearly delimited from healthy skin. Telangiectasia is also observed. Other presentations include verrucous, ulcerated, gummatous, scleroderma-like, macular, and infiltrative lesions [10]. This polymorphic nature is the most frequent finding upon clinical examination, particularly in cases of long duration of the disease. The lesions may have black dots on the surface, which represent transepithelial elimination of the fungus as observed in other deep mycoses such as chromoblastomycosis [11,12] (Fig. 1). The clinical types can be classified as monomorphic or polymorphic. Skin lesions are localized, multifocal, or disseminated and can be classified as mild (b5 cm), moderate (6–15 cm), severe (N16 cm), or a combination of these types [8]. Several clinical classifications have been suggested. Some authors proposed 2 polar groups: hyperergic (macules and gummas) and hypoergic (keloid-like). Although internal organ involvement is rare, there is the case of a patient from Costa Rica who presented testicular involvement and secondary lymphangitis after 47 years of the disease [13].

In the present case report, analysis of cell-mediated immunity and cytokine expression showed differences in the immune response pattern among the different types of lesion. This finding agrees with previous studies showing different cytokine profiles in lacaziosis depending on the type of lesion and clinical presentation of the disease [8,14]. However, previous works with lesions obtained from different patients and skin samples obtained from different body areas were analyzed in most of these studies [14]. It is known that the skin immune system exerts different functions depending on the area analyzed, and the present study evaluated this immune response in different types of lesion obtained from the same body area [8,14,15]. The present results thus confirm those of previous studies showing that the type of lesion in lacaziosis is correlated with the immune response pattern, including a lesion-type characteristic of the Th1 profile (nodular lesions), intermediate lesions or forms, and infiltrative lesions related to the Th2 profile. Based on the analysis of tissue samples from the same body area, these findings permit us to infer that the clinical features of lacaziosis lesions comprise a spectrum that ranges from susceptibility to resistance.

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Role of mast cells as IL10 producing cells in paracoccidioidomycosis skin lesions

Carla Pagliari, Elaine R. Fernandes, Fernanda Guedes, Cleiton Alves & Mirian N. Sotto
University of São Paulo – Medical School, São Paulo, Brazil

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Abstract

Recent works have demonstrated that mast cells may have an important role in immunologic reactions and inflammation once they synthesize and secrete many cytokines including IL4, IL5, IL6 and TNF- α . We have conducted research in order to verify if mast cells would participate in the local inflammatory immune response against *Paracoccidioides brasiliensis* in skin lesions characterized by a Th2 pattern of cytokines. Fifty-nine skin biopsies with previous histopathological diagnosis of paracoccidioidomycosis and immunohistochemical characterization of cytokines present in the inflammatory infiltrate were classified in three groups: group 1 (G1), with compact granuloma and a Th1 pattern of cytokines; group 2 (G2), with loose granuloma and a Th2 pattern of cytokines; group 3 (G3), both kind of granuloma in the same lesion, characterized by cytokines from Th1 and Th2 patterns. Ten biopsies from normal skin were used as control group. Mast cells were visualized and quantified by a toluidine blue/HCl staining and a double immunostaining was performed to detect a co-localization of mast cells and IL10. G2 presented an increased number of mast cells when compared to G1, G3 and control group and we frequently could find mast cells expressing IL10 in G2. The data obtained suggest that mast cells participate in the immune response against *P. brasiliensis* in skin lesions with loose granuloma and a Th2 pattern of cytokines. Considering these results, mast cells could constitute a source of IL10, contributing to a non-effective response against fungal antigens.

Key words: cytokines, granuloma, mast cells, Paracoccidioidomycosis, skin

Introduction

Mast cells are believed to belong to the nonspecific or innate immune system, which provides the first line of defense against tissue damage.

Detailed knowledge of their role in human health and disease is poorly understood. Recent investigations have attempted to address whether mast cells interact with other cell types. These studies suggest that they seem to play a central role in immune response [1, 2].

Human mast cells express class II MHC molecules suggesting their capacity to present antigens [3–5]. Through experiments, it was demonstrated

that this capacity is inhibited by IFN- γ , suggesting that it will be diminished during the development of a Th1 cell-mediated response, i.e., characterized by a T cell subset that secrete predominantly IFN- γ . On the other hand, in immune response with a Th2 profile, characterized by the secretion of IL4 and IL10, mast cells seem to be particularly involved. They are able to synthesize and secrete many cytokines including IL4, IL5, IL6 and TNF- α [6]. TNF- α is stored within cytoplasmic granules of human dermal mast cells and is released upon degranulation [7]. It is known that IL4, in combination with IL10, is capable of enhancing the proliferation of mast cells in experimental conditions [8].

As resident cells in skin, they represent one of the first inflammatory components to get in contact with pathogens. There are evidences that they participate in the recognition and reaction to many microorganisms such as bacteria and parasites [9, 10]. Recently, the interaction between *Mycobacterium tuberculosis* and mast cells was described. Mast cells recognize the mycobacterial antigen, internalize the bacilli and release histamine, TNF- α and IL6. Histamine influences the Th1/Th2 balance, increasing Th1 cytokines [11, 12]. However, other works demonstrate that histamine suppresses IL12 and stimulates IL10 secretion, increasing Th2 response [13]. Considering their role in releasing TNF- α following cell activation, mast cells seem to participate in differentiation and activation of immature monocytes, macrophage activation and granuloma formation [14, 15].

In this paper, we intend to study the role of mast cells in paracoccidioidomycosis (PCM), a granulomatous disease. It is a human systemic mycosis caused by the dimorphic fungus *Paracoccidioides brasiliensis*. Primary lesions frequently occur in the lungs and can disseminate via bloodstream to other organs. The skin is involved in approximately 50% of the patients [16].

The PCM lesions are characterized by compact or loose granulomas. Well organized and compact granulomas are of the sarcoid type, with small number of parasites and are related to the hyperergic pole of the disease. Poorly or loose granulomas are not efficient to contain the infection and are rich in actively multiplying parasites. This kind of granuloma is related to the anergic pole of PCM [17].

We recently described in PCM skin lesions a Th1 pattern of cytokines related to compact granulomas. We demonstrated by immunohistochemistry cells expressing IFN- γ and TNF- α in large numbers in this type of tissue reaction. The biopsies with well organized granulomas were classified as group 1 (G1). Another group of skin lesions was classified as G2 and presented a large number of cells expressing IL5 and IL10 (Th2 pattern). These lesions were characterized by loose granulomas. We also observed that cells that resembled mast cells were immunolabeled with the anti-IL10 antibody and were distributed in the inflammatory infiltrate of lesions with loose granuloma. A third group (G3) was characterized by both kinds of granuloma in the same lesion and cytokines from Th1 and Th2 patterns [18].

Considering the above exposed, our aim was to verify if mast cells could be participating preferentially in skin inflammatory immune response against *P. brasiliensis*, characterized by a Th2 pattern of cytokines, i.e., loose granuloma reaction. We also intend to demonstrate if mast cells express IL10 in PCM skin lesions.

Material and methods

Fifty-nine skin biopsies from patients with clinical and histopathological diagnosis of PCM were selected from the files of the Dermatopathology Laboratory of the Dermatology Department of University of São Paulo Medical School. The biopsies had been fixed in neutral buffered formalin and embedded in paraffin. Twenty specimens presented inflammatory reaction with well organized granulomas (group 1 – G1), 20 biopsies exhibited inflammatory process with poorly organized granulomas (group 2 – G2) and 19 biopsies exhibited inflammatory reaction with both kinds of granuloma (group 3 – G3). Ten biopsies from normal skin were used as control group.

Mast cells were demonstrated by toluidine blue acid staining. Briefly, sections were dewaxed in xylene and hydrated through a graded series of ethanol. Specimens were stained for 5 min in toluidine blue/HCl solution. They were washed in tap water, dehydrated and mounted with glass cover slip and resin. Mast cells were quantified in 15 randomized high-power fields for each specimen with a $\times 10$ ocular lens with a square grid and a $\times 40$ objective. The results of groups were compared in pairs by Mann–Whitney nonparametric test. The level of significance was set at 95%. Statistical analysis was performed using the Graph Pad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA).

In order to study the possible role of mast cells as IL10-producing cells, a double immunostaining was performed using the doublestain Envision system (Dako Corporation, Carpinteria CA). After deparaffinization with xylene and hydration of the tissues, endogenous peroxidase activity was blocked with 0.03% hydrogen peroxide for 10 min. Slides were incubated over-night at 4 °C with the monoclonal mouse antibody anti-IL10 (R&D Systems, Minneapolis, MN) diluted 1:10. Peroxidase labeled-polymer conjugated to affinity

purified goat anti-rabbit antibody was applied for 30 min at 37 °C and the reaction was visualized with diaminobenzidine and NiCl_2 . The first reaction was blocked with doublestain blocking solution and incubated over-night with a 1:100 dilution of anti-CD117 (Dako Corporation, Carpinteria CA) that labels tissue mast cells [19]. Alkaline phosphatase-labeled polymer conjugated to affinity-purified anti-rabbit was applied for 30 min and the reaction was visualized with fast red and naphthol.

Results

Mast cells were distributed throughout the dermis in the inflammatory infiltrate of all groups of PCM and the control group (Figure 1). The group characterized by loose granuloma and Th2 cytokines (G2) presented higher number of mast cells (median 57.87) when compared to the group with compact granuloma and Th1 cytokines (G1) (median 42.6) and the group with both kinds of granuloma in the same lesion in the tissue inflammatory reaction (G3) (median 16.0), the same with the control group (median 13.3) ($P < 0.05$).

The double labeling technique disclosed CD117 positive cells (labeled in red), that also express IL10 (labeled in black) (Figure 2).

The distribution of mast cells per square millimeter in the PCM groups and control group and statistical analysis are presented in Figure 3.

Discussion

An increased mast cell population is observed in different pathologic process, such as the chronic inflammatory ones. However, the function of mast cells in such processes is poorly understood [20].

It has been demonstrated that IL3 and IL4 can influence mast cell proliferation. These cells circulate in peripheral blood. Once reach the tissues, they undergo final maturation influenced by factors of the tissue microenvironment [21, 22].

We verified that mast cells were frequent in PCM skin lesions characterized by loose granulomas (G2) and these cells population were higher when compared to the other groups ($P < 0.05$). Loose granuloma inflammatory response has been described as non-effective against *Paracoccidioides brasiliensis* [23].

Figure 1. Paracoccidioidomycosis – skin lesion. Mast cells in the dermal inflammatory infiltrate associated to well-organized granuloma (A), loose granuloma (B), both kinds of granuloma in the same lesion (C) and control group of normal skin (D) (Toluidine blue/HCl $\times 400$).

Figure 2. Paracoccidioidomycosis – skin lesion. Double immunostaining showing the expression of IL10 (black) in the cytoplasm of mast cells (red) (Envision system $\times 400$).

Figure 3. Increased number of mast cells in skin lesions with loose granuloma (O), when compared to lesions with compact granuloma (●), lesions with both kinds of granuloma (■) and control group (□). Mann-Whitney test, $P < 0.05$, medians 57.87 in (O), 42.6 in (●), 16.0 (■) and 13.33 (□).

The ability of mast cells in producing $\text{TNF-}\alpha$ [7] makes them important components for the granuloma formation. However, as observed in allergic diseases, mast cells can function as effector cells in host defense through the activation of Toll-like receptors 2 and 4. It is known that, the activation by Toll-like receptor 2 leads

to degranulation and production of IL4 and IL5, cytokines that characterize the Th2 immune response [24, 25].

It has also been demonstrated the cooperation between toll-like receptors in the pattern recognition of pathogens. For example, toll-like receptors 2 and 6 coordinate both the activation of macrophages by zymozan (a yeast-cell particle) [26].

Considering that the toll-like receptor 2 is related to recognition of fungal particle and leads to the mast cells degranulation, we could suppose that it would be present in the immune response against *Paracoccidioides brasiliensis* and consequently, they could be contributing to the Th2 pattern of cytokines in the PCM group with loose granulomas (G2), where this receptor would predominate.

We have previously described cells immunolabeled with anti-IL10 antibody in PCM skin lesions that resemble mast cells [18]. This result suggests that, in addition to the cytokines described as released by mast cells, they could also be a source of IL10, another cytokine of the Th2 pattern. In the present paper we could reinforce this assumption by demonstrating CD117 positive cells labeled by anti-IL10 antibody. CD117 is considered a good immunostaining to demonstrate mast cells [19]. Streilein et al (1999) studying UVB skin response in murine experimental model, suggested that mast cells may be a source of IL10 in skin [27].

Considering the results obtained, we could conclude that mast cells play a role in the immune response against *P. brasiliensis* in skin lesions characterized by loose granulomas that is an expression of Th2 cellular immune response. Mast cells participation in PCM lesions may be regarded as a source of cytokines, including IL10. To the best of our knowledge, this is the first description of mast cells expressing IL10 in skin lesions. The predominant IL10 cytokine would contribute to the non-effective response against the fungal antigens in loose granuloma (G2) tissue response.

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Address for correspondence: Carla Pagliari, University of São Paulo – Medical School, São Paulo, Brazil
E-mail: cpagliari@uol.com.br

Characterization of cytotoxic immune response in skin and mucosal lesions of paracoccidioidomycosis

Background: CD8+ T cells and natural killer (NK) cells are involved in the immune response against some pathogens. For this purpose, we investigated the *in situ* paracoccidioidomycosis (PCM) immune response addressing the participation of NK cells, CD8+ T cells, perforin and granzyme B expression.

Methods: Sixty biopsies of PCM skin and mucosa were classified according to the presence of compact granulomas (G1), poorly organized granulomas (G2) and both kinds in the same lesion (G3). CD8+ T cells, NK cells, perforin and granzyme B were showed by immunohistochemistry.

Results: CD8+ T cells were increased over NK cells in cutaneous G1 and G2 lesions. There was no difference regarding such cells in G3 lesions, although they were abundant in such lesions. In mucosa, CD8+ T cells were increased in number over NK cells in all groups. Granzyme B in skin increased in G2 and G3. The number of granzyme did not differ in mucosal lesions in the three groups.

Conclusions: CD8+ T cells and NK cells play a role in PCM cutaneous and mucosal lesions. The predominance of CD8+ T cells over NK cells may represent an effective response against the fungi. Moreover, the high number of granzyme B expressing cells corroborates this possibility.

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Carla Pagliari^{1,2}, Naiura Vieira Pereira^{1,2}, Luciane Kanashiro¹, Felipe Weissaupt Stegun¹, Julio Croda³, Maria Irma Seixas Duarte¹ and Mirian Nacagami Sotto²

¹Laboratory of the Discipline of Pathology of Transmissible Diseases, Department of Pathology, Faculty of Medical Sciences, University of São Paulo, São Paulo, Brazil

²Laboratory of Dermatopathology, Department of Dermatology, Faculty of Medical Sciences, University of São Paulo, São Paulo, Brazil

³Faculty of Health Sciences, University of Grande Dourados, Dourados, Brazil

Carla Pagliari, PhD,
Faculty of Medical Sciences, University of São Paulo,
Avenue Dr Arnaldo, 455, sala 1118, 1^o andar, CEP
01246-903, Cerqueira Cesar, São Paulo, SP, Brazil
Tel: 55 11 3061 7238
Fax: 55 11 3061 7238
e-mail: cpagliari@usp.br

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Paracoccidioidomycosis (PCM) is an important systemic mycosis in Latin America, caused by the fungus *Paracoccidioides brasiliensis* (*Pb*). The chronic form of the disease is characterized by lesions in lungs and subsequently the yeast cells can spread to other organs. The granulomatous response present in PCM lesions is a specialized tissue response against fungal dissemination.^{1,2} Such lesions are characterized by compact or poorly organized granulomas. Compact or well-organized granulomas are of the sarcoid type, with small number of parasites and are related to the hyperergic pole of the disease. Loose or poorly organized granulomas are not efficient to contain the infection and are rich in actively multiplying parasites.

This kind of granuloma is related to the anergic pole of PCM.

We recently described in PCM skin lesions a Th1 pattern of cytokines related to compact granulomas. We showed by immunohistochemistry cells expressing IFN- γ and TNF- α in large numbers in this type of tissue reaction. The biopsies with well-organized granulomas were classified as group 1 (G1). Another group of skin lesions was classified as G2 and presented a large number of cells expressing IL5 and IL10 (Th2 pattern). These lesions were characterized by loose granulomas. A third group (G3) was characterized by both kinds of granulomas in the same lesion and cytokines from both Th1 and Th2 patterns.³

The presence of lesions in skin and oral mucosa is frequent in almost 30% of the cases and the study of such lesions is important considering their value for diagnosis. Indeed, the concept of the compartmentalization of immune system⁴ was an urge to study the secondary organs of infection by *Pb*, in order to evaluate possible differences from the lungs, where the primary focus of infection occurs.

Considering that in PCM the major cellular immune response is mediated by CD4+ T cells, there are few reports regarding CD8+ T cells, the same with natural killer (NK) cells, both of which are able to produce immune mediators. These cells participate in the cytotoxic immune response producing perforin and granzyme.⁵

NK cells, in experimental infection by *Pb*, seem to be related to a depressed immune response with increased lesions late in the infection.⁶ Some studies suggest an important role of CD8+ T cells in the control of fungal load.^{7,8}

In this study we propose to contribute to the understanding of the cutaneous and mucosal immune response in PCM, focusing on the participation of NK and CD8+ T cells, and the cytotoxic mediators, perforin and granzyme B.

Materials and methods

Biopsies

Sixty skin biopsies and 60 mucosal oral biopsies from patients with clinical and histopathological diagnosis of PCM were selected from the files of the Dermatopathology Laboratory of the Dermatology Department of University of São Paulo Faculty of Medical Sciences.

Histopathology

The biopsies had been fixed in neutral buffered formalin and embedded in paraffin. The histopathological study of lesions was performed with hematoxylin-eosin staining to observe the epidermal/epithelial and dermal/stromal alterations. The lesions were classified into three groups: group 1 (G1) was characterized by an inflammatory reaction composed of well-organized and compact epithelioid granulomas with giant cells and surrounded by lymphocytes and few plasma cells. Group 2 (G2) exhibited loosely arranged epithelioid cells intermingled with macrophages, lymphocytes, plasma cells and few polymorphonuclear neutrophils. Group 3 (G3) displayed both kinds of tissue reaction in the same specimen. G1 was constituted by 22 skin and 18 mucosal lesions, group 2 presented 20 skin and 27 mucosa and group 3, 18 skin and 15 mucosa.

Cells and granules analysis

A streptavidin-biotin peroxidase immunohistochemical method was performed with the LSAB®+ kit (LSAB + KIT, K0690, Dako Corporation, Carpinteria, CA, EUA). Following deparaffinization in xylene and hydration in ethanol, antigen recovery was performed in a retrieval solution pH 9.0 (S2368, Dako Corporation) for 20 min at 95°C. The primary antibodies anti-granzyme B (MS-1157, Labvision), perforin (MS-1834, Labvision) and NK cells (MS-136-P, Labvision) were applied and after an overnight incubation at 4°C it was applied the second antibody and the complex LSAB. CD8+ T cells (M7103, Dako) were better visualized by using the catalyzed signal amplification system (K1497, Dako Corporation) based on the peroxidase-catalyzed deposition of a biotinylated phenolic compound followed by a secondary reaction with streptavidin peroxidase.⁹ 3,3-Diaminobenzidine tetrahydrochloride (Sigma) was used as chromogen and the slides were counterstained with hematoxylin. All reactions were performed with positive and negative controls. The second ones were constituted by the use of isotype controls and the omission of the primary antibody.

Quantitative analysis

Cells were quantified by counting the number of immunolabeled cells in nine randomized high-power fields for each specimen with a ×10 ocular lens with a square grid and a ×40 objective.

Statistical analysis

The number of positive cells was statistically analyzed by using Graph Pad Prism version 3.00 for Windows (Graph Pad software, San Diego, CA, USA) to perform the Mann-Whitney test with the level for significance set at 95% ($p \leq 0.05$).

Results

Histopathological analysis

Both cutaneous and mucosal lesions presented similar histological characteristics. Epidermis and epithelium were characterized by hyperkeratosis, parakeratosis and acanthosis. Intraepidermic microabscesses were present in 60% of the cases. The dermis and stroma were characterized by inflammatory infiltrate with plasma cells, lymphocytes and macrophages.

Cutaneous lesions

NK cells were present in all the cases of G1 and G3 and absent in one case in G2. The statistical analysis did not evidence difference among the groups

Fig. 1. Histopathology and immunohistochemistry of representative skin lesion of patient with paracoccidioidomycosis. Granuloma with giant cell and inflammatory infiltrate (A); natural killer cells immunostained in brown, around granuloma (B); CD8+ T cells immunostained in brown, around the granuloma (C) and immunodetection of granzyme B granules in the cytoplasm of giant cell (D) (hematoxylin-eosin and streptavidin-biotin peroxidase $\times 400$).

($p > 0.05$). The CD8+ T cells were visualized in all lesions although they did not present statistical difference among the groups ($p > 0.05$). They were distributed in the inflammatory infiltrate and around the granuloma (Fig. 1).

Comparing the number of CD8+ T cells with NK cells, the predominance of CD8+ T cells over NK cells in the three groups was observed. In patients presented with well-organized and poorly organized granulomas (G1 and G2), these differences were statistically significant ($p = 0.0141$ and $p = 0.0008$, respectively) (Fig. 3).

Cells expressing granzyme B were present in all the skin lesions. It was frequent in the presence of granules in the cytoplasm of giant cells with fungal cells. The quantification evidenced higher number of positive cells in G2, the opposite in G1. The statistical analysis did not evidence difference between G2 and G3 (Fig. 4).

Mucosal lesions

NK cells were present in the inflammatory infiltrate of all the mucosal lesions. The statistical analysis did not evidence significant difference among the groups. The CD8+ T cells were visualized in the inflammatory infiltrate and around the granuloma in all the mucosal lesions with no numerical difference among them (Figs. 2 and 3).

The CD8+ T lymphocytes were increased in number when compared to NK cells in the three groups of mucosal lesions ($p < 0.0001$).

It was possible to detect cells expressing granzyme B in the stroma of all the mucosal lesions; however,

it was not detected numerical difference when the groups were compared (Fig. 4).

The expression of perforin was poorly detected, both in cutaneous and mucosal lesions. For this reason, cells expressing such protein were not quantified. The biopsies were classified as positive or negative for this immunoreaction, as presented in Fig. 5.

Discussion

The study of cutaneous and mucosal lesions in PCM is important considering their frequency and diagnostic value for the disease. Skin and mucosal involvement are considered secondary focus of a lung infection by *Pb*.¹⁰

Similar to our previous report in cutaneous lesions,³ mucosal lesions could also be classified according to the characterization of granulomatous response. The different granulomatous response, reflect *in situ* expression of the immune differences of the host against the *Pb*.

NK cells are subset of lymphocytes that play an important role in protection against infectious agents. NK cells present a diminished activity in patients with active PCM. In experimental PCM, NK cells seem to be activated during the early phase of the infection and are involved in the natural host resistance. However, in later stages of the disease, this activity is decreased owing to a depressed cellular immune response, and increase of lesions. In this phase of experimental PCM, NK cells are unable to control the dissemination of the fungi.^{6,11} The

Fig. 2. Histopathology and immunohistochemistry of representative mucosal lesion of patient with paracoccidioidomycosis. Granuloma with giant cell and inflammatory infiltrate (A); natural killer cells immunostained in brown (B); CD8+ T cells immunostained in brown in the inflammatory infiltrate (C) and immunodetection of granzyme B granules in granuloma (D) (hematoxylin-eosin ($\times 100$) and streptavidin-biotin peroxidase, $\times 400$).

Fig. 3. Distribution of NK cells and CD8+ T lymphocytes in paracoccidioidomycosis cutaneous (A) and mucosal (B) lesions. Bars represent mean values. Mann-Whitney test.

Fig. 4. Distribution of cells expressing granzyme B in paracoccidioidomycosis cutaneous (A) and mucosal (B) lesions. Bars represent mean values. Mann-Whitney test.

participation and interaction of NK cells with other cells remain to be determined in human PCM lesions.

The present study did not display any correlation with the number of NK cells and the type of tissue response in skin and mucosal PCM lesions.

Considering the CD8+ T cells, it was also possible to verify that such cells seem to play an important role in the tissue response, both in skin and mucosa lesions. Otherwise, the number of this lymphocyte subset could not correlate with the different kinds of granulomatous response observed in PCM lesions.

Cytotoxic response in paracoccidioidomycosis

Fig. 5. Perforin expression in paracoccidioidomycosis cutaneous and mucosal lesions.

In animal models of PCM, it is described that deficiency of CD8+ T cells lead to increased mortality rates and, irrespective of the host susceptibility pattern, fungal loads are mainly controlled by this group of cells. The genetic resistance to PCM is associated with concomitant CD4+ and CD8+ T cell immunity secreting cytokines of Th1 and Th2 patterns.¹²

The CD8+ T cells play a role in the control of other intracellular pathogens, such as in tuberculosis and histoplasmosis.^{13–15}

It is well known that CD4+ T cells subset play a key role in controlling Pb infection. PCM oral lesions displayed predominance of CD4+ T cells associated with well-organized granulomas, and a balance in CD4+ and CD8+ T cells in non-granulomatous areas.¹⁶

Because the role of CD8+ T cells in a host with no deficiency in CD4+ T cell numbers is comparatively minor, not much is known about the function of CD8+ T cells. The increased number of CD8+ T cells observed in the present study suggests that these cells may play a role in the *in situ* immune response, both in cutaneous and mucosal PCM lesions.

The main mechanism that CD8+ T cells kill target cells is through the secretion of cytotoxic perforin and granzyme granules. Perforin was described as playing an important role for primary immunity in fungal infection.¹⁷

The cytotoxic immune response seems to be another important mechanism to control *Pb* infection. Cells expressing perforin and granzyme B were observed in both cutaneous and mucosal PCM lesions. Our data are in accordance with some recent results that showed cytotoxic granules and CD8+ T cells in bronchoalveolar lavage of PCM patients.¹⁸

Although cutaneous and mucosal PCM lesions display tissue response characteristics that are related to the Th1 and Th2 patterns of cytokines,³ the number of NK and CD8+ T cells did not correlate to the characteristics of tissue reaction observed in such lesions.

The results obtained may contribute to the better characterization of cutaneous and mucosal immune response in PCM. We could provide evidences that CD8+ T cells display a cytotoxic function in PCM, based on the demonstration of their expression of perforin and granzyme B. The cytotoxic response may play a role in the *in situ* immune response against the fungal infection. The predominance of CD8+ T cells over NK cells may represent an effective response against the fungi. Moreover, the high number of granzyme B expressing cells corroborates to this possibility.

CD8+ T cells are able to produce granzyme B that can target infected macrophages and giant cells that form the granulomas. We may suggest that, although CD4+ T cells are essential for host defense against Pb, CD8+ T cells play a role in the elimination of the fungus.

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Original Article

The cytotoxic T cells may contribute to the *in situ* immune response in Jorge Lobo's Disease human lesions

Ariane Fernandes Alexandre^{1,2}, Juarez Antonio Simões Quaresma^{3,4},
Tânia Cristina Barboza^{1,2}, Arival Cardoso de Brito³, Marília Brasil Xavier³,
Clivia Maria Moraes de Oliveira³, Deborah Aben Athar Unger³,
Luciane Kanashiro-Galo¹, Mirian Nacagami Sotto¹,
Maria Irma Seixas Duarte¹ and Carla Pagliari^{1,2,*}

¹Faculdade de Medicina, Universidade de São Paulo, Departamento de Patologia, ²Programa de Pós-graduação em Ciências da Saúde/Instituto de Assistência Médica ao Servidor Público Estadual, ³Núcleo de Medicina Tropical, Universidade Federal do Pará and ⁴Universidade do Estado do Pará

*To whom correspondence should be addressed. Dra. Carla Pagliari, Universidade de São Paulo, Faculdade de Medicina, Departamento de Patologia, Av. Dr. Arnaldo, 455, sala 1118, CEP 01246–903, Cerqueira Cesar, São Paulo, SP, Brasil.
Tel/Fax: +(55 11) 3061–7238; E-mail: cpagliari@usp.br

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Abstract

Jorge Lobo's Disease (JLD) is a cutaneous chronic granulomatous disease caused by the pathogenic fungus *Lacazia loboi*. It is characterized by a granulomatous reaction with multinucleated giant cells and high number of fungal cells. In order to contribute to the comprehension of immune mechanisms in JLD human lesions, we studied the cytotoxic immune response, focusing on TCD8+ and NK cells, and granzyme B. Forty skin biopsies of lower limbs were selected and an immunohistochemistry protocol was developed to detect CD8+ T cells, NK cells and Granzyme B. In order to compare the cellular populations, we also performed a protocol to visualize TCD4+ cells. Immunolabeled cells were quantified in nine randomized fields in the dermis. Lesions were characterized by inflammatory infiltrate of macrophages, lymphocytes, epithelioid and multinucleated giant cells with intense number of fungal forms. There was a prevalence of CD8 over CD4 cells, followed by NK cells. Our results suggest that in JLD the cytotoxic immune response could represent another important mechanism to control *Lacazia loboi* infection. We may suggest that, although CD4+ T cells are essential for host defense in JLD, CD8+ T cells could play a role in the elimination of the fungus.

Key words: Jorge Lobo's disease, cytotoxic response, skin, human lesions, immunohistochemistry.

Introduction

Jorge Lobo's Disease (JLD) is an important cutaneous chronic granulomatous disease caused by the pathogenic

fungus *Lacazia loboi*. The higher numbers of cases are reported in Brazil, although the disease is described in other Latin American countries.^{1–5}

Lesions are commonly found on the ears and upper and lower limbs and can present different clinical aspects.⁶ Some aspects are the keloid-like type, verrucous, ulcerated, gummy, scleroderma or macular infiltrative ones.^{7–9}

The histopathological diagnosis is an important tool through which it is possible to observe a granulomatous reaction with multinucleated giant cells and high number of fungal cells. The local immune response has been explored, but some aspects of immunopathogenesis are still little understood. Studies focusing on the *in situ* immune response in human lesions of JLD characterized some cells and cytokines.¹⁰

Regarding the cytotoxic immune response in fungal infections, we recently studied the role of TCD8+ cells, NK cells, and granzyme B in cutaneous and mucosal lesions from patients with Paracoccidioidomycosis, a disease that share some characteristics with JLD. It was evidenced the cytotoxic function of CD8+T cells based on the detection of perforin and granzyme B and suggested a role in the *in situ* immune response to eliminate the fungus.¹¹

Studies on the cellular immune response in JLD demonstrate the presence of macrophages (CD68+) and intense expression of TGF- β , associated to fibrosis and absence of well organized granulomas.⁷

The ineffective immune response against *L. loboi* seems to be related to a Th2 pattern of cytokines.¹² It is described an expressive number of CD4+ T cells producing IL17 over TCD4+Foxp3+ cells in JLD cutaneous lesions and suggested that the Th17 pattern of cytokines could be present as an effort to modulate the local immune response.¹³ In another approach, it was possible to demonstrate that JLD lesions comprise a clinical and immunological spectrum that ranges from susceptibility to resistance.¹⁴

Some studies suggest an important role of CD8+ T cells in the control of fungal load.^{15,16,17} In order to contribute to the best comprehension of the immune mechanisms in JLD human lesions, we intended to explore the cytotoxic immune response, focusing on CD8+ and NK cells, and granzyme B.

Material and methods

Forty skin biopsies of lower limbs from 40 patients were selected from the files of the Nucleo de Medicina Tropical, Universidade Federal do Para, Belem, PA, Brazil. The diagnosis was based on the direct mycological examination and histological analysis.

Immunohistochemistry

Following deparaffinization in xylene and hydration in ethanol, antigen recovery was performed in a TRIS/EDTA

solution pH9.0 for 20 minutes at 95°C. The primary antibodies anti-CD8 (DakoCytomation, M7103, CA, USA), anti-NK (Labvision, MS-136, CA, USA) and anti-Granzyme B (Santa Cruz, SC1969, TX, USA) were applied at 1/20, 1/200, and 1/50 dilutions, respectively. In order to compare the cellular populations, we also performed an immunohistochemistry protocol to visualize TCD4+ cells (1/1000) (DakoCytomation, M834, CA, USA). Following an overnight incubation at 4°C, it was applied to the second antibody and a Streptavidin-Biotin peroxidase system (LSAB, Dako, Carpinteria, CA, USA). 3, 3-diaminobenzidine tetrahydrochloride was used as chromogen, and the slides were counterstained with hematoxylin. All reactions were performed with positive and negative controls. The second ones were constituted by the use of isotype controls and the omission of the primary antibody. Immunolabeled cells were quantified in nine randomized fields in the dermis using an x10 ocular lens with a square grid and an x40 objective. The sum of the number of immunostained cells was divided by the number of fields (nine) and the result was divided by 0.0625 (area of the grid), providing the number of cells/mm². Statistical analysis was performed using the GraphPad Prism 5.0 program, using the Mann–Whitney test. A level of significance of 5% ($P < .05$) was adopted for all tests. The Spearman test was used to evaluate the correlation coefficients (r).

Results

The patients' age ranged from 25 to 80 years, mean age of 52.5 years. Thirty-five were male (age range 31–80 years) and 5 were female (age range 25–65 years).

The lesions were of keloid-like type ($n = 34$) and verrucous ($n = 6$), 8 localized in the ear, 3 on the face, 6 in upper limb, and 23 in lower limb.

The lesions were characterized by inflammatory infiltrate constituted by macrophages, lymphocytes, rare asteroid corpuscles, epithelioid cells, and multinucleated cells of Langhans and foreign body types, intense number of fungal cells (Figure 1A).

The populations of CD4 and CD8 T lymphocytes were visualized in all the specimens of JLD, distributed in the inflammatory infiltrate, the same to NK cells and granzyme B (Figure 1B–E).

The quantification of immunolabeled cells evidenced the prevalence of CD8 (mean $107.2 \pm \text{SD } 153.4$) over CD4 cells (mean 59.24 ± 154.1), followed by NK cells (mean $20.0 \pm \text{SD } 31.28$). The mean value of cells expressing granzyme B was $39.40 \pm \text{SD } 61.52$. Figure 1F presents the quantitative results, considering nine randomized fields. The analysis of quantitative results (Mann–Whitney test) evidenced

Figure 1. Jorge Lobo's Disease, skin lesions. **A)** Inflammatory infiltrate constituted by macrophages (arrow), lymphocytes (arrow), epithelioid cells (EC) and multinucleated cells, intense number of fungal cells (F). Detail: giant cell with asteroid body (Hematoxylin-Eosin, $\times 200$); **B)** High number of CD8+ T lymphocytes in the inflammatory infiltrate and in the granuloma reaction (Streptavidin-Biotin HRP, $\times 200$); **C)** CD4+ T lymphocytes in the granuloma reaction (Streptavidin-Biotin HRP, $\times 200$); **D)** Rare NK cells in the inflammatory infiltrate (Streptavidin-Biotin HRP, $\times 200$); **E)** Some cells intermingled to the fungal cells expressing granzyme B (Streptavidin-Biotin HRP, $\times 600$); **F)** Quantitative analysis evidencing the mean value of immunolabeled cells: 1-CD8 (SD = 153.4), 2-CD4 (SD = 154.1), 3-NK (SD = 31.28), 4-cells expressing granzyme B (SD = 61.52).

statistically significant difference only when the populations of CD8 $\times$ CD4 and CD8 $\times$ NK were compared ($P < .05$). The casuistic was also divided in lesions of verrucous and keloid-form types and values were compared for each cell population, evidencing no statistical differences between both groups ($P > .05$).

The correlation coefficients between the expression of granzyme B and CD4, CD8 T cells and NK cells is in Table 1.

Discussion

Jorge Lobo's Disease is an important disease, characterized by a chronic granulomatous reaction and generally is full of

Table 1. Jorge Lobo's disease.

	Mean $\pm$ SD – JLD Spearman test		
	CD4+T cells	CD8+T cells	NK cells
	59.24 $\pm$ 154.1	107.2 $\pm$ 153.4	20.00 $\pm$ 31.28
Granzyme B	r = 0.07	r = 0.29	r = 0.05
39.40 $\pm$ 61.52	P = .63	P = .07	P = .78

Note: Mean values $\pm$ standard deviation and correlation coefficients between the expression of granzyme B and CD4 and CD8 T cells, NK cells in human cutaneous lesions. (Spearman test).

JLD, Jorge Lobo's disease; SD, standard deviation.

parasites in the dermis. It is frequent in exposed populations of endemic areas in Latin America.¹⁸

In the last few years, some studies regarding the characterization of the *in situ* immune response in patients with Jorge Lobo's Disease have been performed. Previous studies demonstrated poorly organized granuloma constituted by CD68+ macrophages and multinucleated giant cells, CD3+ T lymphocytes, CD4+ and CD8+ lymphocytes, CD57+ natural killer cells, CD79+ plasma cells and CD20+ B lymphocytes.^{19,20}

Xavier et al.⁷ correlated the strong presence of collagen bands as an effect of TGF- β and consequent inhibition of macrophages, inducing fibrosis. Authors speculated that TGF- β could play a role in the pathogenesis of JLD, either by inhibiting the cellular immune response mainly mediated by macrophages or by inducing fibrosis.

TGF- β 1 is produced by macrophages and Th3 lymphocytes and is considered a potent immunosuppressive cytokine that suppresses the phagocytic activity of macrophages.²¹ However, this cytokine plays a role in promoting the proliferation of CD8+ T cells. Considering the characterization of JLD lesions regarding the phenotype of cells and cytokines, our results evidencing the predominance of CD8+ T cells over CD4+ T cells are in accordance with those previous data. The increased number of CD8+ T cells observed in the present study suggests that these cells may play a role in the *in situ* immune response in JLD lesions.

It may also be considered that TGF-beta, as a regulatory factor, affects cells proliferation, differentiation and is involved in some processes of immune response and wound healing.¹⁸ It is demonstrated the potent inhibitor role over TCD4+, TCD8+, and NK cells, considering the function. This could explain at least in part the lower number of TCD4+ cells in JLD lesions when compared to TCD8+ cells. Some studies have shown that TCD8+ cells activated in the presence of TGF-beta do not present cytotoxic function.^{22,23} Considering that, besides the presence of high number of TCD8+ cells, it could be suggested that they have some deficit in their cytotoxic role in JLD.

NK cells play an important role in protection against different infectious agents. NK cells present a diminished activity in patients with active PCM, a disease that share some characteristic with JLD. Although in the early phase of PCM these cells are related to resistance, in later stages this activity is decreased and unable to control the dissemination of the fungi.²⁴

The main mechanism that CD8+ T cells kill target cells is through the secretion of perforin and granzyme granules. Not only CD8+ T cells but also NK cells produce granzyme B as an important ability to kill their targets.²⁵

Previously, studying skin and mucosal lesions of PCM, we evidenced that CD8+ T cells display a cytotoxic function through the expression of perforin and granzyme B and speculated that the cytotoxic response may play a role in the *in situ* immune response against that fungal infection.¹¹

Allied to the expression of granzyme B, other cytotoxic elements are important in the interaction of these groups of cells in fungal infections. In cryptococcosis, CD8 cells use granulysin to destroy yeast cells and in histoplasmosis, it is verified the importance of granzyme A and B.^{26,27}

NK cells, also in cryptococcosis, present cytotoxic activity mediated by perforin, however, the immune response against *Aspergillus fumigatus* these cells did not mediate cytotoxicity through perforin and granulysin or granzyme and present other factors not yet well elucidated.^{27,28}

Our results suggest that in JLD the cytotoxic immune response could represent another important mechanism to control *Lacazia loboi* infection. Cells expressing granzyme B were observed in the inflammatory infiltrate. CD8+ T cells are able to produce granzyme B that can target infected macrophages and giant cells that form the granulomas. We may suggest that, although CD4+ T cells are essential for host defense in JLD, CD8+ T cells could play a role in the elimination of the fungus.

The present results contribute to the existing knowledge on the immune responses to the *Lacazia loboi* infection and could lead to the development of new strategies for patient management and remission of the lesions.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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Paracoccidioidomycosis: Cells expressing IL17 and Foxp3 in cutaneous and mucosal lesions

Carla Pagliari^{a,*}, Elaine Raniero Fernandes^a, Felipe Weissaupt Stegun^a, Wellington Luiz F. da Silva^a, Maria Irma Seixas Duarte^a, Mirian N. Sotto^{a,b}

^aLaboratório da Disciplina de Patologia de Moléstias Transmissíveis, Departamento de Patologia, Faculdade de Medicina da Universidade de São Paulo, Brazil

^bLaboratório de Dermatopatologia, Departamento de Dermatologia, Faculdade de Medicina da, Universidade de São Paulo, Brazil

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ABSTRACT

We demonstrated and quantified by immunohistochemistry the population of cells expressing IL17 and Foxp3 in cutaneous and mucosal paracoccidioidomycosis lesions, associating these populations of cells with different presentations of granulomatous response. For this purpose, 61 skin biopsies and 55 oral mucosal biopsies were evaluated. Cells expressing IL17 were distributed in the inflammatory infiltrate in both groups of lesions and were found in the vessels' wall too. Foxp3+ expression was limited to the nuclei of lymphocytes in the inflammatory infiltrate. The distribution of IL17 was similar among the groups; however, Foxp3+ cells were increased in mucosal lesions that displayed compact granulomas. The results suggest that IL17 seems to play a role in paracoccidioidomycosis cutaneous and mucosal lesions, probably as secondary cells in the clearance of the fungal antigens. The presence of Foxp3+ cells both in skin and mucosa corroborates some previous researches that suggest the role of this group of cells in the modulation of local immune response.

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1. Introduction

Paracoccidioidomycosis (PCM), a human systemic mycosis caused by the dimorphic fungus *Paracoccidioides brasiliensis* (Pb), is very important in Latin America, with endemic areas in Brazil, Argentina, Colombia and Venezuela. Primary lesions occur in lungs and the skin and mucosa are affected in approximately 50% of the cases [1].

The pattern of cellular immune response mediated by CD4 + T cells determines the resistance or susceptibility during the course of the infection. Cells producing Th1 cytokines and the histological pattern of compact granulomas characterize resistance against the fungus. On the other hand, a Th2 pattern of cytokines and poorly-organized granulomas are related to susceptibility to the fungi [2–7]. The concept of a Th1/Th2 paradigm of T helper cells was first introduced almost 25 years ago [8] and elucidated many immunological phenomena. However, recently it was discovered a third set of T helper cells that produce mainly IL17, with distinct functions from Th1 and Th2 [9,10].

Th17 cells seem to have been developed to assist in the clearance of pathogens that are not effectively destroyed by Th1 or Th2 lineages. Their development needs TGF-beta, establishing a link between T regulatory lymphocytes (Treg) and Th17. It is known that IL17- producing CD4 + T cells present reactivity against fungal antigens, suggesting that Th17 cells may play an important role in the defense against fungal infections [11,12]. Its importance against fungi was first described in *Candida albicans* infection in 2004 [13]. Although IL17 is involved in innate immunity against *C. albicans*, memory T cells specific for fungal antigens were present in human Th17 subsets, suggesting its importance in acquired immunity [11]. A decrease in IL17 was related to susceptibility to infection by *Cryptococcus neoformans* [14]. Otherwise, protection in *Pneumocystis carinii* pneumonia also seems to be related to IL17 [15].

Regulatory T cells (Treg) constitute an important subpopulation of T lymphocytes in the induction and maintenance of immune tolerance and has emerged as a regulator key in the finalization of the immune response. Deficiency or dysfunction of those cells can aggravate pathogen-induced inflammation. The expression of TGF-beta plus IL6 or IL21 is required for early differentiation of Th17 cells [16,17]. However, TGF-beta was previously related to the development of Foxp3+ Treg cells. Bearing that in mind, it was proposed a reciprocal relationship between Tregs and Th17 cells [12,18]. In a recent study, Cavassani and colleagues found that the number of Treg is elevated in blood and oral lesions of patients with PCM.

* Corresponding author. Faculdade de Medicina – USP, Av Dr Arnaldo, 455 sala 1118, Cerqueira Cesar – São Paulo-SP-Brasil, CEP 01246-903, Brazil. Tel./fax: +55 11 3061 7238.

E-mail address: cpagliari@usp.br (C. Pagliari).

According to these authors, Treg accumulate in sites of infection, limiting the effector immune response and allowing the persistence of the pathogen and induction of chronicity [19].

Considering that there are few studies focusing the expression of CD4 + T cells with a Th17 pattern of response in fungal infections, the objective of the present study was to contribute to the study of tissue response of skin and oral mucosa in PCM, addressing the involvement of cells expressing IL17 and Treg lymphocytes in the lesions. The study also intends to verify the number of Treg cells in different types of tissue response of PCM: well organized granulomas, loosely organized and in lesions with both types of granulomatous response.

2. Materials and methods

2.1. Characterization of the samples

Sixty-one skin biopsies and fifty-five mucosal oral biopsies from patients with clinical and histopathological diagnosis of PCM were selected from the files of the Dermatopathology Laboratory of the Dermatology Department of University of São Paulo Faculty of Medicine.

The biopsies had been fixed in neutral buffered formalin and embedded in paraffin. The previous histopathological study of lesions, performed with hematoxylin-eosin (HE) staining, permitted the classification of the biopsies in three groups: group 1 (G1) was characterized by an inflammatory reaction composed of well organized and compact epithelioid granulomas with giant cells and surrounded by lymphocytes and few plasma cells. Group 2 (G2) exhibited loose arranged epithelioid cells associated with macrophages, lymphocytes, plasma cells and few polymorphonuclear neutrophils. Group 3 (G3) displayed both kinds of tissue reaction in the same specimen. G1 was constituted by 21 skin and 16 mucosal lesions, G2 presented 22 skin and 21 mucosa and G3, 18 skin and 18 mucosa fragments.

2.2. Immunohistochemistry

It was performed a Streptavidin-biotin peroxidase immunohistochemical method with the LSAB[®] + kit (LSAB[®] + KIT, K0690, Dako Corporation, Carpinteria, California, EUA). Following deparaffinization in xylene and hydration in ethanol, antigen recovery was performed in a retrieval solution pH9.0 (S2368, Dako Corporation) for 20 min at 95 °C. The primary antibodies anti-IL17 (AF-317-NA, RD Systems) and anti-Foxp3 (14-4776, E-Bioscience) were applied at a 1:20 and 1:50 dilution respectively and after an overnight incubation at 4 °C it was applied the second antibodies and

the LSAB complex. 3, 3-diaminobenzidine tetrahydrochloride (DAB-Sigma) was used as chromogen and the slides were counterstained with hematoxylin. All reactions were performed with positive and negative controls. The second ones were constituted by the use of isotype controls and the omission of the primary antibody.

In order to define the phenotype of cells expressing IL17, it was performed a double immunostaining reaction to detect the co-expression of CD4/IL17 and CD8/IL17. The fragments were incubated overnight at 4 °C with the anti-IL17 primary antibody and were developed with the LSAB complex. The peroxidase reaction was revealed with DAB. Following, it was performed the second reaction with the anti-CD4 or anti-CD8 antibody. The reaction was developed with Envision alkaline phosphatase (AP) (code K4018 – Dako) and revealed with Permanent Blue (code K 051-110, Diagnostic BioSystems).

2.3. Quantitative analysis

Cells immunostained were quantified by counting the number of positive cells in nine randomized high-power fields for each specimen at $\times 400$ magnification using a square grid of 0.0625 mm². The double immunostaining for detection of co-localization of CD4/IL17 and CD8/IL17 was quantified considering only mononuclear cells.

2.4. Statistical analysis

The number of positive cells of the three groups of tissue reaction was compared using Graph Pad Prism version 5.00 for Windows (Graph Pad software, San Diego, CA, USA) to perform a Mann-Whitney test with the level for significance set at 95%.

3. Results

The histological analysis of the lesions from both groups of skin and mucosa disclosed epidermis an epithelium with hyperkeratosis, parakeratosis and mild acanthosis. The dermis and corium displayed diffuse inflammatory infiltrate with lymphocytes, plasma cells, macrophages and giant cells (Fig. 1) and well organized and loose granulomas, or both types of granulomas in the same lesion.

Cells expressing IL17 were distributed among the inflammatory infiltrate and within and around the granulomas, near multinucleated giant cells in both skin and mucosa biopsies. It was frequent neutrophils expressing IL17 in the microabscesses. Interesting was the detection of some positive cells in the vessels walls (Fig. 2A–D).

Fig. 1. PCM – Inflammatory infiltrate with lymphocytes, plasma cells and macrophages. (A) Compact granuloma ($\times 400$) AND (B) Poorly-organized granuloma ($\times 200$). Hematoxylin-Eosin.

Fig. 2. PCM - Immunohistochemistry evidencing IL17 + cells in skin (A,B) and oral mucosa (C,D). Cells distributed in the inflammatory infiltrate and in the granulomas, near multinucleated giant cells. Note the presence of positive cells in the wall vessels (arrow). In E and F, immunohistochemical expression of Foxp3 presenting a nuclear pattern in lymphocytes in skin and mucosa. Streptavidin-biotin peroxidase, $\times 400$.

The quantitative analysis evidenced a similar number of IL17-positive cells ($p > 0.05$) in the three groups of both cutaneous and mucosal lesions (Fig. 3A,B).

Foxp3+ lymphocytes were distributed among the inflammatory infiltrate in cutaneous and mucosal lesions (Fig. 2E,F).

The population of Treg cells did not differ in the three groups of inflammatory reaction (G1, G2 and G3) of cutaneous lesions ($p > 0.05$). However, the comparative analysis among the mucosal lesions presented an increased number of Foxp3+ cells in G1 when compared to G2 ($p = 0.046$) (Fig. 3C,D).

The double immunostaining disclosed both CD4 + T cells and CD8 + T cells expressing IL17. However, the comparative analysis evidenced a higher number of CD4 + IL17 + cells over CD8 + IL17 + cells ($p = 0.0006$) (Fig. 4).

4. Discussion

The cutaneous and mucosal lesions in PCM are consequence of hematogenic dissemination of the fungi. The clinical and histological morphology of such lesions depend on the host–fungus interaction

that can give rise to different local immune response. The immunopathogenesis of fungal infections is explained primarily through the balance Th1/Th2 immune reaction. However, recent studies point to the presence of Treg cells and Th17 cells playing a role in the immune response against fungal infections [20–22].

Th17 cells are involved in the recruitment of neutrophils and Th1 lymphocytes to the tissues. Recently these cells have been related to the defense against *Mycobacterium tuberculosis*, another granulomatous disease [23,24]. Th17 cells are activated by many fungal components and are associated with fungal persistence and disease at mucosal sites [25,26].

In our study, the quantitative analysis of mononuclear cells expressing IL17 displayed no significant difference according to the characteristics of the tissue reaction both in skin and mucosal PCM lesions. The double staining technique disclosed a higher number of CD4 + T cells expressing IL17 over CD8 + T cells. We could also detect the expression of IL17 in polymorphonuclear neutrophils (PMN) in the microabscesses. Similar results were previously described by Loures et al in a pulmonary model of PCM (2009). Those authors described a prevalent activation of Th17 cells and

Fig. 3. PCM — Quantitative analysis of cells expressing IL17 (A,B) and Foxp3 (C,D) in skin and oral mucosa (c = G1; - = G2; * = G3).

pulmonary inflammation associated to high number of PMN and diminished presence of Treg cells in the lesions [27].

IL17 induces macrophages to release IL1- β and TNF- α and consequently, the up-regulation of the expression of endothelial adhesion molecules [28,29]. The increased expression of IL17 was related to vascular inflammation in systemic lupus erythematosus [30]. In experimental PCM model the expression of cellular adhesion molecules correlates to the intensity of the inflammatory response [31].

Mononuclear cells expressing IL17 were frequently observed in the vessels walls of skin and mucosa PCM lesions. Considering the above mentioned report [31] we could speculate if these IL17-expressing cells localized in the vessels would contribute, at least in part, to the tissue injury, inducing a high expression of endothelial adhesion molecules in PCM lesions.

Fig. 4. PCM — Double immunostaining evidencing the co-expression of CD4 + T cells/IL17 (arrows). Peroxidase-labeled streptavidin-biotin detection of IL17 is indicated by brown staining, and alkaline phosphatase-labeled polymer detection of CD4 + T cells is evidenced in blue ($\times 400$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Regulatory T cells present activity at the time of pathogen invasion or can be induced as a consequence of the infection. The natural occurring CD4 + Foxp3+ T cells may recognize either self-antigens or those from pathogens [32,33]. Treg cells may interfere with the efficacy of host immunity or represent a beneficial to the host, once they are able to diminish the damage that often follows an immune response [34]. In viral infection the blunting of immune response is related to the presence of Treg cells [35,36].

We demonstrated an expressive number of Foxp3+ cells in both cutaneous and mucosal PCM lesions. Increased Foxp3+ cells in PCM lesions have been described before [19]. The number of Treg cells did not differ according to the pattern of granulomatous response in skin. Otherwise, in oral mucosa, the number of Foxp3+ cells was higher in lesions with compact granulomas. This group of tissue reaction in PCM was previously described as associated to a Th1 pattern of cytokines [7]. Treg cells could represent a local contributor to diminish the tissue damage contributing to the maintenance of the granulomatous reaction, as demonstrated to other granulomatous infectious conditions. Treg cells present in PCM chronic lesions must be involved in the modulation of local immune response [19]. The decreased expansion of Treg cells is related to the maintenance of pathogenic inflammatory reactions and deleterious effects in experimental PCM lung lesions [27,37].

It was unexpected the similar number of Treg cells in all skin lesions groups, beside the differences in the characteristics of the tissue reaction related to the immune response. It has been speculated the plasticity of Treg cells and their role in the control of infections. They can express specific transcription factors of Th1 or Th2 cells according to the microenvironment of effector T cells [38–40]. The expressive presence of cells Foxp3+ in cutaneous and mucosal lesions is in accordance with results previously described in blood of patients with PCM.

The beneficial or detrimental role of Treg cells seem to depend on some factors. In chronic infectious diseases Treg cells are involved in the control of infection and keeping of immunity to reinfection due to a balance between Treg and T effectors cells [41,42].

Another interesting role of Treg cells is their capacity to up regulate granzyme B expression. Treg cells play a role as cytotoxic

cells in a similar way as CD8⁺ cells [43]. In PCM skin lesions there is a high number of cells expressing granzyme B and TCD8⁺ cells [44]. In order to improve the knowledge about a possible expression of granzyme by Foxp3⁺ cells in PCM lesions, our group intend to develop a double immunostaining to demonstrate it.

Overall, our data demonstrated the expressive presence of Treg cells in mucosal and cutaneous PCM lesions. In those sites, Treg cells would represent local immunoregulatory mechanisms in the pathogenesis of PCM.

To the best of our knowledge it is the first time that Th17 cells were shown in human PCM skin and mucosal lesions.

Considering that Treg and Th17 cells have reciprocal developmental pathways and opposite roles in the generation and control of inflammation [18], we believe that further studies are necessary to better understand the role of these groups of cells in such an important disease as PCM, characterized by a polarized immune response and different pathological expression.

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Regulatory T cells in cutaneous lesions of patients with Paracoccidioidomycosis

Aline Alves de Lima Silva ^{a,b}, Mirian N. Sotto ^{a,b}, Maria Irma Seixas Duarte ^a, Carla Pagliari ^{a,b,*}

^a Laboratório da Disciplina de Patologia de Moléstias Transmissíveis, Departamento de Patologia, Faculdade de Medicina da Universidade de São Paulo, Brazil

^b Laboratório de Dermatopatologia, Departamento de Dermatologia, Faculdade de Medicina da Universidade de São Paulo, Brazil

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ABSTRACT

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by the dimorphic fungus *Paracoccidioides brasiliensis*, with high incidence in Brazil and very significant in Latin America. The disease is clinically classified as acute, subacute or chronic where the primary lesion initiates in the lungs and can spread to other organs such as the skin and mucous membranes. The lesions are characterized by granulomatous formation, organized according to the type of pattern of host immune response. We demonstrated and quantified by immunohistochemistry the expression of Foxp3, CD25, TGF- β and IL-10 in thirty cutaneous lesions with different presentation of granulomatous response. Cells expressing Foxp3 and CD25 were increased in lesions with compact granulomas. The expression of TGF- β and IL-10 was similar in all PCM lesions. As previous studies, our data suggest the correlation of Treg cells with the chronicity of the disease and the participation in suppressing mechanism as a possible source of IL-10. TGF- β and IL-10, two important suppressor cytokines, are expressed in great amounts in the lesions but our results do not allow correlating with the differences in the granulomatous response.

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1. Introduction

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by the dimorphic fungus *Paracoccidioides brasiliensis* [1]. The route of infection is the inhalation of fungal conidia and the lungs constitute the primary focus, from which the infection disseminates to other organs [2].

PCM presents high endemicity in Latin America, especially in Brazil. The disease is set as the eighth leading cause of death from infectious disease among the predominantly chronic infectious and parasitic diseases. The chronic form affects mostly males, resident or linked to activities in rural areas [3].

Although it is known that the inoculum volume and virulence of the infecting strain play a role in the infection process, the determining factor to disease progression is the cellular immune response of the host, through the expression of cytokines in the microenvironment [4].

The *in situ* immune response in patients with cutaneous lesions of PCM is characterized by a predominance of IFN- γ associated to compact granulomas (Th1) and IL5 and IL10 associated to poorly-organized granulomas (Th2) [5]. Also, high serum levels of IL12 and TNF- α play a prominent role [4]. Some studies, however, demonstrate that it is possible a transition from one pattern to another, depending on the immune status of the patient [5–7].

Regulatory T (Treg) cells are characterized by the constitutive expression of some molecules, such as CD25, CTLA-4, and specifically the transcription factor Foxp3. This group of cells suppresses the immune response by the production of cytokines, among other mechanisms.

Studies suggest that although regulatory T cells play a role of promoting the control of the immune response while avoiding overreaction, its presence in infection by *P. brasiliensis* promotes the permanence of the fungus thus maintaining latent foci and the immunosuppression. The treatment with anti-CD25 in mice promotes a significant decrease in the amount of fungi in pulmonary lesions of PCM, which is consistent with observations that Tregs migrate to the site of infection, benefit the pathogen and induce chronic disease [8,9].

Patients with PCM present a negative delayed-type hypersensitivity to fungal antigens, high rate of apoptosis of lymphocytes

* Corresponding author. Faculdade de Medicina – USP, Av Dr Arnaldo, 455 sala 1118, Corqueira Cesar, São Paulo, SP, CEP 01246-903, Brazil. Tel./fax: +55 11 3061 7238.

E-mail address: cpagliari@usp.br (C. Pagliari).

Fig. 1. Paracoccidioidomycosis skin lesion. A, B, C: immunohistochemistry detection of T reg cells with nuclear expression of Foxp3. D, E, F: Cells in the inflammatory infiltrate expressing CD25. G, H, I: TGF- β immunohistochemistry pattern in cells of the inflammatory infiltrate. J, K, L: Cells expressing IL-10 in the inflammatory infiltrate. Group 1, group 2 and group 3 are represented from left to right, respectively, in each column of the different groups of immunostained cells. Streptavidin–Biotin peroxidase ($\times 400$).

and high suppressive cytokine production (IL-10 and TGF- β). Considering the population of Treg cells, it is observed that patients with active PCM present high levels of regulatory markers in peripheral blood, suggesting that regulatory T cells can adopt an immunosuppressive profile [10].

We previously correlated the pattern of cytokines with granulomatous response in skin lesions. It was observed in patients with the adult form a better tissue response associated to a Th1 pattern of cytokines and a predominance of Th2 cytokines in lesions with poorly-organized granulomas. The aim of this study was to focus on

the regulatory response and a possible correlation with the different histological presentation of cutaneous lesions in PCM.

2. Material and methods

2.1. Casuistic

Thirty skin biopsies taken from patients (87% males, mean age 37 years old) with clinical and histopathological diagnosis of PCM were selected from the files of the Dermatopathology Laboratory of

Fig. 2. Paracoccidioidomycosis skin lesion. Quantitative analysis of cells expressing Fxp3, CD25, TGF-beta and IL10. G1 displayed an increased number of cells expressing both Fxp3 and CD25 when compared to G3 ($p < 0.05$). The expression of TGF-beta and IL10 was similar among the three groups. Correlation of Fxp3 and CD25 — Pearson's linear correlation test ($p = 0.02$).

the Dermatology Department of University of São Paulo Medical School. The biopsies were classified in three groups: group 1 (G1) was characterized by an inflammatory reaction composed of well organized and compact epithelioid granulomas with giant cells and lymphocytes and few plasma cells. Group 2 (G2) exhibited loose arranged epithelioid cells associated with macrophages, lymphocytes, plasma cells and few polymorphonuclear neutrophils. Group 3 (G3) displayed both kinds of tissue reaction in the same specimen. Ten specimens of skin with no inflammatory activity constituted the control group.

2.2. Immunohistochemistry

It was performed a Streptavidin-biotin peroxidase immunohistochemical method with the LSAB[®]+ kit (LSAB[®]+ KIT, K0690, Dako Corporation, Carpinteria, California, EUA). Briefly, following deparaffinization in xylene and hydration in ethanol, antigen recovery was performed in a retrieval solution pH9.0 (S2368, Dako Corporation) for 20 min at 95 °C. The primary antibodies anti-Fxp3 (14-4776, E-Bioscience), anti-CD25 (NCL-CD25, Novocastra), anti-IL-10 (MAB217, R&D Systems) and anti-TGF-β (NCL-TGF, Novocastra) were applied and after an over-night incubation at 4 °C it was applied the second antibodies and the LSAB complex. 3, 3'-diaminobenzidine tetrahydrochloride (DAB-Sigma) was used as chromogen and the slides were counterstained with hematoxylin. All reactions were performed with positive and negative controls. The last ones were constituted by the use of isotype controls and the omission of the primary antibody.

2.3. Quantitative analysis

Immunostained cells were quantified considering the number of positive cells in nine randomized high-power fields for each specimen at a $\times 400$ magnification using a square grid of 0.0625 mm².

2.4. Statistical analysis

The number of positive cells was compared among the groups using Graph Pad Prism version 5.00 for Windows (Graph Pad software, San Diego, CA, USA) to perform a Mann–Whitney test with the level for significance set at 95%.

3. Results

The histological classification of granulomatous response in the lesions was based on the definitions proposed by De Brito and Franco [11].

The histological analysis of the lesions disclosed epidermis with hyperkeratosis, parakeratosis and mild acanthosis. The dermis displayed diffuse inflammatory infiltrate with lymphocytes, plasma cells, macrophages and giant cells and well organized and loose granulomas, or both types of granulomas in the same lesion.

Fxp3+ cells were distributed in the inflammatory infiltrate and associated to granuloma. Cells expressing CD25 were visualized in the dermal infiltrate and granulomas. The expression of TGF-β was present in mononuclear cells among the inflammatory infiltrate

Fig. 3. Paracoccidioidomycosis skin lesion. Double immunostaining demonstrating co-expression of Foxp3/CD25 in group 1. Peroxidase-labeled streptavidin–biotin detection of Foxp3 was limited to nucleus and is evidenced in brown staining (black arrow); alkaline phosphatase-labeled detection of CD25 is evidenced in red (white arrow) ($\times 1000$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and within and around the granulomas, the same to IL-10 (Fig. 1). The studied cells were sparse or even absent in the control group.

The quantitative analysis of Foxp3+ lymphocytes evidenced an increased number of this group of cells in G1 when compared to G3 ($p < 0.05$). Also, the expression of CD25 was increased in G1 when compared to G3 ($p < 0.05$) (Fig. 2).

The quantitative analysis of cells expressing TGF- β and IL-10 did not differ among the groups of PCM lesion (Fig. 2).

It was evidenced a positive correlation between the population of cells expressing CD25 and Foxp3 (Pearson's linear correlation test, $p = 0.02$).

In order to verify if CD25+ cells were, at least in part, the same population of Foxp3+ cells, we performed a double immunostaining and could verify the co-localization of both markers in some cells (Fig. 3).

4. Discussion

Cutaneous lesions in PCM are considered a consequence of hematogenic dissemination of the fungi from the primary pulmonary involvement. Patients with chronic PCM present high serum levels of Th1 cytokines (IL-12, IFN- γ and TNF- α) and the acute form is characterized by high serum levels of IL-4, IL-5, IL-10 [4]. The Th2 pattern of cytokines (IL-10, IL-4 and IL-5) observed in cutaneous lesions seems to be inefficient to contain the fungal load and is related to poorly-organized granulomas [5,6].

In addition to the Th1 and Th2 pattern of cytokines that characterizes the immune response against *P. brasiliensis*, recent studies pointed the importance of Treg cells and Th17 cells in the immune response against fungal infections [12]. Treg cells are important components of the immune response and present a key-role in the tolerance to endogenous antigens and regulation of induced immune response against exogenous antigens [13]. Treg cells are CD4+CD25+ and express specifically the foxp3 gene [14,15].

In the present work we focused on this cell population, studying different components that characterize the regulatory immune response. Treg cells can have a suppressor function through production of cytokines with an inhibitory profile, such as IL-10. This cytokine inhibits antigen presenting cells and is antagonist to IFN- γ [16]. Regulatory T cells are increased in number in blood and oral

lesions of patients with PCM and seem to limit the effector immune response and promote the chronicity [9]. Corroborating these results, in previous studies we found that mucosal lesions presented an expressive number of Foxp3+ cells and this group of cells were related to well organized granulomas [12]. In the present work, we also detected an increased number of foxp3+ cells related to compact granulomas in PCM skin lesions, a possible role in keeping the chronicity.

TGF- β is an interesting factor with different immune functions and in the context of regulatory response it seems to play a role. This factor suppresses CD8+T lymphocytes [17], induces the production of Foxp3 by naive T cells [18], transforms CD4+CD25- T cells into CD4+CD25+ T cells [19] and is also involved in tissue repair [20].

We could observe cells expressing TGF- β and IL-10 in all PCM cutaneous lesions. Although the quantitative analysis of mononuclear cells expressing IL-10 displayed no statistically significant difference according to the characteristics of the tissue reaction, we could observe a slight predominance in the group with poorly organized granulomas and higher number of yeast cells.

It is observed that *P. brasiliensis* in lymph node uses TGF- β and IL-10 to convert the host immune response and reactivate the quiescent lesions [21].

Overall, our data support previous studies suggesting that the presence of Treg cells is related to the chronicity of the disease, although these cells may also play the role of promoting the control of immune response [8].

We could suppose that regulatory T cells in Paracoccidioidomycosis participate in suppressing mechanism as a possible source of IL-10, regardless of the profile of immune response.

Our data demonstrated the local immunoregulatory mechanisms in the pathogenesis of PCM, characterized by cells expressing foxp3, CD25, IL-10 and TGF- β . The frequent presence of those cells in the inflammatory infiltrate permits the conclusion that they play an essential role in the immune response against the *P. brasiliensis*. Different granulomatous response seems also to be related to the quantity of cells expressing Foxp3+/CD25+, that we could demonstrate both with single and double immunostaining in group 1 of well organized granuloma and, consequently, a better immune response against the fungus. However, TGF- β and IL-10, two important suppressor cytokines, are expressed in great amounts in the lesions but our results do not allow correlating with the differences in the granulomatous response.

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In Situ Immune Response in Human Chromoblastomycosis – A Possible Role for Regulatory and Th17 T Cells

Aline Alves de Lima Silva^{1,2*}, Paulo Ricardo Criado², Ricardo Spina Nunes², Wellington Luiz Ferreira da Silva^{1,2}, Luciane Kanashiro-Galo¹, Maria Irma Seixas Duarte¹, Mirian N. Sotto^{1,2}, Carla Pagliari^{1,2}

¹ Laboratório da Disciplina de Patologia de Moléstias Transmissíveis/Departamento de Patologia, Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil,

² Laboratório de Dermatopatologia/Departamento de Dermatologia, Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil

Abstract

Background: Chromoblastomycosis is a chronic fungal infection that affects skin and subcutaneous tissue. Lesions can be classified in tumorous, verrucous, cicatricial and plaque type. The cellular immune response in the severe form of the disease seems to correlate with a Th2 pattern of cytokines. The humoral immune response also seems to play a role. We intended to explore the populations of regulatory T cells and the Th17 pattern.

Methodology: Twenty-three biopsies of verrucous form were obtained from patients with clinical, culture and histopathological diagnostic of chromoblastomycosis, without treatment. It was performed an immunohistochemistry method to detect Foxp3, CD25, TGF- β , IL-6, IL-17 and IL-23.

Principal findings: IL-17 was the only cytokine with high expression in CBM when compared to normal skin. The expression of Treg cells, TGF- β , IL-6 and IL-23 were similar to normal skin.

Conclusions/Significance: The constitution of a local immune response with high expression of IL-17 and low expression of other cytokines could be at least in part, an attempt to help the immune system against fungal infection. On the other hand, high levels of local immune response mediated by Th17 profile could overcome the role of Treg cells. The inefficient immunomodulation as a consequence of the unbalance by Treg/Th17 cells seems to corroborate with the less effective immune response against fungi.

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* Email: alinealves@usp.br

Introduction

Chromoblastomycosis (CBM) is a chronic granulomatous fungal infection that affects skin and subcutaneous tissue, especially the lower limbs. It is a cosmopolitan disease, but classically it is found in tropical and subtropical regions [1].

The causative agents of CBM are dimorphic fungi [2,3] which have a brownish color due to the presence of melanin pigments in their cell wall and the spherical morphology that allow easy histological and mycological identification [4–8].

The infection occurs following traumatic inoculation of conidia or mycelial fragments from dematiaceous fungus [9]. The most frequently isolated species are *Fonsecae pedrosoi*, *Phialophora verrucosa*, *Cladophialophora carrionii* and eventually *Rhinocladiella aquaspersa* [10].

Clinically, the lesions can be classified in two instances: one that takes into account the appearance (tumorous, verrucous, cicatricial and plaque type) and the other, considering the gravity (mild,

moderate or severe, according to the number and size of lesions) [11,12,13].

In general, CBM begins with the eruption of papules or nodules that develop slowly into warts. Regularly lesions appear in the lower limbs, knees and hands. There are reports of involvement of the face, chest, buttocks and other areas; dissemination via the lymphatic system is possible but infrequent [12].

The mechanisms of host defense in CBM are not fully elucidated. It is known that the immune response against the CBM is primarily cellular, where the process is ordered by phagocytic macrophages and humoral response also plays a role. Langerhans cells and Factor XIIIa+ dermal dendrocytes appear to be involved to a lesser extent in phagocytosis and antigen presentation against *F. pedrosoi* [6,14–16].

Some studies have investigated the polarity of CBM and demonstrated, both experimentally and *in situ*, that the severe form of the disease, or warty lesions, correlates with a Th2 pattern of immune response by presenting the production of IL-10, high

Author Summary

Chromoblastomycosis (CBM) is a chronic infection that affects skin and subcutaneous tissue, caused by some fungi which have a brownish color due to the presence of melanin pigments. The most frequent lesions are of verrucous type. Here we describe the participation of regulatory T cells and cells with the Th17 pattern of cytokines. High levels of Th17 cells participate in chronic inflammatory conditions, once at least in part they could improve the immune response and act in concomitance to the Th1 and Th2 patterns. Our results indicate a predominance of the Th17 pattern over Treg cells in verrucous lesions. We speculate that the local immune imbalance in CBM lesions characterized by exacerbated Th17 response, probably by suppressing the Treg response, is not effective for total fungal elimination. Even after long-period treatments, most patients has no absolute cure and often there is recurrence of the lesions. We believe that our study could contribute to the understanding of the immunopathogenesis of CBM and in such a way, presents some aspects to new possible therapies.

fungal burden, and also TNF- α . The average form is related to the Th1 profile, with high production of IFN- γ , low levels of IL-10, scarce number of fungi and relates to the better granulomatous immune response, resulting in less severe injuries (plaque type lesion) [17,18].

Several studies have been conducted to better understand the Th1/Th2 paradigm of immune response or, in some diseases, the presence of both patterns of cytokines in the host. It is known that there is a subset of T lymphocytes that can modulate the immune response against pathogens, self-antigens and allergens which is also a constituent of immunological tolerance, called regulatory T cells (Treg). They are characterized by the expression of high levels of CD25 (α chain of the IL-2) whose function depends directly on the transcription factor Foxp3 [18–20].

A cytokine of high value for the studies of the immune response is TGF- β , since it is involved in the healing process in order to minimize tissue damage [21] and suppresses CD8⁺ cells [22], transform CD4⁺CD25[−] cells into CD4⁺CD25⁺ [23], induce naive T cells to differentiate into Foxp3+ [24] and still participates in differentiation of CD4 T cells in Th17 cells [25].

High concentrations of TGF- β added to the absence of pro-inflammatory cytokines direct the immune response to the development of regulatory T cells. Similarly, low concentrations of TGF- β associated with pro-inflammatory cytokines such as IL-1 β , IL-6, IL-21 and IL-23 promote expression of the IL-23 receptor (IL-23R), factor that allows the differentiation of CD4 T cells in Th17 [26,27].

The Th17 lineage is a subpopulation of CD4⁺ T cells characterized by the secretion of IL-17 that seems to reinforce the protection of the host when the immune profiles of Th1 and Th2 cells are not totally effective against intracellular pathogens. Target of scientific spotlight, these cells appear to be protagonists in chronic inflammatory conditions, such as psoriasis [28,29].

The study of both cell lines was performed by Pagliari *et al.* [30] in specimens from patients with paracoccidioidomycosis skin and mucous membranes lesions. The analysis revealed the involvement of both cellular profiles, identifying both the presence of immunoregulatory mechanism as the strengthening of effector T cells expressing IL-17.

Taking into account that, although the Th17 cells and regulatory T have similar ontogeny but distinct roles in the

generation and control of infections [31], exploring their relationship could improve the understanding of the immunopathology of chromoblastomycosis and contribute to the most effective therapeutic strategies against the disease.

Materials and Methods

Biopsies

Twenty-three biopsies from skin lesions were kindly provided by Dermatopathology Laboratory, Division of Clinical Dermatology, Hospital das Clínicas, Faculty of Medicine, University of São Paulo, obtained from patients with clinical, culture and histological diagnostic of chromoblastomycosis by *F. pedrosoi* (86.96% males, mean age 61 years old, SD 15.28). The control group was constituted by ten specimens of normal skin, free of infectious or inflammatory activity at the time of surgery.

In addition to the skin control group without inflammatory activity (n = 10), it was also used a group of twenty skin lesions of paracoccidioidomycosis (PCM). This disease is caused by the dimorphic fungus *Paracoccidioides brasiliensis* and the host immune response against this fungus shares some similarities with chromoblastomycosis. Moreover, some markers of immune response proposed in this work have been described and discussed in PCM.

The use of the material that constituted the casuistic was approved by the ethics committee of Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, under the number 0317/11.

Immunohistochemistry

It was performed a streptavidin-biotin peroxidase method. The specimens were deparaffinized and hydrated in ethanol, the antigens were retrieved in TRIS/EDTA buffer pH 9.0 for 20 minutes at 95°C. The primary antibodies anti-Foxp3 (clone 236A/E7), anti-CD25 (clone 4C9), anti-TGF- β (clone TGFB17), IL-6 (clone 10C12), IL-17 (clone IL17A) and IL-23 (clone HLT2736) were diluted in 1% bovine albumin solution and incubated over-night at 4°C. Following, it was applied the second antibody and Streptavidin-peroxidase complex. 3,3-diaminobenzidine tetrahydrochloride was used as chromogen and the slides were counterstained with hematoxylin. All reactions were performed with positive and negative controls. The second ones were constituted by the use of isotype controls and the omission of the primary antibody.

Quantitative analysis

Cells were quantified by counting the number of immunolabeled cells in nine randomized high-power fields for each specimen with an $\times 10$ ocular lens with a square grid area of 0.0625 mm². The number of positive cells was statistically analyzed with the Mann-Whitney test with the level of significance set at 95%.

Results

The group of CBM specimens consisted of lesions with a verrucous aspect. The histopathological analysis evidenced epidermal changes as hyperkeratosis, irregular acanthosis and microabscesses. The dermis was constituted by suppurative granulomas, inflammatory infiltrate consisting of giant cells, epithelioid cells, macrophages, lymphocytes, plasma cells, and eosinophils. The semi-quantitative analysis of parasitism ranged from moderate to intense. Eventually it was possible to identify ulceration of the skin, pseudocarcinomatous hyperplasia and fibrosis (Fig. 1).

Figure 1. Chromoblastomycosis – Histopathology of skin lesions. A: epidermis presenting pseudocarcinomatous hyperplasia and acanthosis (×40); B: presence of acanthosis and intraepidermal microabscess (×100); C: dermal inflammatory infiltrate characterized by granulomatous response with giant cells, high number of fungal cells, eosinophils, macrophages and lymphocytes (×200) (hematoxylin-eosin).
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The immunohistochemical method allowed observing cells expressing Foxp3 and CD25, both in the inflammatory infiltrate and around granulomas. The expression of TGF- β was present in mononuclear cells of the inflammatory infiltrate. There was a discrete expression of IL-6. The expression of IL-17 was visualized in mononuclear and polymorphonuclear cells, mainly in granulomatous areas. Cells expressing IL-23 were present in the inflammatory infiltrates (Fig. 2 and 3).

The group of PCM was characterized by the presence of all markers, expressed in mononuclear cells in the inflammatory infiltrate similar to the CBM group.

The control group of normal skin also presented the markers. However, the expression was low or even absent in some cases.

Cells expressing IL-23 were detected in 66% of the normal skin specimens. Those cells were observed only in the dermis.

The statistical analysis of cells expressing Foxp3 evidenced a decreased number of such cells in CBM group when compared to PCM group ($p < 0.001$) and similar number when compared to normal skin.

The expression of CD25 was similar between CBM and PCM groups, however CBM group presented a statistically significant higher number of positive cells when compared to normal skin ($p < 0.001$).

The number of cells expressing TGF- β was similar among the three groups ($p = 0.05$), the same to IL-6 and IL-23 ($p > 0.05$).

The three groups presented cells expressing IL-17. However, CBM group had an increased number when compared to the two other ($p < 0.001$).

The quantitative analysis can be visualized in figure 3 and table S1.

Discussion

The study of CBM skin lesions is important because these injuries can reflect the immune status of the patient. The cutaneous tissue is often responsible for the initiation of immune cascade and, in the case of chromoblastomycosis, it is the structure to which the fungus has tropism.

Studies concerning immune response in CBM show that verrucous lesions present the mycotic granuloma formation with a pattern of Th2 response consistent with worse response against fungus. On the contrary, lesions of plaque type are characterized by a Th1 pattern of cytokines and therefore a better immune response, according to Minotto (unpublished data) and D'ávila [17]. However, there are no reports studying the immune dynamics of the lesions or inferences about what it takes to develop such responses.

We evidenced the predominance of cells expressing IL-17 and the presence of TGF- β and IL-23, although in low number. According to the literature published, this pattern of cytokines characterizes the Th17 immune response. The concomitant presence of a Th17 pattern could represent at first, an attempt of the *in situ* immune system to restrain the fungal infection.

With respect to the presence of regulatory T cells, although to a lesser extent when compared to cells expressing IL-17, we were also able to verify considerable number of Foxp3+ cells (40% of cases) and CD25+ cells (90%).

According to Melo and Carvalho [32], regulatory T cells have a key role in maintaining tolerance and regulation of the immune response. In the same way, in a work of Weaver [28] it is discussed that Th17 cells appear to be the translation of adaptability of the immune system favoring the protection of the host, so that the elucidation of the characteristics of both groups of cells, i.e. Treg and Th17 cells, could not only identify the mechanisms of

invading organisms, but also to assist in the development of more effective therapies for numerous diseases.

Treg cells are characterized by a CD4+CD25+ phenotype [33], however the CD25 molecule is expressed by others populations of cells, such as others T cells and B lymphocytes or activated macrophages. Studies demonstrated that the gene Foxp3 has a nuclear expression in Treg cells and therefore a reliable marker of this cell line [34].

As already seen, the cytokine TGF- β plays a dichotomy role and mediates the targeting of Treg and Th17 responses. This selection between both profiles seems to depend on the levels of cells and the cytokines present in the microenvironment. High levels of TGF- β and low expression of pro-inflammatory cytokines favor the regulatory T-profile. On the contrary, low levels of TGF- β associated to significant presence of pro-inflammatory cytokines promote the synthesis of IL-23, development and maintenance of Th17 lymphocytes [35–41].

The cytokine IL-17 has received a considerable attention in many contexts since its discovery in 1993 [42]. The cytokine IL-23 promotes the secretion of IL-17 produced mainly by CD4+ T cells [43,44].

According to many studies, high levels of Th17 cells are protagonists in chronic inflammatory conditions, once this cell population, at least in part, could improve the immune response and act in concomitance to the Th1 and Th2 patterns of response [28]. The performance of this cell line is characterized by secretion of IL-6, IL-17, IL-22 and TNF- α [45].

In the same context of investigation we consider patients affected by paracoccidioidomycosis (PCM), a systemic mycosis similar in some aspects to CBM. The disease follows inhalation of conidia of *Paracoccidioides brasiliensis*, a dimorphic fungus [46], and the primary focus are the lungs. It is described the cutaneous involvement at about 30% of cases. Unlike CBM, the lympho hematogenous spread of fungi is more frequent [47].

The cellular immune response in PCM is mainly mediated by macrophages and CD4+T cells [48,49]. The Th1 profile of cytokines is considered the most important and some studies have also demonstrated the role of Treg cells and the profile of Th17 cytokines [30].

Considering previous investigations on the role of Treg cells in PCM, our results could suggest that this cell population not only have the capacity to interfere in the efficient immune response against fungi in chromoblastomycosis, but also benefit the host, by being able to reduce the tissue damage that follows a local immune response [30,50].

In a recent study, the authors discussed the interaction of cells producing IL-17 and Treg cells and the homeostasis of the intestinal mucosal tissue [51]. The unregulated interaction of pro-inflammatory activity of IL-17 with pathogens seems to change the balance between regulatory and effector response predisposing the individual to the chronicity of the disease. It is noteworthy that even being subjected to long-period treatments, most patients affected by CBM has no absolute cure and often there is recurrence of the lesions. Thus, the unbalance between the populations of Treg/Th17 cells seems to restrain the effective immune response against the fungus.

Finally, it was interesting the similar number of cells expressing TGF- β , IL-6 and IL-23 when we compared the groups of lesions and normal skin. We expected that both CBM and PCM specimens presented more cells expressing those markers. We speculate that, at least in part, the presence of such cytokines in normal skin could be produced by the components of skin immune system [52].

Interestingly, in a previous work, Esterre *et al.* (1994) observed an overexpression of TGF-beta mainly at the periphery of the granulomas in areas of fibrosis [53].

Figure 2. Immunohistochemistry of representative chromoblastomycosis skin lesion. A: Nuclear expression of Foxp3 in regulatory T cells. B: expression of CD25 in lymphocytes of the inflammatory infiltrate. C: Low number of cells expressing TGF- β . D: Scarce presence of IL-6. E: Intense number of T cells with expression of IL-17 in the inflammatory infiltrate. F: Low number of cells expressing IL-23 distributed in the lesion. Streptavidin-biotin peroxidase, immunostaining in brown ($\times 200$ and inset $\times 400$).
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Statistical analysis

Figure 3. Comparative analysis among chromoblastomycosis, paracoccidioidomycosis and normal skin groups. Distribution of number of cells/mm² expressing Foxp3, CD25, TGF-β, IL-6, IL-23 and IL-17. Mann-Whitney test, * p<0.05. doi:10.1371/journal.pntd.0003162.g003

In this work, we could suggest that the low number of cells with TGF-beta in lesions of CBM, with no difference from normal skin, could be explained by the randomized counting of positive cells throughout the dermis and not specifically in areas of fibrosis where such cells were also observed.

We suggest that our study could contribute to the understanding of the immunopathogenesis of chromoblastomycosis and in such a way, presents some aspects that could assist in the possibility of new therapies to modulate the immune system to the most effective immune profile of patients.

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Supporting Information

Table S1 Quantitative analysis: Numerical results of the markers studied. Results are given as mean ± standard deviation. (TIF)

Author Contributions

Conceived and designed the experiments: CP MNS. Performed the experiments: AAdLS LKG. Analyzed the data: AAdLS CP MISD. Contributed reagents/materials/analysis tools: WLFdS PRC RSN. Wrote the paper: AAdLS CP.

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Original Article

Th17 and regulatory T cells contribute to the *in situ* immune response in skin lesions of Jorge Lobo's disease

Luciane Kanashiro-Galo¹, Carla Pagliari^{1,2,*}, Tania Cristina Barboza^{1,2}, Arivaldo Cardoso de Brito³, Marilia Brasil Xavier^{3,4}, Clivia Maria Moraes de Oliveira³, Deborah Aben Athar Unger³, Mirian Nacagami Sotto¹, Juares Antonio Simões Quaresma^{3,4} and Maria Irma Seixas Duarte¹

¹Universidade de São Paulo, Faculdade de Medicina, Laboratório da Disciplina de Patologia de Moléstias Transmissíveis, São Paulo, SP, Brasil, ²Instituto de Assistência Médica ao Servidor Público Estadual, Programa de pós-graduação em Ciências da Saúde, São Paulo, SP, Brasil, ³Núcleo de Medicina Tropical, Universidade Federal do Pará, Belém, PA, Brasil and ⁴Universidade do Estado do Pará, PA, Brasil

*To whom correspondence should be addressed. Carla Pagliari, BSc, PhD, Universidade de São Paulo, Faculdade de Medicina, Departamento de Patologia, Av. Dr. Arnaldo, 455, sala 1118, CEP 01246-903 - Cerqueira Cesar, São Paulo, SP, Brasil. Tel/Fax: +(55 11) 3061-7238; E-mail: cpagliari@usp.br

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Abstract

Jorge Lobo's disease (JLD) is a chronic granulomatous mycosis described in various Latin American countries. The main objective of the present study was to investigate the possible role of Th17 and Foxp3+ Treg cells in the pathogenesis of Jorge Lobo's disease. Human skin biopsies were submitted to an immunohistochemistry protocol to detect Foxp3, interleukin (IL)-1beta, CD25, IL-6, IL-17, and IL-23. The epidermis presented acanthosis, hyperkeratosis, and frequent presence of fungi. The dermis presented inflammatory infiltrate comprising macrophages, lymphocytes, epithelioid and multinucleated cells, and an intense number of fungi. Foxp3+ Treg cells and IL-17+ cells were visualized in lymphocytes in the inflammatory infiltrate. IL-1, IL-2R (CD25), IL-6, and IL-23 were visualized in the dermis, intermingled with fungal cells, permeating or participating of the granuloma. Following IL-17, the most prominent cytokine was IL-6. IL-23 and cells expressing CD25 were present in fewer number. The comparative analysis between IL-17 and Foxp3 demonstrated a statistically significant increased number of IL-17+ cells. Th17 cells play a role in the immune response of JLD. IL-1beta and IL-6 added to the previously described increased number of TGF-beta would stimulate such pattern of response. Th17 cells could be present as an effort to modulate the local immune response; however, high levels of a Th17 profile could overcome the role of Treg cells. The unbalance between Treg/Th17 cells seems to corroborate with the less effective immune response against the fungus.

Key words: Jorge Lobo's Disease, immune response, Th17 cells, regulatory T cells.

Introduction

Jorge Lobo's disease (JLD) is a chronic granulomatous mycosis caused by the pathogenic fungus *Lacazia loboi*. It is usually characterized by lesions that predominate on the ears and upper and lower limbs. The disease is described in various Latin American countries, with great importance in Brazil's Amazon region and predominates in males of rural activity [1–5].

Lacazia loboi is unicellular, yeast type and is found in lesions either alone or in chains of cells connected resembling a “pearl necklace” [6].

Clinically there are differences related to the time of evolution, as well as extension of the lesions [7]. The classic clinical aspect is the keloid-like type, with isolated or confluent lesions that can form multilobular aggregates, probably related to the high expression of transforming growth factor (TGF)-beta in the lesions [8–10].

TGF-beta is an important cytokine involved in healing processes, and it also plays a role in the suppression of CD8+ T cells and differentiation of naïve T cells in Foxp3+ regulatory or Th17+ cells, depending on the stimulus of pro-inflammatory cytokines [11–15].

Studies focusing on the cell-mediated immune response in JLD demonstrate differences according to the type of lesion and clinical presentation [5,16,17]. The dermal immune response is mainly characterized by Langerhans cells, Factor XIIIa+ dermal dendrocytes, macrophages, T lymphocytes and the cytokines IL-1-beta, tumor necrosis factor (TNF)-alpha, TGF-beta, IL-10, and IFN-gamma.

The Th17 and regulatory T cells have been studied in other cutaneous mycosis that share some characteristics with JLD, for example, chromoblastomycosis and paracoccidioidomycosis and their role in both immunopathogenesis have been speculated [18,19].

We intended to contribute to the characterization of the *in situ* immune response in cutaneous lesions in Jorge Lobo's Disease, focusing on Th17 and regulatory T cells and exploring their role in the pathogenesis of this disease.

Materials and Methods

Biopsies: Forty-one skin biopsies of lower limbs from 41 patients were selected from the files of the Nucleo de Medicina Tropical, Universidade Federal do Para, Belem, PA, Brazil. The diagnosis was based on the clinical presentation, direct mycological examination, and histological analysis according to the report of each sample. Samples from patients

coinfected with human immunodeficiency virus (HIV) and patients receiving any type of treatment at the time of the biopsy were excluded of the casuistic. Ten biopsies from normal skin were used as control. The mean age of patients was 52 years old, 92% male.

Immunohistochemistry: Following deparaffinization in xylene and hydration in ethanol, antigen recovery was performed in a TRIS/EDTA solution pH9.0 for 20 minutes at 95°C. The primary antibody anti-Foxp3 (EBioscience, San Diego, CA, USA) was applied at a 1:50 dilution; anti-IL-1beta (RD Systems, Minneapolis, MN, USA), anti-CD25 (Novocastra, Newcastle, UK) anti-IL-6 (Novocastra, Newcastle, UK) at a 1:20 dilution; anti-IL-17 (RD Systems, Minneapolis, MN, USA) was applied at a 1:10 dilution and anti-IL-23 (BioLegend, London, UK) at a 1:50 dilution. Following an over-night incubation at 4°C, it was applied the second antibody and a Streptavidin-Biotin peroxidase system (LSAB, Dako, Carpinteria, CA, USA). 3, 3'-diaminobenzidine tetrahydrochloride was used as chromogen and the slides were counterstained with hematoxylin. All reactions were performed with positive and negative controls. The second ones were constituted by the use of isotype controls and the omission of the primary antibody.

In order to verify if cells expressing Foxp3 were CD4+ T cells, we performed a double immunostaining using the above protocol to detect Foxp3 followed by an immunohistochemistry with phosphatase and a blue chromogen to detect CD4+ T cells.

Immunolabeled cells were quantified in nine randomized fields in the dermis using a $\times 10$ ocular lens with a square grid and a $\times 40$ objective. The sum of the number of immunostained cells was divided by the number of fields (nine) and the result was divided by 0.0625 (area of the grid), providing the number of cells/mm².

Statistical analysis

The number of positive cells was statistically analyzed by using the Mann-Whitney test with the level for significance set at 95% ($P \leq .05$).

Results

The lesions were characterized by an inflammatory infiltrate, nodular and diffuse, comprising macrophages, rare asteroid corpuscles, lymphocytes, epithelioid cells, high number of multinucleated cells of Langhans and foreign

Figure 1. Jorge Lobo's disease. Histopathology aspect of lesion, characterized by inflammatory infiltrate with macrophages, lymphocytes, some eosinophils, multinucleated giant cells and high number of fungal cell and fibrosis. Note neutrophils and fungal cells infiltrating the epidermis. (Hematoxylin-Eosin, magnification $\times 200$)

body types, with an intense number of fungal cells, in the epidermis and in granuloma or macrophages in the dermis (Figure 1).

The nuclear expression of Foxp3 was visualized in lymphocytes in all the specimens of JLD, distributed in the inflammatory infiltrate. Also, cells expressing IL-17 were detected in all specimens of lesions, in lymphocytes and neutrophils.

The control group did not present Foxp3+ cells, but a small number of cells expressing IL-17 were observed.

The double immunostaining enabled the visualization and confirmation that the predominating cells that were Foxp3+ also expressed CD4.

Cells expressing the cytokines IL-1, IL-2R (CD25), IL-6, and IL-23 were visualized in the dermis, intermingled with fungal cells, permeating or participating of the granulomatous formation. Figure 2A-G presents the immunohistochemistry expression of cells and cytokines studied.

The quantitative analysis of immunolabeled cells evidenced that, following IL-17, the most prominent cytokine was IL-6. The cytokine IL-23 and cells expressing CD25 were present in less number. Figure 2H presents the mean values for each molecule evaluated, considering nine randomized fields.

The comparative analysis between cells expressing IL-17 and Foxp3 in the group of lesions demonstrated a statistically significant increased number of IL-17+ cells when compared to Foxp3+ cells ($P = .034$). The mean values and standard deviation of each element studied both in JLD lesions and control group of normal skin are described in Table 1.

Table 1. Mean values and standard deviation of the cytokines and cells studied both in Jorge Lobo's Disease and control group of normal skin.

	Mean $\pm$ SD – JLD	Mean $\pm$ SD – Normal skin
Foxp3	61.36 $\pm$ 42.67	0.00
IL-1 beta	15.93 $\pm$ 49.73	0.87 $\pm$ 1.35
CD25	13.44 $\pm$ 17.67	0.88 $\pm$ 0.96
IL-6	57.73 $\pm$ 50.91	7.46 $\pm$ 15.72
IL-17	114.5 $\pm$ 93.40	36.44 $\pm$ 36.50
IL-23	13.63 $\pm$ 9.95	37.92 $\pm$ 53.07

Discussion

The mean age that characterized the present casuistic was 52 years old with a predominance of males (92%). The biopsies were obtained from patients of the Amazon region in Brazil, in which rubber gatherers, gold prospectors, and rural workers are frequent activities. This is in accordance with previous works that related such activities to the predominance of the disease [20]. Similarly, in a previous study of 249 patients, the mean age was 53 years old. However, in “Caiabi” Brazilian Indians, the disease is very frequent among women and predominates in young people up to 20 years old, suggesting an earlier infection when compared to other population [21].

The histopathologic findings were constituted by an intense histiocytic infiltrate of the dermis, with a prominent number of parasites, similar to other fungal infections such as chromoblastomycosis and paracoccidioidomycosis. The epidermis presented areas of acanthosis, hyperkeratosis, spongiosis, and frequent presence of neutrophil and fungus, revealing their capacity of transepidermal elimination.

In the last few years, many studies about the characterization of the *in situ* immune response in cutaneous lesions of patients with Jorge Lobo's disease have been performed. The classification of lesions is frequently done according to the distribution: local or isolated and disseminated forms [20].

It is speculated that the genesis of symptoms of JLD is related to the host defective cellular immune response [17,18]. The lesions begin as papules and can result in plaques or nodules. Also, they can present a verrucous, gummatous, ulcerated, or infiltrative aspect [22].

It was demonstrated that the cell-mediated immune response and the pattern of cytokines is different according to the clinical presentation of lesions and can vary from a Th1 profile, intermediate and Th2 pattern [17].

Some elements of the local immune response were suggested as modulating this infection, such as the Langerhans cells, which could help the fungus to escape the immune system [23]. The Th2 pattern of cytokines, mainly IL-4 and

Figure 2. Jorge Lobo's disease. Immunohistochemistry characterization of inflammatory infiltrate. **A)** Foxp3+ regulatory T cells intermingled with fungal cells. **B)** Double stain evidencing the colocalization (arrows) of CD4+ T cells (in blue) and Foxp3 nuclear expression (in brown). **C)** High number of cells expressing IL-1beta and granulomatous area with intense expression of this cytokine. **D)** Expression of IL-2R (CD25). **E)** Expression of IL-6. **F)** Cells expressing IL-17 in superficial and reticular dermis. **G)** Expression of IL-23 in mononuclear and giant cells. (Streptavidin-Biotin peroxidase, $\times 400$). **H)** Quantification of cells expressing cytokines and Foxp3 in the dermis considering nine randomized fields. Values expressed as number of cells/mm².

IL-6, and also the presence of melanin in the fungus wall could also collaborate to a noneffective response, keeping the infection. It is noteworthy that fungal viability indices range from 20% to 50% [4].

In this study, we intended to explore the participation of both Treg and Th17 cells in JLD and could observe the high number of cells expressing IL-6, a proinflammatory cytokine, expressive number of IL-17 over Foxp3+ cells and the presence of IL-23. Previously, it was described that high numbers of cells expressed TGF-beta in skin lesions of JLD [8]. In this context of cytokines, IL-10 is also present and acts with TGF-beta by inhibiting the effective cellular immune response [10].

The Th17 cells are characterized by the production of IL-17 and are involved in processes in which the immune profiles of Th1 and Th2 cells are not effective in the host defense against intracellular pathogens. They have been considered in many pathologic processes such as fungal infections [18,19,24,25]. This group of cells plays an inflammatory role and seems to be associated with the chronic inflammatory response and fungal persistence in candidiasis and aspergillosis, with defective fungal clearance.

The induction of a Th17 response is mediated by IL-23, which promotes the expression of IL-17 in T cells [26]. IL-23, on the other hand, shares the p40 subunit to IL-12, a Th1-related cytokine [27]. Also, TGF-beta, IL-1beta and IL-6 constitute an important group of cytokines that induces the activation of Th17 cells [28,29]. The high concentration of TGF-beta is also crucial for the development of regulatory T cells, in the presence of low expression of proinflammatory cytokines.

Our results allow us to conclude that Th17 cells participate and seem to play a role in the immune response of Jorge Lobo disease in human skin lesions. The presence of the proinflammatory cytokines IL-1beta and IL-6 added to the previously described increased number of cells expressing TGF-beta would stimulate such pattern of response. Some works have also demonstrated that TGF-beta can play a role in the genesis of the intense collagen tissue observed in injuries [8].

The Th17 pattern of cytokines could be presented as an effort to modulate the local immune response; however, it is also important to consider that the effects of Th17 immunity can also contribute to pathology with detrimental effects [30], which is still a controversial field, as we recently verified in Chromoblastomycosis, another important human fungal infection that shares some characteristics with JLD [21].

Similar to that, in JLD we could also observe the presence of Foxp3+ T cells, but it seems to have an imbalance with respect to the Th17 response. The Foxp3+ T regulatory cells are important inducer of an appropriate environment

for the development and chronicity of the lesion, since such cells control negatively the immune response.

The characterization of a local immune response with high expression of IL-17 could be at least in part, an attempt to help the immune system against this fungal infection. However, high levels of a Th17 profile could overcome the role of Treg cells. The unbalance between Treg/Th17 cells seems to corroborate with the least effective immune response against the pathogenic fungus *Lacazia loboi*.

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Declaration of interest

None of the authors have any potential conflict of interest or financial support in the subject matter.

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Short communication

Revisiting Langerhans cells in paracoccidioidomycosis: expression of CD207/langerin in human cutaneous and mucosal lesions

Carla Pagliari ^{a,*}, Elaine Raniero Fernandes ^a, Wellington Luiz Ferreira da Silva ^a,
Aline Alves de Lima Silva ^a, Felipe Weissaupt Stegun ^a, Maria Irma Seixas Duarte ^a,
Mirian Nacagami Sotto ^b

^a Departamento de Patologia, Faculdade de Medicina, Universidade de São Paulo, Brazil

^b Departamento de Dermatologia, Faculdade de Medicina, Universidade de São Paulo., Av. Dr. Arnaldo, 455 sala 1118, cep 01246-903, Cerqueira Cesar, São Paulo SP, Brazil

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Abstract

Langerhans cells are identified by the expression of langerin. We detected this molecule in cutaneous and mucosal lesions in paracoccidioidomycosis, an important infection in Latin America. Langerin+ cells were scarcely distributed, with short dendrites in epidermis and epithelium and were frequent in the dermis and corium, in the inflammatory infiltrate and granulomas. Mucosal lesions presented a higher expression of langerin in lesions with loose granulomas. For the first time we presented the expression of langerin in paracoccidioidomycosis. Positive cells in dermis and corium could represent migrating Langerhans cells or a new subset of langerin+ cells with a role in paracoccidioidomycosis.

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Keywords: Paracoccidioidomycosis; Langerin; Immune response

1. Introduction

Paracoccidioidomycosis (PCM) is a fungal infection caused by *Paracoccidioides brasiliensis* and represents an important health problem in Latin America. Cutaneous and mucosal lesions are very frequent and important due to their value for diagnosis [1].

Langerhans cells (LC) are constitutively present dendritic cells in skin and mucosa, such as nasal and oral ones. They are ultrastructurally identified by the presence of a unique organelle, the Birbeck granule [2] and are considered a very important group of epidermal cells with immunological characteristics.

LC are recognized by the expression of HLA-DR and CD1a and recently, it was identified the expression of langerin/CD207 as a molecular component of Birbeck granules that plays a role in their formation. As a C-type lectin, langerin is characterized as a pathogen recognition receptor. It binds sugar using carbohydrate recognition domains [3].

Recently, a new population of langerin+ dendritic cells was found in the dermis [4,5]. They are rare and are distinct from LCs in transit through the dermis to the lymph nodes. Evidence indicates that they are not related to LC. This subset of dermal Langerin+ cells has not yet been confirmed for human skin, even though preliminary evidence suggests that a human counterpart may exist [6]. LCs play a key role in the induction of immune response against some pathogens. This group of cells expresses toll-like receptors that recognize bacterial and viral antigens. Moreover, it was demonstrated the interaction between HIV-1, HPV and *Mycobacterium leprae* and langerin [7–9]. Another evidence of a possible role of

* Corresponding author. Faculdade de Medicina – USP, Av Dr Arnaldo, 455 sala 1118, Cerqueira Cesar, CEP 01246-903, São Paulo, SP, Brazil. Tel./fax: +55 11 3061 7238.

E-mail address: cpagliari@usp.br (C. Pagliari).

LCs in the pathogenesis of *M. leprae* infection, considering the CD1a expression, was demonstrated by Quaresma et al. [10]. Considering other intracellular pathogens, LCs were best studied in experimental leishmaniasis but some details remain to be clarified [11].

The capacity of recognize mannose, fucose and N-acetylglucosamine monosaccharides (GlcNAc) [12] led to a boost to study the interaction of langerin with fungal species. In an elegant research of de Jong and colleagues, it was observed the recognition of *Candida*, *Malassezia* and *Saccharomyces* species by LCs. The authors suggest that langerin is the major fungal receptor in this group of human cells [13]. We have presented some data about the role of epidermal LC (at that time identified through the expression of CD1a) and Factor XIIIa+ dendritic cells in PCM cutaneous lesions and could demonstrate the interaction between the last ones and the *P. brasiliensis* antigens. LCs were decreased in number in the lesions when compared to normal skin [14,15]. Previously, Gimenez et al. studied the density of LC in the skin of patients with disseminated form of *P. brasiliensis* using adenosine triphosphatase staining of epidermal sheets. They observed a reduction in the number of LCs and morphological alterations [16]. Some morphological changes were described by Sandoval and colleagues although they didn't find fungal exoantigens in the cytoplasm of LC with an anti-S100 antibody and a double-immunostaining method [17].

An antibody against langerin was proposed as the main and unequivocal tool to identify Langerhans cells by immunohistochemistry [3,18].

The development of that antibody to study langerin+ cells in human tissue was an urge to revisit the LCs both in cutaneous and mucosal lesions in PCM and try to identify dermal langerin+ cells in this model of intracellular pathogen.

2. Material and methods

2.1. Biopsies

Sixty-one skin biopsies and fifty-five mucosal oral biopsies from patients with clinical and histopathological diagnosis of PCM were selected from the files of the Dermatopathology Laboratory of the Dermatology Department of Universidade de São Paulo, Faculdade de Medicina. Ten biopsies from normal skin were used as control.

2.2. Histopathology

The biopsies had been fixed in neutral buffered formalin and embedded in paraffin.

It was performed a streptavidin-biotin peroxidase immunohistochemical method with the LSAB[®]+ kit (LSAB[®]+ KIT, K0690, Dako Corporation, Carpinteria, California, EUA). Following deparaffinization in xylene and hydration in ethanol, antigen recovery was performed in a retrieval solution pH9.0 (S2368, Dako Corporation) for 20 min at 95 °C. The primary antibody anti-langerin (clone 12D6-NOVOCASTRA) was applied at a 1:400 dilution and after an over-night

incubation at 4 °C it was applied the second antibody (a polyvalent anti rabbit and anti-mouse Immunoglobulin, from the LSAB kit, Dako, K0690) and the complex LSAB. 3, 3-diaminobenzidine tetrahydrochloride (Sigma) was used as chromogen and the slides were counterstained with hematoxylin. All reactions were performed with positive and negative controls. The second ones were constituted by the use of isotype controls and the omission of the primary antibody.

2.3. Quantitative analysis

Cells were quantified by counting the epidermal/epithelial area fraction using an $\times 10$ ocular lens with a square grid and an $\times 40$ objective. It consisted in counting all the intersection of lines from the grid that coincided with the positive reactions to Langerin, over all the intersection of lines that coincided with the epidermis [19].

2.4. Statistical analysis

The number of positive cells was statistically analyzed by using Graph Pad Prism version 5.00 for Windows (Graph Pad software, San Diego, CA, USA) to perform the Mann–Whitney test with the level for significance set at 95% ($p \leq 0.05$).

3. Results

3.1. Histopathological analysis

The histological analysis of the lesions from both groups of skin and mucosa evidenced epidermis an epithelium characterized by hyperkeratosis, parakeratosis and acanthosis. Intraepidermic microabscesses were present in 60% of the cases. The dermis and corium were characterized by inflammatory infiltrate with lymphocytes, plasma cells and macrophages. The histopathological study of lesions, performed with hematoxylin-eosin (HE) staining, permitted the classification of the biopsies in three groups: group 1 (G1) was characterized by an inflammatory reaction composed of well-organized and compact epithelioid granulomas with giant cells, surrounded by lymphocytes and few plasma cells. Group 2 (G2) exhibited loose arranged epithelioid cells, intermingled with macrophages, lymphocytes, plasma cells and few polymorphonuclear neutrophils. Group 3 (G3) displayed both kinds of tissue reaction in the same specimen. G1 was constituted by 21 skin and 16 mucosal lesions, G2 presented 22 skin and 21 mucosa and G3, 18 skin and 18 mucosa lesions (Fig. 1).

3.2. CD207/langerin expression

Langerhans cells were distributed along the epidermis in the specimens of control group and presented either long or short dendrites. However, in the PCM lesions those cells were scarcely distributed or, in some cases, absent and presented short dendrites. It was not detected any relation in the expression of langerin with the microabscesses present in the epidermis.

Fig. 1. Paracoccidioidomycosis – Inflammatory infiltrate with lymphocytes, plasma cells and macrophages. (A) Well-organized, compact granuloma with giant cells, surrounded by lymphocytes ($\times 100$); (B) Poorly-organized granuloma, loose arranged epithelioid cells and giant cells intermingled with lymphocytes and macrophages ($\times 200$). Hematoxylin-Eosin.

Fig. 2. Immunohistochemical detection of langerin+ cells. Langerin immunoreactivity in normal skin showing distribution of cells along the epidermis, with either long or short dendrites (A,B). In PCM cutaneous lesions the dendrites were short and cells were scarcely distributed (C,D). It was interesting the frequent detection of langerin+ cells in the interface epidermis/dermis (E) and dermis, distributed in the inflammatory infiltrate (F). Streptavidin-biotin peroxidase method – $\times 200$ (C) and $\times 400$ (A,B,D,E,F).

Interesting was the frequent detection of langerin+ cells in the dermis and corium of sections from the PCM lesions. In those sites, the positive cells were distributed in the inflammatory infiltrate and were frequently detected in the granulomas (Figs. 2 and 3).

The comparison among the groups of skin lesions presented similar numbers of positive cells ($G1 \times G2$ $p = 0.5656$; $G1 \times G3$ $p = 0.6111$; $G2 \times G3$ $p = 0.0997$). On the other hand, when compared to the group of normal skin, we observed a statistically significant decreased number of langerin+ cells in the epidermis of the three groups of lesions ($p = 0.0166$, 0.0060 and 0.0466) (Fig. 4A). Inversely, numbers of dermal langerin+ cells were markedly increased in lesion skin as compared to healthy control skin, in which those cells were practically absent ($p = 0.001$).

The statistical analysis among the groups of mucosal lesions revealed an increased expression of langerin in G2 and G3 when compared to G1 ($p = 0.0024$ and 0.0471 , respectively) and a similar expression when G2 and G3 were compared ($p = 0.9326$). The comparison with the group of normal skin revealed a decreased number in the epithelium of the three groups of mucosal lesions ($p = 0.0005$, 0.0018 and 0.0102) (Fig. 4B). However, langerin+ cells were increased in the corium when compared to normal skin ($p = 0.001$).

4. Discussion

Langerhans cells are antigen presenting cells, important in the induction of primary immune response [20]. Langerin is a C-type lectin and potent inducer of Birbeck granules, the hallmark of Langerhans cells.

Once they are located in a strategic site, they are constantly exposed to external antigens and pathogens. It was previously demonstrated the interaction of LCs mediated by langerin with *Candida*, a pathogenic fungus. However, in the same study, it was not observed the interaction with *Cryptococcus*, an important agent causative of infections mainly in immunocompromised patients. It was suggested that this fungus is able to express some evading immune recognition by LCs [13]. In a previous study of Langerhans cells with an anti-CD1a antibody in PCM skin lesions, we demonstrated a decreased number of positive cells in lesions with well-organized granulomas (here defined as G1) and with poorly-organized granulomas (G2) when compared to normal skin. The reduced number and morphological alterations described at that time, were considered as a result of factors produced by *P. brasiliensis* or as a migration to the dermis, with immunophenotypical alterations in the membrane expression of CD1a [15]. According to Gimenez et al the reduction in the number

Fig. 3. Immunohistochemical detection of langerin+ cells in mucosal lesions. Langerhans cells in epithelium presenting short and scarce dendrites (A,B). The presence of langerin+ cells in corium was very frequent (C) and positives cells were frequently detected associated to granulomas. In D, two positives cells next to a parasitized (arrow) giant cell. Streptavidin-biotin peroxidase method — $\times 200$ (A,C) and $\times 400$ (B,D).

Fig. 4. Quantitative analysis of cells expressing langerin in epidermis and epithelium in PCM lesion. The number of cells was similar among PCM cutaneous lesions, but decreased when compared to normal skin (A). In the mucosal group of lesions, an increased expression of langerin in G2 and G3 when compared to G1 and a similar expression when G2 and G3 were compared. The three groups of mucosal lesions presented a decreased number of LCs when compared to normal skin (B).

of LCs may reflect the depressed cellular immunity secondary to the infection with *P. brasiliensis* [16].

When we used the anti-CD1a antibody to study LCs, we did not observe positive cells in the dermis. In the present study, it was frequent the detection of langerin+ cells, both in dermis and corium. This data could suggest that LCs are present in the immune response against *P. brasiliensis* and suffer alterations losing the expression of CD1a, but keeping the expression of langerin when migrate from their typical place, i.e., supra-basal layer of epidermis. It is known that langerin persists during the maturation and migration of LCs [21] and so, in the present study, the positive cells localized both in dermis and corium could be migrating LCs.

On the other hand, a number of researches have evidenced the dermal langerin+ cells as a novel subset of dendritic cells [22–24]. If in PCM lesions the positive cells that we found are in fact this new group of cells, that is another interesting result and new researches need to be developed in order to better characterize them. Considering our previous results describing

for the first time the Factor XIIIa+ dermal dendrocytes as antigen presenting cells and/or fungal target cells, we are now tempted to follow our investigation in this new subset of cells that can play a role in PCM lesions too.

Recently it was speculated that LCs may not enhance a protective immune response against pathogens and that keratinocytes and mainly dermal dendritic cells should be taken into consideration [25].

Bearing in mind the still necessary efforts to better draw the entire picture of cutaneous and mucosal immunity regarding dendritic cells, we now intend to develop a double-immunostaining to investigate the presence of fungal antigen in the cytoplasm of LCs and contribute to the characterization of such cells by using an anti-EpCam antibody in order to identify if they are migrating epidermal LCs.

Overall, our present data reinforce the numerical and morphological changes of LCs in PCM cutaneous and mucosal lesions, presenting, to the best of our knowledge for the first time, results that show langerin+ dendritic cells both in dermis and corium of such lesions.

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Short communication

Immunohistochemical study of Langerhans cells in cutaneous lesions of the Jorge Lobo's disease

Juarez Antonio Simoes Quaresma^a, Debora Unger^b, Carla Pagliari^c, Mirian Nacagami Sotto^c, Maria Irma Seixas Duarte^c, Arival Cardoso de Brito^{a,b,*}

^a Laboratório de Imunopatologia, Núcleo de Medicina Tropical, UFPA, Belem-PA, Brazil

^b Disciplina de Dermatologia, Instituto de Ciências da Saúde, UFPA, Belem-PA, Brazil

^c Departamento de Patologia, Faculdade de Medicina, USP, São Paulo-SP, Brazil

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ABSTRACT

Jorge Lobo's disease is a chronic infection caused by the fungus *Lacazia loboi* endemic in South America. The infection is characterized by the appearance of parakeloidal, ulcerated or verrucous nodular or plaque-like cutaneous lesions. The histopathological aspect is characterized by poorly organized granulomas with histiocytes and multinucleated giant cells. Little is known about local immune response in lobomycosis skin lesions. Thirty-three skin biopsies from patients with Jorge Lobo's disease were selected from Ambulatory of Dermatology, UFPA. The control group was constituted by ten biopsies from normal skin. Langerhans cells were identified by immunohistochemistry using anti-CD1a antibody (Serotec). The number of positive cells was statistically analyzed. Langerhans cells were visualized along the epidermis in biopsies from Jorge Lobo's disease and the morphology and the number of Langerhans cells did not differ from normal skin ($p > 0.05$). In Jorge Lobo's disease, this cell population probably presents some escape mechanism of the local immune system to evade the antigen presentation by those cells.

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1. Introduction

Jorge Lobo's disease, or lacaziosis, is a chronic mycosis caused by uncultivated fungi pathogen *Lacazia loboi*, an agent phylogenetically closely related to the dimorphic pathogen *Paracoccidioides brasiliensis* (Taborda et al., 1999; Vilela et al., 2005). The disease is more frequent in males, mainly affecting individuals in the age range of 21–40 years. Cases of lacaziosis have been described in various South American countries, but it assumes great importance in the Brazilian Amazon region where the largest numbers of these cases are found (Rodríguez-Toro, 1993; Pecher, 1994; Silva et al., 1996). Clinical progression is slow and characterized by parakeloidal cutaneous lesions mainly located in the auricle and lower limbs of humans (Rodríguez-Toro, 1993; Silva et al., 1996). Pecher and Funchs (1988) suggested the presence of cellular immunodeficiency in patients with Jorge Lobo's disease and few studies are available in the literature characterizing the *in situ* cellular and humoral immune response profile (Pecher and Funchs, 1988; Yates et al., 2007). The role of the immune response

in the evolutions of many skin infections disease has been described (Kharkar et al., 2007; Yates et al., 2007). The Langerhans cells (LC) have a very important role in these pathological evolutions (Nestle and Nickoloff, 1995; Azadeh and Dabiri, 2004). Langerhans cells initiate immune response by capturing and processing foreign antigens and then emigrating to the regional lymphnodes, where they present the processed antigen to naïve T cells (Hunger et al., 2004). Langerhans cells have been implicated in the maintenance of cutaneous homeostasis by inducing tolerance as well as stimulating immune responses. In many skin diseases with a enhanced influx of T lymphocytes, the total number of LCs increases in the epidermis and decreases after exposure to ultraviolet rays or toxic substances (Aberer et al., 1981; Cestari et al., 1995; Steinman and Germain, 1998; Koch et al., 2006). These cells are located in epidermis and are CD1a positive (CD1a+) by immunohistochemistry. CD1 proteins comprise a family of transmembrane glycoproteins expressed in association with β -2-microglobulin (Martin et al., 1987; Zeng et al., 1997), which are structurally related to antigen-presenting molecules of the major histocompatibility complex (MHC) class I but are encoded by a different gene locus.

The main objective of the present study was to investigate the possible role of Langerhans cells in the pathogenesis of Jorge Lobo's disease in tissue samples of skin lesions using immunohistochemistry technique.

* Corresponding author at: Núcleo de Medicina Tropical/UFPA, Av. Generalíssimo Deodoro 92, Umarizal, Belem-PA, 66055-240, Brazil.

E-mail address: acdebrito@uol.com.br (A.C. de Brito).

Fig. 1. Jorge Lobo's disease—Histopathological aspects shown inflammatory infiltrate of the dermis consisted of histiocytes, epithelioid cells, giant cells with parasitic corpuscles (A) frequently associated with asteroid corpuscles. Transepidermal release of the fungus in epidermis (B). Grocott stain shown fungus structures in the dermis (C) (A and C: $\times 400$; B: $\times 200$).

2. Materials and methods

2.1. Diagnostic procedures and histological examination

Samples of skin lesions of Jorge Lobo's disease were obtained by biopsy specimen of thirty-three patients from Ambulatory of Dermatology of Federal do Para University and Santa Casa Hospital (Belem, Brazil), with age between 25 and 80 years, 24% female and 76% male. The diagnosis was made by histopathologic aspects and identification of the agents in the lesions. Samples were fixed

in 10% neutral-buffered formalin, followed by paraffin embedding, micron-thick sectioned and stained by hematoxylin and eosin method and grocott stain.

2.2. Immunologic markers

We performed an immunohistochemistry reaction using an anti-CD1a antibody (Serotec) and a streptavidin–biotin method. The primary antibody is a monoclonal antibody (isotype IgG1) produced in mouse. A mouse primary antibody is first added, over-night at 4°C. The primary antibody is detected with a peroxidase-conjugated secondary antibody, applied for 15 min at room temperature. The next step is the bound peroxidase to catalyze oxidation of fluorescyl-tyramide for 15 min and finally a peroxidase-conjugated anti-fluorescein for 15 min. The negative

Fig. 2. Jorge Lobo's disease—Quantitative analysis of Langerhans cells in Jorge Lobo's disease and normal skin (Mann–Whitney test).

Table 1
Jorge Lobo's disease—Statistic analysis of the Langerhans cells in Jorge Lobo's disease and normal skin (Mann–Whitney test).

	JLD	NS
Mean $\pm$ SD	0.07644 $\pm$ 0.05902	0.1182 $\pm$ 0.07141
Median	0.0639	0.0992
Variation	0–0.2428	0.0325–0.2474

JLD = Jorge Lobo's disease; NS = normal skin.

Fig. 3. Jorge Lobo's disease—Immunohistochemistry for Langerhans cells with anti-CD1a antibody shown positive cells in Jorge Lobo's disease (A) and normal skin (B). The morphology of Langerhans cells is similar to the ones in normal skin. Streptavidin–biotin method ($\times 200$).

controls were performed by omitting the primary antibody. For quantitative analysis of the positive cells we used a grid-scale with 10×10 subdivisions in an area of 0.0625 mm^2 . Cells were evaluated by determining the epidermal area fraction with positivity for CD1a (Pagliari and Sotto, 2003). The control group was constituted by ten biopsies from normal skin.

2.3. Statistical analysis and ethics aspects

The number of positive cells was statistically analyzed by the Graph Pad Prism version 3.00 for Windows using a Mann–Whitney test with the level of significance set at 95%. The project was approved by the Ethics and Research boards of the Tropical Medicine Center of Federal do Para University.

3. Results

The patients, 26 men and 7 women ranging in age from 25 to 80 years (mean of 59 years), were from the Amazon region, State of Para, Brazil, and predominantly consisted of farmers or laborers. The time of progression of the disease ranged from 4 to 64 years (mean time of 19.56 years). Clinically, the lesions were predominantly located on the lower limbs and face, and consisted of keloid infiltrative nodular or vegetative-verrucous lesions, frequently showing exulcerations covered with hematic crusts or discharging a purulent secretion.

Histopathological analysis revealed a granulomatous inflammatory infiltrate dispersed through the deep dermis and subcutaneous tissue. The granulomatous infiltrate consisted of frequent xanthomatous histiocytes, epithelioid cells, Langhans and foreign body type giant cells, lymphocytes and asteroid corpuscles (Fig. 1a). Transepidermic release of fungus also was a characteristic finding (Fig. 1b). Parasitic corpuscles surrounded by a double membrane were abundant especially in the cytoplasm of giant cells (Fig. 1c). Expansions of collagen areas were observed in all cases.

Langerhans cells were visualized along the epidermis in all the cases. The morphology of Langerhans cells in lobomycosis they are presented morphologically similar to the ones in normal skin, the same aspects is observed when we consider its number ($p > 0.05$) (Table 1) (Figs. 2 and 3a, b).

4. Discussion

Jorge Lobo's disease patients present evolutive symptoms of various years with multiple parakeloidal lesions plaques predominantly located on the lower limbs (De Brito and Quaresma, 2007). Some authors believe that the genesis of symptoms of Jorge Lobo's

disease is associated with cellular immune deficiencies (Vilani-Moreno et al., 2004a,b).

The number and morphology of Langerhans cells in PCM skin lesions, has demonstrated probable inactivation by fungal products or migration to the dermis to process antigens (Pagliari and Sotto, 2003). However, in Jorge Lobo's disease they are presented morphologically similar to the ones in normal skin, the same with their number. A similar aspect was demonstrated in previous study where the number of CD1a positive cells within the epidermis of lepromatous leprosy was not significantly different when compared with normal skin (Sieling et al., 1999). Some works have demonstrated that dendritic skin cells, when activated, are thought to have the function to regulate T and B responses (Nestle and Nickoloff, 1995). Moreover, dendritic cells may be important for the induction of immunological tolerance as well as for the regulation of the type of T-cell-mediated immune response (Yates et al., 2007). Vilani-Moreno et al. (2005) analyzing biopsy specimens obtained from 15 patients with Jorge Lobo's disease had shown that the poorly organized granuloma was constituted by histiocytes and multinucleated giant cells, and the identified immune cells showed CD68+ histiocytes, CD3+ T lymphocytes, CD4+ lymphocytes, CD8+ lymphocytes, CD57+ natural killer cells, CD79+ plasma cells, CD20+ B lymphocytes. In addition, Xavier et al. (2008) had shown high expression of TGF- β , an immunosuppressive cytokine, in the skin lesions of Jorge Lobo's disease. These findings, together with our data, suggest a possible immunoregulatory disturbance in the Jorge Lobo's patients. However, more investigations are necessary to elucidate the antigen presentation mechanism and the role of the innate immune response in Jorge Lobo's disease.

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Original Article

Plasmacytoid dendritic cells in cutaneous lesions of patients with chromoblastomycosis, lacaziosis, and paracoccidioidomycosis: a comparative analysis

Carla Pagliari^{1,2,3,*}, Luciane Kanashiro-Galo¹, Aline Alves de Lima Silva^{1,2}, Tânia Cristina Barboza^{2,3}, Paulo Ricardo Criado², Maria Irma Seixas Duarte¹, Arival Cardoso de Brito⁴, Marília Brasil Xavier^{4,5}, Deborah Unger⁴, Clivia Maria Moraes Oliveira⁴, Juarez Antonio Simões Quaresma^{4,5} and Mirian Nacagami Sotto²

¹Universidade de São Paulo, Faculdade de Medicina, Departamento de Patologia, ²Universidade de São Paulo, Faculdade de Medicina, Departamento de Dermatologia, ³Instituto de Assistência Médica ao Servidor Público Estadual—Programa de pós-graduação em Ciências da Saúde, São Paulo, Brazil, ⁴Universidade Federal do Pará, Núcleo de Medicina Tropical and ⁵Universidade Estadual do Pará, Belém, Para, Brazil

*To whom correspondence should be addressed. Carla Pagliari, Faculdade de Medicina da Universidade de São Paulo, Departamento de Patologia, Av Dr Arnaldo, 455, sala 1118, Cerqueira Cesar, São Paulo, Brasil, CEP 01246-903. Tel/Fax: (55 11) 3061-7238; E-mail: cpagliari@usp.br

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Abstract

Plasmacytoid dendritic cells (pDCs) are characterized by expression of CD123 and BDCA-2 (Blood Dendritic Cell Antigen 2) (CD303) molecules, which are important in innate and adaptive immunity. Chromoblastomycosis (CBM), lacaziosis or Jorge Lobo's disease (JLD), and paracoccidioidomycosis (PCM), are noteworthy in Latin America due to the large number of reported cases. The severity of lesions is mainly determined by the host's immune status and *in situ* responses. The dendritic cells studied in these fungal diseases are of myeloid origin, such as Langerhans cells and dermal dendrocytes; to our knowledge, there are no data for pDCs. Forty-three biopsies from patients with CBM, 42 from those with JLD and 46 diagnosed with PCM, were evaluated by immunohistochemistry. Plasmacytoid cells immunostained with anti-CD123 and anti-CD303 were detected in 16 cases of CBM; in those stained with anti-CD123, 24 specimens were obtained from PCM. We did not detect the presence of pDCs in any specimen using either antibody in JLD. We believe that, albeit a secondary immune response in PCM and CBM, pDCs could act as a secondary source of important cytokines. The BDCA-2 (CD303) is a c-type lectin receptor involved in cell adhesion, capture, and processing of antigens. Through the expression of the c-lectin

receptor, there could be an interaction with fungi, similar to other receptors of this type, namely, CD207 in PCM and CD205 and CD209 in other fungal infections. In JLD, the absence of expression of CD123 and CD303 seems to indicate that pDCs are not involved in the immune response.

Key words: plasmacytoid dendritic cells, paracoccidioidomycosis, chromoblastomycosis, lacaziosis.

Introduction

Some mycoses constitute a major public health problem in Latin America. Because diagnostic methods are not available in all medical centers, the lesions that result from such infections often become exacerbated and cause serious consequences in immunocompromised patients.

There are some mycoses that are noteworthy due to the relatively large number of reported cases and the severity of clinical manifestations. Here we describe the following three major mycoses because they share some epidemiological, clinical, and histological characteristics: chromoblastomycosis (CBM), lacaziosis, or Jorge Lobo's disease (JLD), and paracoccidioidomycosis (PCM). The etiologic agents of these infections are *Fonsecaea pedrosoi* (the most common in Latin America), *Paracoccidioides brasiliensis*, and *Lacazia loboi*, respectively. There is no requirement to report the diseases caused by these agents; most primarily cause skin lesions, the severity of which is usually determined by the host's immune status and the *in situ* response developed against the fungus. The main form of transmission is the traumatic implantation of fungal elements from contaminated soil or, in the case of PCM, the inhalation of propagules, which form a primary pulmonary focus and later spread to other organs including the skin [1–4].

CBM lesions are characterized by the presence of muriform cells. In warty lesions, a mixed inflammatory infiltrate composed of neutrophils, histiocytes, fewer lymphocytes, plasma cells, eosinophils, mast cells, and many Th2 cytokines is present. In addition, suppurative and poorly formed granulomas with extensive tracts of fibrosis, high parasitism, and fungi intact and dividing within microabscesses could be present. The plate-type atrophic lesions show inflammatory infiltrate in the upper dermis with well-organized granulomas, giant cells, and lymphocytes with Th1 cytokines [5–7].

JLD is a chronic disease that presents with keloid lesions. It is commonly found in tropical regions, with a large number of cases found in the Amazon region, especially among males. The dermal infiltrate is of the granulomatous type, with macrophages, giant Langerhans-type, and foreign bodies with many fungal forms. Neutrophils and necrosis are rare; nerve structures are intact in the interstices of the tissue and the granuloma [8–11].

PCM cutaneous lesions also consist of granulomas, giant cells, and macrophages with fungal forms and lymphocyte infiltrate. The best tissue response is associated with Th1 cytokines, while the worst injuries are noted in the presence of Th2 cytokines [12–14].

Considering dendritic cells in these three mycoses in association with the skin, it has been verified in CBM that macrophages, in particular, and also Langerhans cells and factor XIIIa+ dermal dendrocytes function as antigen-presenting cells. Also, no numerical difference in the population of Langerhans cells has been noted between the forms of plate-type atrophic or verrucous lesions [15].

In JLD, it has been reported that Langerhans cells do not present trophy or numerical changes when compared with normal skin, which seems to represent some mechanism for escape and evasion of antigen presentation [16]. Factor XIIIa+ dermal dendrocytes are present in cutaneous lesions but seem to be related to a small number of yeast-like cells. The phagocytic or antigen presentation capacities seem to be altered in the presence of high fungal parasitism [C. Pagliari, personal communication]. In PCM, Langerhans cells demonstrate trophy and numerical reduction, whereas factor XIIIa+ dermal dendrocytes are activated to initiate phagocytic activity [17,18].

Plasmacytoid dendritic cells (pDCs) are characterized by expression of the CD123 molecule, which binds the receptor of interleukin (IL)-3 α . This cell population is involved in the antiviral immune response by producing interferon-alpha (IFN- α) and is important in both innate and adaptive immunity. Another important marker that is typically expressed is CD303 (BDCA-2), a lectin type II [19].

Massone and colleagues studied such cells in leprosy, a granulomatous disease that shares some pathogenic characteristics with the mycoses we present here, such as granulomatous reaction, chronicity, and lymphohistiocytic infiltrate, among others [20]. This cell population was also described as participating in the immune response in American tegumentary leishmaniasis skin lesions [21].

To our knowledge, no data exist in the literature regarding pDCs in these three diseases. Thus, we explored this cell population and present our findings here.

Material and methods

Forty-three biopsies from CBM patients and 46 from PCM patients were retrieved from the files of the Departamento de Dermatologia, Faculdade de Medicina da Universidade de São Paulo (USP), Brazil. Forty-two biopsies from JLD patients came from the Núcleo de Medicina Tropical, Universidade Federal do Pará (UFPA), Belém, Brazil. The biopsy samples had been fixed in formalin and embedded in paraffin.

We observed the histopathological characteristics of lesions using hematoxylin-eosin staining. An immunohistochemical reaction based on polymers was performed. Briefly, following deparaffinization in xylene and hydration in ethanol, the antigen was recovered in a retrieval solution (pH 6.0); the process was conducted at 95°C. The primary monoclonal antibodies, that is, anti-CD123 (E-bioscience, 14-1239-82, CA, USA) and anti-CD303 (Dendritics, A546, Lyon, France), were applied and, following overnight incubation at 4°C, two polymer systems were used (Advanced-Dako, Carpinteria, CA, USA and Novolink-Leica, Newcastle, UK), respectively. The chromogen used to develop the immunohistochemistry was 3,3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) and the specimens were counterstained with hematoxylin.

The immunolabeled cells were quantified considering nine randomized high-power fields for each specimen using a $\times 10$ ocular lens with a square grid and a $\times 40$ objective. Results were expressed as number of cells per square millimeter, compared and statistically analyzed using Graph Pad Prism, version 6.0 for Windows, and the Mann-Whitney test was performed ($P < 0.05$).

The procedures used to obtain the biopsy materials were in accordance with the ethical standards of the responsible committees on human experimentation at Universidade de São Paulo, Faculdade de Medicina and Universidade Federal do Pará, Núcleo de Medicina Tropical.

Results

CBM lesions were clinically and histologically classified as verrucous and characterized by inflammatory infiltrates with neutrophils, macrophages, lymphocytes, plasma cells, and eosinophils. The granulomas were poorly organized, with extensive tracts of fibrosis and extensive parasitism (Fig. 1A). Plasmacytoid cells immunostained with anti-CD123 and anti-CD303 antibodies were observed in 16 cases (Fig. 2A and 2B).

The JLD lesions presented granulomatous inflammatory infiltrates with extensive parasitism, lymphocytes, and frequent asteroid corpuscles (Fig. 1B). We did not detect the

presence of plasmacytoid cells in any specimen immunostained with either antibody.

Lesions of PCM were characterized by the presence of compact granulomas, loose granulomas, or both types of granulomas in the same lesion. There was infiltration of lymphocytes and macrophages (Fig. 1C). pDCs were visualized in 24 specimens with anti-CD123 (Fig. 2C), but cells expressing CD303 were rarely visualized.

The comparative analysis between groups using the nonparametric Mann-Whitney test demonstrated a similar number of pDCs when CBM and PCM groups were compared ($P = 0.06$). However, there was a statistically significant difference between CBM and PCM lesions when compared with those of JLD ($P < 0.0001$). Figure 3 presents the statistical analysis, showing the numerical similarity between CBM and PCM.

Discussion

The three fungal infections investigated here have a major impact on public health in Latin America; however, their immunopathogenic mechanisms have not been fully elucidated. These fungal infections are a frequent health problem in tropical and subtropical areas in the world and are difficult to control. The health programs do not require the notification of new or recurrent cases and so, they are considered neglected [1,22,23].

CBM progresses slowly and is usually limited to skin and subcutaneous regions. It develops with different clinical manifestations, such as nodular, plaque, and warty lesions that have the potential to develop into squamous cell carcinoma [24,25,26]. CBM is common among people who work in rural environments [23,25]. JLD also develops slowly, forming keloid lesions and is also common among those who work in rural environments. There is no effective treatment for JLD [20]. PCM is also common in rural areas and presents different clinical results of its infection.

Histologically, CBM lesions are characterized by chronic granulomatous inflammatory reaction and large numbers of neutrophils. The sclerotic bodies are seen in the cytoplasm of giant multinucleate Langhans cells or appear free in the tissue. In our evaluation, the lesions were of the verrucous type and characterized by the presence of eosinophils and plasma cells [27,28]. In the context of dendritic cells, *in vitro* studies demonstrate the inability of Langerhans cells to contain a primary infection [29], although by immunohistochemical method it was possible to demonstrate the interaction of this cell population with fungal forms [15].

The role of pDCs in host immune response against fungal infections has not been explored adequately. These cells were visualized in 37% of the CBM biopsies and about

Figure 1. Histological evaluation of lesions. Inflammatory infiltrate in chromoblastomycosis (A) with neutrophils, macrophages, lymphocytes, plasma cells, and eosinophils. Fungal forms with natural pigmentation (arrow) in the cytoplasm of giant cells. (B) Lesion from lacaziosis, presenting granulomatous infiltrate, lymphocytes, macrophages, and intense parasitism (arrow). (C) Lesion from paracoccidioidomycosis with granulomas and intense inflammatory infiltrate of lymphocytes and macrophages. Fungal forms in the cytoplasm of macrophages and giant cells (arrow). Staining with hematoxylin-eosin; $\times 200$. This Figure is reproduced in color in the online version of *Medical Mycology*.

Figure 2. (A) Immunohistochemical detection of cells expressing CD123 in chromoblastomycosis distributed in the inflammatory infiltrate; (B) cells expressing CD303 in chromoblastomycosis; (C) cells expressing CD123 in paracoccidioidomycosis lesion in the inflammatory infiltrate near the dermal-epidermal junction. Immunohistochemistry (arrow), polymer system; $\times 200$. This Figure is reproduced in color in the online version of *Medical Mycology*.

Figure 3. Quantitative analysis of cells expressing CD123 in chromoblastomycosis (CBM), paracoccidioidomycosis (PCM), and lacaziosis (JLD). The comparative analysis evidenced similar number of cells between CBM and PCM ($P = 0.06$) and a statistically significant difference between CBM and JLD or PCM and JLD ($P < 0.0001$). Mann-Whitney test was used. The values as mean $\pm$ standard deviation were CBM, 4.322 ± 11.17 ; PCM, 4.998 ± 7.94 ; and JLD, 0.00.

50% of PCM specimens. However, in JLD, there was no evidence of pDCs using both protocol to visualize reaction to CD123 or CD303 molecules. The populations of dendritic cells studied to date in these three fungal infections

are classified as being of myeloid origin and, to our knowledge, there are no data in the literature about pDCs in these three diseases. pDCs function as antigen-presenting cells, expressing CD123 and BDCA-2 (CD303), among other markers. They are well studied in malignant melanoma, carcinoma of the head and neck, and ovarian cancer, in addition to inflammatory conditions such as systemic lupus erythematosus, psoriasis, and contact dermatitis [30–33]. Under these conditions, they are able to release cytokines and chemokines and activate lymphocytes [34]. Pathogen-mediated activation is seen in viral infections such as human immunodeficiency virus-1, herpes simplex, influenza virus, and others. There are also reports of plasmacytoid cells being activated by *Plasmodium falciparum* malaria and some bacteria [35,36]. As IFN- α/β producing-cells, pDCs would be able to participate in the modulation of immune response such as differentiation of myeloid dendritic cells, activation of Th1 cells, natural killer cell activation, and induction of antibody response [37,38]. The BDCA-2 (CD303) expressed by the pDCs is a c-type lectin receptor that is involved in cell adhesion, signaling and antigen capture and processing [39].

We believe that, albeit a secondary immune response to PCM and CBM, the pDCs, in response to lesions, could act as a secondary source of production of important cytokines, as previously described [5]. We suggest that, at least in CBM and considering expression of c-lectin receptor (CD303), pDCs could interact with fungal forms, similar to what has been described with other receptors of this type, namely, CD207 in PCM [18] and CD205 and CD209 in other fungal infections [40].

In JLD, as in leprosy, which shares some features of tissue injury, the absence of CD123 and CD303 expression seems to indicate that such cells are not involved in the immune response against *Lacazia loboi*. *Lacazia loboi* is not highly pathogenic, which relates to the high number of fungal forms, chronicity, number of the skin lesions, and absence of systemic compromise. The antifungal role of pDCs was previously demonstrated [41]. In JLD, the difficulty of eliminating the agent from skin lesions may be due to possible local immune-deficient mechanisms, which may include the absence of pDCs in the tissue response. Added to the immune issues discussed above, pDCs displayed in CBM and PCM could also be important to the inhibition of fungal growth in skin.

Although we made numerous efforts to detect CD303 in lesions of PCM, the results were negative. This may be due to the length of time the specimens were conserved. However, after performing the same protocol with a newer group of PCM lesions, the results were also negative. We intend to study this molecule in other specimens from PCM patients, such as lungs and lymph nodes.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper. The findings and conclusions of this article are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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Original Article

***Paracoccidioides brasiliensis* interacts with dermal dendritic cells and keratinocytes in human skin and oral mucosa lesions**

Wellington Luiz Ferreira da Silva¹, Carla Pagliari^{1,2}, Maria Irma Seixas Duarte² and Mirian N. Sotto^{1,2,*}

¹Department of Dermatology, University of São Paulo Medical School, São Paulo, Brazil and ²Department of Pathology, University of São Paulo Medical School, São Paulo, Brazil

*To whom correspondence should be addressed. Mirian N. Sotto, Faculdade de Medicina da Universidade de São Paulo, Avenida Dr. Arnaldo, 455 – sala 1118, 01246–903, São Paulo – SP, Brazil. Tel: +55-11-3061-7238; Fax: +55-11-2661-3361; E-mail: mnsotto@usp.br

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Abstract

Paracoccidioidomycosis (PCM) is a systemic disease caused by the fungus *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii*. In PCM the skin and oral mucosa are often affected. Dendritic cells and keratinocytes of the integument play a role in innate and adaptive immune response against pathogens, due to their function as antigen presenting cells. Aiming to verify the interaction of *P. brasiliensis* with these cell populations, we studied 52 skin and 47 oral mucosa samples taken from patients with proven diagnosis of PCM. The biopsies were subjected to immunohistochemical and/or immunofluorescence staining with anti-factor XIIIa (marker of dermal dendrocytes), anti-CD207 (marker of mature Langerhans cells), anti-pan cytokeratins (AE1-AE3) and anti-*P. brasiliensis* antibodies. Analyses with confocal laser microscopy were also performed for better visualization of the interaction between keratinocytes and the fungi. In sum, 42% of oral mucosa samples displayed yeast forms in Factor XIIIa dermal dendrocytes cytoplasm. Langerhans cells in skin and oral mucosa samples did not show yeast cells in their cytoplasm. In sum, 54% of skin and 60% of mucosal samples displayed yeast cells in the cytoplasm of keratinocytes. The parasitism of keratinocytes may represent a possible mechanism of evasion of the fungus to local immune mechanisms. Factor XIIIa dendrocytes and keratinocytes may be acting as antigen-presenting cells to fulfill the probably impaired function of Langerhans cells in skin and oral mucosa of human PCM.

Key words: Paracoccidioidomycosis, dendritic cells, keratinocytes, immunohistochemistry, confocal laser microscopy, skin and oral mucosa.

Introduction

Paracoccidioidomycosis (PCM) is caused by dimorphic fungus *Paracoccidioides brasiliensis* (S1, PS2, and PS3) and

Paracoccidioides lutzii.¹ PCM is a systemic disease that affects various organs, mainly the lungs. The involvement of skin and oral mucosa is very frequent.²

The fungus is observed in the lesions where it is recognized by the characteristic pilot wheel appearance with more than four budding yeasts adhering to the mother cell. The yeast cells parasitize macrophages and giant cells of the tissue causing an inflammatory reaction.³

Dendritic cells, as well as keratinocytes, in skin and integument are cells that play a role in innate and adaptive immunity against pathogens.⁴ Langerhans cells are the main antigen-presenting cells of the integuments. Other dendritic cells present in normal skin and mucosa also play a role in integument immunity against pathogens.⁵ Among other dendritic cells factor XIIIa dendrocytes are characterized by the cytoplasmic expression of factor XIIIa which is a pro transglutaminase involved in hemostasis.⁶ Normal skin displays factor XIIIa dendrocytes at the dermal-epidermal junction, around the dermal superficial blood vessels and in the adventitia of skin appendages. These cells are scarce in reticular dermis and display spindle morphology.⁷ Factor XIIIa dermal dendrocytes display antigen presenting function and influence lymphocyte migration in skin through tumor necrosis factor alpha production.^{8,9}

There are few reports addressing the interaction of *P. brasiliensis* yeast cells with dendritic cells and keratinocytes at the site of human PCM lesions^{10,11} and in experimental conditions;¹² thus, we intended to verify the interaction of *P. brasiliensis* yeast cells with these cells in skin and oral mucosa lesions.

Material and methods

Oral mucosa and skin samples

Forty-seven oral mucosa and fifty-two-skin paraffin embedded samples taken from PCM patients for diagnostic purpose were selected from the files of the Dermatopathology Laboratory of Dermatology Department of University of São Paulo Medical School. All patients were from the State of São Paulo, where *Paracoccidioides brasiliensis* is the agent of PCM.¹

The samples were classified by the characteristics of tissue response.¹⁰ G1 group comprised samples displaying well-organized granulomas (Figure 1a), G2 group exhibited poorly organized granulomas (Figure 1b) and G3 group displayed both types of granuloma.

Immunohistochemical staining for demonstration of P. brasiliensis yeast cells interaction with factor XIIIa dendrocytes, Langerhans cells and keratinocytes.

In sum, 4 μ m thick histological sections of each sample were submitted to an immunohistochemical staining technique modified from Pagliari and Sotto.¹⁰ Table 1 displays the antibodies, dilution, visualization systems, and substrate chromogens used.

Figure 1. Paracoccidioidomycosis. Well-organized granulomatous tissue reaction (A). Poorly organized granulomatous tissue response (B). Hematoxylin and eosin stain.

After the immunohistochemical procedures the slides were counterstained with Mayer's hematoxylin and mounted with Permount resin (SP15-100, Fisher Scientific) and glass coverslips.

Only the mucosal samples were subjected to double labeling to demonstrate Factor XIIIa dendrocytes/*P. brasiliensis* co-localization, because the parasitism of dermal dendrocytes in PCM skin lesions was demonstrated previously elsewhere.¹⁰

All reactions were run with positive controls. The negative controls of the reactions were obtained by the omission of the primary antibodies that were replaced by isotypic nonreagent antibodies or phosphate buffer saline solution (PBS) pH 7.4.

Double immunofluorescence technique to demonstrate P. brasiliensis within keratinocytes cytoplasm

In sum, 4 μ m thick dewaxed histological sections of seven skin and one oral mucosa samples were incubated for two days at 4°C, with a mixture of AE1-AE3 and anti-*P. brasiliensis* antibodies (dilution 1/250). After washing the

Table 1. Antibodies, visualization systems, and substrate chromogens used in the immunohistochemical double staining techniques for demonstration of *Paracoccidioides brasiliensis* yeast cells co-localization with dermal dendritic cells, Langerhans cells (CD207), and keratinocytes.

Antibody	Dilution	Visualization system	Substrate chromogen
Anti- <i>P. brasiliensis</i> rabbit polyclonal antibody ¹⁰	1:300	EnVision™ G2 System/AP (K5355, Dako)	PermaGreen/AP, PermaBlue/AP (K052, K051, Diagnostic ByoSystems)
Anti-Factor XIIIa (RB1464-P, Labvision)	1:200	Novolink™ Polymer (7159, Novocastra)	3,3' - Diaminobenzidine (D5637, Sigma)
CD207 (clone 12D6, Novocastra)	1:100	Novolink™ Polymer (7159, Novocastra)	3,3' -Diaminobenzidine (D5637, Sigma)
AE1/AE3 (multi-cytokeratin) (NCL-L-AE1/AE3, Novocastra)	1/400	LSAB Biotinylated-Link (K0690, Dako)	3,3' - Diaminobenzidine (D5637, Sigma)

material was submitted to incubation with the mixture of donkey anti-mouse antibody (Alexa Fluor 546, Invitrogen) and goat anti-rabbit antibody (Alexa Fluor 488, Invitrogen) diluted 1/250, during two hours in dark chamber. The slides were then washed in PBS followed by incubation with 4', 6-diamidino-2-phenylindole (Sigma-Aldrich) diluted 1/100, for 30 min in dark chamber, and mounted with glass coverslips and mounting medium (Hydromount, National Diagnostics). Controls included staining with isotype-matched irrelevant antibodies.

Fluorescent images were acquired using UV/ Laser excitation on an LSM/Meta 510 Zeiss microscope and LSM Image Examiner software (Carl Zeiss, Standort Gottingen, Germany).

Statistical analysis

The number of samples of each group of tissue response (G1, G2. and G3) displaying *P. brasiliensis* co-localization with dendritic cells and keratinocytes was analyzed by Fisher's exact test. The level of significance was set at 95%.

Results

The 47 oral mucosa samples comprised 13 biopsies displaying well-organized granuloma (G1) (Figure 1a), 19 samples with poorly organized granulomas (G2) (Figure 1b), and 15 samples with both types of granulomas (G3). Eleven skin samples displayed well-organized granulomas (G1), 22 poorly organized granulomas (G2), and 19 presented both kinds of granuloma formation (G3).

In sum, 42% of mucosal samples displayed *P. brasiliensis* within hypertrophic factor XIIIa dermal dendrocytes cytoplasm (Figure 2a). These cells were intermingled between the diffuse inflammatory infiltrate and involved the granulomas. Multinucleated macrophages also exhibited yeast cells within their cytoplasm (Figure 2b). Table 2 displays the statistical analyses of these results.

Figure 2. Paracoccidioidomycosis. Factor XIIIa+ dendrocytes (arrows) around granuloma and *Paracoccidioides brasiliensis* yeast cells within giant cells' cytoplasm (A). Yeast cells (green) within factor XIIIa+ dendrocyte cytoplasm (brown) (B). Double staining with anti-factor XIIIa antibody (brown) and anti-*Paracoccidioides brasiliensis* antibody (green).

Fifty-two skin and forty-seven oral mucosa samples displayed scarce Langerhans cells labeled for CD207 antibody in the spinous layer of epidermis/epithelium (Figure 3a). CD207 immune stained cells were also distributed between the inflammatory infiltrate and within granuloma in skin

Table 2. Comparison of the number of oral mucosa samples with *Paracoccidioides brasiliensis* yeast cells within factor XIIIa+ dendrocytes' cytoplasm.

Group of tissue response	Number of samples with <i>P. brasiliensis</i> yeast cells within factor XIIIa+ dendrocytes cytoplasm	Number of samples without <i>P. brasiliensis</i> yeast cells within factor XIIIa+ dendrocytes cytoplasm	<i>P</i> value*
G1	1/13 (8%)	12/13 (92%)	.0003 (G2 vs G1)
G2	14/19 (74%)	5/19 (26%)	.003 (G2 vs G3)
G3	5/15 (33%)	10/15 (67%)	> .05 (G3 vs G1)

Note: G1 – well-organized granulomas; G2 – poorly organized granulomas; G3 – both types of granulomas. * Fisher's exact test.

Figure 3. Paracoccidioidomycosis. Langerhans cells with short dendrites scattered among epidermal keratinocytes. Yeast cell (arrow) within macrophage cytoplasm in the dermal inflammatory infiltrate (A). Oral mucosa sample displaying Langerhans cells among the inflammatory infiltrate of the lamina propria. Yeast cells (arrow) within giant cells cytoplasm (B). Double staining with anti-CD207 antibody (brown) and anti-*Paracoccidioides brasiliensis* antibody (green).

(29/52 samples) and in 28/47 of oral mucosa samples (Figure 3b). No samples exhibited yeast cells within the cytoplasm of CD207 labeled cells.

Yeast cells within the cytoplasm of keratinocytes were observed in 28/52 (54%) skin and 28/47 (60%) oral mu-

Figure 4. Paracoccidioidomycosis. *Paracoccidioides brasiliensis* yeast cells within keratinocytes cytoplasm (arrows) labeled for cytokeratin (A e B). Double staining with AE1-AE2 antibody (brown) and anti-*Paracoccidioides brasiliensis* antibody (blue).

cosa samples (Figure 4). Table 3 displays the comparison of the double staining positivity between samples classified according to the type of tissue response.

The confocal laser microscopy examination displayed *P. brasiliensis* yeast cells within the cytoplasm of keratinocytes and fungal antigenic deposits within the intercellular space of the squamous epithelia of the eight samples examined (Figure 5).

Table 3. Comparison of the number of samples displaying *Paracoccidioides brasiliensis* yeast cells within keratinocytes' cytoplasm.

Group of tissue response	Oral mucosa samples (n = 47)	
	Number of samples with <i>P. brasiliensis</i> yeast cells within keratinocytes cytoplasm	Number of samples without <i>P. brasiliensis</i> yeast cells within keratinocytes cytoplasm
G1	6/13 (46%)	7/13 (54%)
G2	12/19 (63%)	7/19 (37%)
G3	10/15 (67%)	5/15 (33%)
Group of tissue response	Skin samples (n = 52)	
	Number of samples with <i>P. brasiliensis</i> yeast cells within keratinocytes cytoplasm	Number of samples without <i>P. brasiliensis</i> yeast cells within keratinocytes cytoplasm
G1	5/11 (45%)	6/11 (55%)
G2	11/22 (50%)	11/22 (50%)
G3	12/19 (63%)	7/19 (37%)

Note: G1 – well-organized granulomas; G2 – poorly organized granulomas; G3 – both types of granulomas. No difference when comparing the results of the groups of tissue response (Fisher's exact test).

Discussion

We demonstrated *P. brasiliensis* parasitism of factor XI-IIa dermal dendrocytes in 42% of oral mucosa samples. These cells' parasitism was observed in 40% of skin lesions in another study.¹⁰ Factor XIIIa dermal dendrocytes are involved in the pathogenesis of other infectious diseases. These cells are abundant and hypertrophic in chromoblastomycosis skin lesions and displayed fungal antigen in their cytoplasm.¹³ Parasitism of Factor XIIIa dendrocytes by *Leishmania* amastigotes was demonstrated by our group.¹⁴ Dermal dendrocytes are cells that display phagocytic function and antigen presenting capacity.⁸ Considering the results obtained we speculate that this group of cells may be acting as antigen presenting cells or are only cellular targets for the fungi in human PCM. We decided to observe the frequency of co-localization of fungus yeast cells with factor XIIIa dermal dendrocytes in PCM samples showing the three types of tissue response because we formerly verified that these groups of lesions (G1, G2, and G3) displayed different cytokine profiles.¹⁵ It was interesting to observe that the number of factor XIIIa immune labeled cells displaying *P. brasiliensis* yeast cells within their cytoplasm was higher in the mucosal samples exhibiting tissue response with poorly organized granuloma (G2 group) than samples with organized granulomas (G1) and samples with tissue response with both kind of granuloma (G3 group). This result could be related to the high number of yeast cells in G2 type of tissue reaction, which is characterized by Th2 cytokines profile.¹⁵

Langerhans cells did not co-localize with *P. brasiliensis* yeast cells in both skin and oral mucosa lesions. This

result is in accordance to what was observed by Sandoval et al.¹⁶ These authors demonstrated, by immune electron microscopy, that *P. brasiliensis* antigens accumulated in the macrophages but not in the Langerhans cells in human PCM mucocutaneous lesions. Langerhans cells are potent antigen presenting cells that internalize small antigen particles (0.5–1 µm in size), but the capacity of these cells to internalize great particles is reduced.⁴ This fact may explain why we did not observe the parasitism of Langerhans cells in PCM lesions. Otherwise, we previously demonstrated fungi antigens in S100 protein (another marker of Langerhans cells) immune labeled cells in chromoblastomycosis skin lesions.¹³ In the present study Langerhans cells were scarce and displayed trophic alterations in both skin and oral mucosa PCM lesions. Other studies demonstrated a decrease of Langerhans cells in the skin of human PCM lesions.^{15,17} The reduced number of Langerhans cells and their morphological alterations were considered the result of the action of factors produced by *P. brasiliensis*, and may reflect the depressed cellular immunity secondary to the infection.¹⁷ CD207 expressing cells were previously observed reduced in number in epidermis and epithelium of oral mucosa in human PCM lesions.¹⁸

In sum, 52% of skin samples and 60% of oral mucosa samples displayed yeast cells within the cytoplasm of keratinocytes. The frequency of co-localization of fungus cells and keratinocytes did not differ in the three groups of tissue response (G1, G2, and G3) of skin and oral mucosa lesions. De Brito et al.¹¹ demonstrated by transmission electron microscopy parasitism of keratinocytes in PCM lesions. These authors considered the parasitism of keratinocytes another

Figure 5. Paracoccidioidomycosis. Double immunofluorescence and laser confocal microscopy displaying yeast cells (green) within epidermis (red) and papillary dermis (A). *Paracoccidioides brasiliensis* yeast cell within keratinocyte cytoplasm and fungi antigen with intercellular fluorescence pattern (B).

mechanism of infection in human PCM. The infection of keratinocytes in PCM occurs through yeast cells, which is different than the primary infection due to conidia/mycelial fragments in the lungs. The deposit of *P. brasiliensis* antigen in the intercellular space of the epithelia was an interesting result obtained by the two immune labeling techniques performed. This same aspect was previously observed. It was considered as diffusion of the fungus antigen into the intercellular epithelial space.¹⁶ The parasitism of keratinocytes may reflect a mechanism of immunological tolerance against *P. brasiliensis*.¹² Otherwise we may suggest

that yeast cells within the cytoplasm of keratinocytes in skin and oral mucosa of PCM lesions is related to the activity of keratinocytes as antigen presenting cells.¹⁹ This possibility is supported since keratinocytes express many receptors for a wide variety of pathogen components. They also produce chemokines and cytokines in response to pathogenic stimuli.^{20,21}

In the literature there are speculations that Langerhans cells may not enhance a protective immune response against pathogens and that keratinocytes and mainly dermal dendritic cells play a role in the protective immune response.²² Based on our results, it is suggested that in human PCM integument lesions, dermal dendrocytes and keratinocytes may play a role in immunological recognition of infective agents because of the impaired function of Langerhans cells.

Ethical approval

The Ethics Committee of University of São Paulo Medical School Hospital approved the study. For this type of study formal consent is not required.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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Analysis of microvasculature phenotype and endothelial activation markers in skin lesions of lacaziosis (Lobomycosis)

Juarez A.S. Quaresma^{a,b,*}, Maysa V. Brito^a, Jorge R. Sousa^a, Luciana M. Silva^b, Kelly E. Hirai^b, Rafael S. Araujo^b, Arival C. de Brito^a, Francisca R.O. Carneiro^b, Hellen T. Fuzii^a, Carla Pagliari^c, Mirian N. Sotto^c, Maria I.S. Duarte^c

^a Nucleo de Medicina Tropical, Universidade Federal do Para, Belem, PA, Brazil

^b Centro de Ciencias Biologicas e da Saude, Universidade do Estado do Para, Belem, PA, Brazil

^c Departamento de Patologia, Faculdade de Medicina, Universidade de Sao Paulo, Sao Paulo, SP, Brazil

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Jorge Lobo's disease is a rare mycosis characterized by chronic inflammation, which causes skin lesions in the absence of visceral dissemination. The disease occurs mainly in hot and humid climates and most cases have been registered in the Brazilian Amazon region. This study investigated possible microvascular alterations in skin lesions caused by infection with *Lacazia loboi* which may interfere with the clinical progression of the disease. Immunohistochemistry was used to evaluate the density of blood and lymphatic vessels, as well as expression of the cell adhesion molecules ICAM-1, VCAM-1 and E-selectin. The results showed a reduced number of blood (62.66 ± 20.30 vessels/mm²) and lymphatic vessels (3.55 ± 5.84 vessels/mm²) in Jorge Lobo's disease when compared to control skin (169.66 ± 66.38 blood vessels/mm² and 8 ± 2.17 lymphatic vessels/mm²). There were a larger number of vessels expressing ICAM-1 (27.58 ± 15.32 vessels/mm²) and VCAM-1 (7.55 ± 6.2 vessels/mm²). No difference was observed in the expression of E-selectin (4.66 ± 11 vessels/mm²). Taken together, the results indicate changes in the local microvasculature which may interfere with the development of an efficient cell-mediated immune response and may explain restriction of the fungus to the site of injury.

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1. Introduction

Lobomycosis is a chronic granulomatous infection caused by *Lacazia loboi*. The disease affects the skin and subcutaneous tissue of humans and members of the family Delphinidae (dolphins) through inoculation of the fungus, probably associated with trauma [1,2].

Lobomycosis was described for the first time in 1931 by the Brazilian dermatologist Jorge de Oliveira Lobo in a rubber tapper from the Amazon Valley [3]. The disease is therefore also known as Jorge Lobo's disease or Jorge Lobo's mycosis. Other synonyms include lacaziosis, keloidal blastomycosis, Jorge Lobo type blastomycosis, miraip or piraip ("that which burns" in Tupi language), Caiabi leprosy, false leprosy, blastomycoid granuloma, and

Amazonia blastomycosis [2]. This mycosis is typical of the Neotropical region and most cases have been reported in the Brazilian Amazon region. Since notification is not mandatory, it is difficult to establish the exact number of cases of the disease; however, there is an estimated 490 cases in the world, 318 of them in Brazil, including 61 Indians of the Caiabi tribe [4,5].

Little is known about the physiopathological features of this disease, probably because it has so far been impossible to grow the etiological agent in artificial media [6,7]. The lesions in Jorge Lobo's disease generally exhibit marked fibrosis, absence of well-formed granulomas, and large numbers of the fungus. These features indicate a deficient cell-mediated immune response and a well-established humoral response, which seem to lead to clinical manifestation of the disease [5].

The maintenance of infection and establishment of a predominant cell-mediated response are influenced by the activation of vessels. Several inflammatory diseases are associated with a hyperactive or insufficient vasculature, including dengue, psoriasis, atopic dermatitis, and ultraviolet light-induced skin damage [8].

* Corresponding author. Nucleo de Medicina Tropical, Universidade Federal do Para, Av. Generalissimo Deodoro 92, Umarizal Belem, PA, Brazil.

E-mail address: juarez@pesquisador.cnpq.br (J.A.S. Quaresma).

Endothelial and lymphatic vessels seem to play an important role in acute and chronic inflammatory diseases. In inflamed skin, vascular remodeling consists of an increase in permeability, expansion of the vessel network accompanied by increased blood flow, and inflammatory cell influx. During chronic inflammation, the activated endothelium expresses chemokines, cytokines and adhesion molecules that induce rolling stable adhesion and leukocyte migration to the skin [9].

The skin undergoes different histopathological changes during infection with *L. loboi* and establishment of the disease. In Jorge Lobo's disease, the activity of endothelial and lymphatic vessels during development of chronic inflammation in response to infection with the fungus has not been investigated. Therefore, the objective of the present study was to evaluate possible microvascular alterations associated with *L. loboi* infection in skin lesions of patients with a confirmed diagnosis of Jorge Lobo's disease.

2. Materials and methods

2.1. Patients

Paraffin-embedded skin lesion of the lower limbs biopsies from 24 patients with a diagnosis of Jorge Lobo's disease obtained before therapeutic intervention were selected from the archive of the Nucleus of Tropical Medicine, Federal University of Pará (Núcleo de Medicina Tropical, Universidade Federal do Pará). The diagnosis was based on the clinical presentation of the patients, direct mycological examination and histopathological analysis according to the report of each sample.

Excluded were samples of patients not diagnosed with Jorge Lobo's disease, patients co-infected with HIV, and patients receiving some type of treatment at the time of biopsy. 20 lower limbs skin biopsies, 10 of the young and 10 of the old subjects without histopathological alterations served as controls. These biopsies were selected from the archive of the Department of Pathology, University of São Paulo School of Medicine (Departamento de Patologia da Faculdade de Medicina da Universidade de São Paulo e FM-USP) and Tropical Medicine Center of Federal do Pará University (Núcleo de Medicina Tropical da Universidade Federal do Pará).

2.2. Sample processing

The paraffin blocks of cases of Jorge Lobo's disease and control cases were cut into 4-mm thick histological sections with a microtome and mounted on glass slides previously prepared with 3-aminopropyltriethoxy-silane adhesive (Sigma Chemical Co., St. Louis, MO, USA). Some slides were stained with hematoxylin-eosin for morphological analysis and the remaining slides were submitted to immunohistochemistry using specific antibodies against ICAM-1 (Novocastra/NCL-CD54/diluted at 1:50), VCAM-1 (RD Systems/BBA19/diluted at 1:1000), E-selectin (Novocastra/NCL-CD62E-382/diluted at 1:50), CD34 (Novocastra/NCL-END-CD34/diluted at 1:50), and podoplanin (D2-40) (Dakocytomation/M3619/diluted at 1:600).

2.3. Immunohistochemistry

Immunostaining for endothelial antigens and cell adhesion molecules was performed according to the method of Hsu et al. [10], partially modified and standardized by the laboratory of the Discipline of Pathology of Transmissible Diseases, Department of Pathology, FM-USP. For this purpose, the histological sections were deparaffinized by incubation in an oven at 56 °C for 24 h, immersed in xylene for 20 min at room temperature, and again immersed in

xylene for 10 min at room temperature. Next, the sections were hydrated in a decreasing ethanol series (100% 95% 70%) for 5 min each and then washed under running water and in distilled water and phosphate-buffered saline (PBS), pH 7.4, for 5 min. Endogenous peroxidase was blocked by three incubations in 3% hydrogen peroxide for 10 min in a dark chamber. The slides were then washed as described above. Antigen retrieval, if necessary, was achieved by wet heat treatment for 20 min at 95 °C in Target Retrieval Solution (Dako), pH 9 or 6 according to antibody standardization. The sections were again washed as described above. Nonspecific proteins were blocked by incubating the sections in 10% skim milk for 30 min at 37 °C, followed by washing under running water, distilled water and PBS, pH 7.4, for an average of 5 min each. Next, the sections were incubated with the antigen-specific primary antibody diluted in 1% bovine serum albumin for 12 h at 4 °C.

After incubation with the primary antibodies, the sections were washed twice with PBS, pH 7.4, for 5 min each. The method of detection and amplification differed for each primary antibody. The LSAB method was used for slides labeled with the anti-VCAM-1 antibody. The sections were incubated with the biotinylated secondary antibody (Biotinylated link universal, Dako A/S, Denmark) for 30 min at 37 °C, followed by incubation with the streptavidin-biotin HRP complex (Dako A/S) for 30 min at 37 °C. For slides labeled with the anti-CD34, anti-ICAM-1 and anti-E-selectin antibodies, an indirect polymer method using the Novolink kit (Novocastra, USA) was applied. The sections were incubated with the secondary antibody for 30 min at 37 °C, followed by incubation with the tertiary antibody labeled with peroxidase-conjugated polymer at 37 °C. An indirect polymer method using the Envision kit (Dako) was used for slides labeled with the anti-D2-40 (podoplanin) antibody. The sections were incubated with the secondary antibody labeled with peroxidase-conjugated polymer for 30 min at 37 °C. After washing, the reaction was developed by incubating the slides with 0.03% 3,3'-diaminobenzidine as chromogen plus 1.2 ml 3% hydrogen peroxide. Color intensity was monitored under a light microscope using positive controls (amygdala) for each reaction. The slides were then washed under running water for 10 min and counterstained with Harris hematoxylin for 15 s, washed under running water, and dehydrated in ethanol. Finally, the slides were mounted in Permount resin (Fisher Scientific, Fair Lawn, NJ, USA).

2.4. Quantitative analysis of immunostaining

Positive staining for the antigens in all samples was defined as cells exhibiting brown staining in the cytoplasm. Immunoreactive vessels were quantified by a single observer. For this purpose, immunostained vessels were counted using a 1-cm² grid divided into 10 portions of 1 mm², which was adapted to the eyepiece of a Nikon Eclipse 200 light microscope. Vessels were counted at 400 $\times$ magnification. The grid was positioned over the entire dermis and 12 fields were selected randomly per sample. The sum of the number of immunostained vessels was divided by the number of fields (12) and the result was divided by 0.0625 (area of the grid), providing the number of cells/mm².

2.5. Statistical analysis

Quantitative variables were compared between patients with Jorge Lobo's disease and controls using the nonparametric Mann-Whitney test. Differences between individual groups were considered to be significant when the probability of equality was less than 0.0001 ($p < 0.0001$) and when the probability of not having a relationship was less than 0.05. All statistical analyses

were performed with the GraphPad Prism 6.0 for Windows program (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Clinical and pathological characteristics of the sample studied

The mean age of the patients was 52 years (range: 22e89 years). There were 8.34% women and 91.66% men.

3.2. Anatomopathological analysis of cases of Jorge Lobo's disease

Large numbers of parasites with an individual double membrane were identified in all preparations. Individual chain-like forms were frequently observed. In addition, live and dead fungi were present in the lesions (Fig. 1). Histological analysis revealed cases exhibiting an atrophic epidermis, acanthosis/hyperkeratosis, or preserved epidermis (Fig. 2).

As shown in Table 1, an atrophic rectified epidermis was seen in 66.66% (n ¼ 16) of cases which exhibited formation of the so-called grenz zone, a thin layer of subepidermal collagen. The opposite

events, i.e., acanthosis and hyperkeratosis characterized by thickening of the epidermis due to a larger number of cells in the spinous layer (acanthosis) and corneal layer (hyperkeratosis), were observed at a lower frequency in 25% of cases (n ¼ 6). Trans-epidermal elimination of the fungus was seen in some lesions. The epidermis was preserved in 8.33% (n ¼ 2) of cases.

Histological analysis of the dermis revealed intense and massive infiltration of macrophages in all cases. Other features included the presence of macrophages filled with finely granular material, the formation of giant cells in the absence of granuloma formation, and the sparse presence of lymphocytes. Asteroid bodies were observed in two cases. Plasma cells and eosinophils were present in some lesions (Fig. 3).

As can be seen in Table 2, macrophages filled with finely granular material were rare in 37.5% (n ¼ 9) of cases, moderate macrophage infiltration was observed in 33.33% (n ¼ 8), intense infiltration in 16.66% (n ¼ 4), and macrophages were absent in 12.5% (n ¼ 3). Giant cell formation was observed in all cases and was rare in 41.66% (n ¼ 10), moderate in 29.16% (n ¼ 7) and intense in 29.16% (n ¼ 7). The formation of asteroid bodies was seen in two cases. Lymphocyte infiltration ranged from rare (41.66% n ¼ 9) to moderate (45.66% n ¼ 11) and was absent in 12.5% (n ¼ 3) of cases. Eosinophils and plasma cells were identified at a lower frequency. Eosinophil infiltration was rare in 16.66% (n ¼ 4) of cases, moderate in 4.16% (n ¼ 1), and absent in 79.16% (n ¼ 19). Plasma cells were mainly absent (83.33% n ¼ 20). A rare plasma cell infiltrate was observed in one case (4.16%), a moderate infiltrate in 2 (8.33%), and an intense infiltrate in one (4.16%). A moderate number of neutrophils were present in only one case (4.16%). Moderate fibrosis was observed in 37.5% (n ¼ 9) of cases and collagen fibers were detected in 33.33% (n ¼ 8).

Fig. 1. Clinical aspects and histopathology with presence of the fungus in human *Lacazia loboi* injury. Intense presence of the fungus in the lesion in individual forms (arrows) and catenulada (ellipse). Note the presence of live yeast (thin arrow) and dead (large arrow). Histological sections stained with hematoxylin and eosin. Magnification: 400 $\times$.

3.3. Microvascular density

The density of blood and lymphatic vessels was determined by immunohistochemical staining of CD34 (pan-endothelial) and D2-40 (lymphatic endothelium) endothelial antigens (Fig. 4).

First, the total number of vessels stained was compared between lesions and normal skin. Vessel density was lower in lesions of patients with Jorge Lobo's disease (66.22 ± 20.65 vessels/mm²) when compared to normal skin samples (177.66 ± 64.5 vessels/mm²) (Fig. 5).

Next, the density of blood and lymphatic vessels was evaluated. Blood vessels were detected in all cases studied and were concentrated in the papillary layer of the dermis. Immunohistochemical quantification showed a mean blood vessel density of 62.66 ± 20.30 vessels/mm² in lesions of patients with Jorge Lobo's disease and of 169.66 ± 66.38 vessels/mm² in normal skin samples. Comparative analysis of blood vessel density between cases and controls showed a reduction in lesions caused by *L. loboi* infection (Fig. 6).

Lymphatic vessels were less frequent in patients with Jorge Lobo's disease. No lymphatic vessels were detected in 41.66% (n ¼ 10) of cases. If present, lymphatic vessels were observed in the reticular layer of the dermis. Immunohistochemical quantification showed a mean density of 3.55 ± 5.84 lymphatic vessels/mm² in lesions of patients with Jorge Lobo's disease and of 8 ± 2.17 vessels/mm² in normal skin samples. A lymphatic vessel density of about 25 vessels/mm² was observed in only one case. Comparative analysis with the control group showed that infection with *L. loboi* reduced the density of lymphatic vessels in the lesions (Fig. 6).

Fig. 2. Histological alterations found in the epidermis during Jorge Lobo's disease. (A) Epidermis conserved. (B) rectified atrophic epidermis with formation of subepidermal band Grenz (arrows). (C) Elimination epidermal transmigration of fungi. (D) acanthosis/hyperkeratosis. Histological sections stained with hematoxylin-eosin. Magnification 200 $\times$ in (A), (B) and (D), 400 $\times$ magnification in (C).

Table 1

Distribution of the histopathological aspects of the epidermis in the lesion of patients with Jorge Lobo's disease.

Histopathological aspects	Histopathological aspects of the epidermis		
	Atrophic epidermis rectified	Acanthosis/hyperkeratosis	Normal epidermis
%	66.66	25	8.33
N	16	6	2

3.4. Evaluation of expression of cell adhesion molecules

Endothelial activation was evaluated by immunohistochemical staining for ICAM-1, VCAM-1 and E-selectin (Fig. 7). A significantly larger number of vessels with endothelial cells expressing ICAM-1 on their surface were observed in lesions of patients with Jorge Lobo's disease when compared to the control group ($p < 0.0001$). The mean number of vessels expressing ICAM-1 was 27.58 ± 15.32 vessels/mm² in lesions of patients, whereas no expression of this adhesion molecule was observed in normal skin (Fig. 8). In addition to endothelial cells, other cell types also exhibited ICAM-1 staining.

The mean number of vessels with cells expressing VCAM-1 was also significantly higher ($p = 0.0375$) in lesions of patients with Jorge Lobo's disease (7.55 ± 6.2 vessels/mm²) compared to normal skin (1.6 ± 1.6 vessels/mm²) (Fig. 8).

In contrast, the number of E-selectin-stained vessels did not differ significantly between control and infected skin. No E-selectin staining was observed in 46% (n = 11) of cases. The mean number of E-selectin-stained vessels was 4.66 ± 11 vessels/mm² in lesions of patients, whereas no stained vessels were detected in normal skin (Fig. 8).

For better evaluation of the level of expression of the cell adhesion molecules in vessels of patients with Jorge Lobo's disease, the percent expression of each molecule was determined in relation to total vessel density. The percent expression of ICAM-1 was significantly higher than the expression of VCAM-1 and E-selectin (Fig. 9).

4. Discussion

Jorge Lobo's disease is a rare mycosis of the skin caused by the fungus *L. loboi*. The dynamic interaction between the pathogenic agent and the host results in a chronic inflammatory disease whose physiopathological aspects are still not well understood. To our knowledge, no studies have investigated the cutaneous vascular system in Jorge Lobo's disease and some aspects of this system were therefore addressed in the present study.

The sample of Jorge Lobo's disease cases studied predominantly consisted of male patients with a mean age of 52 years. This finding agrees with a survey involving 125 cases, in which 92% (n = 115) of the patients were males and 8% (n = 10) were females [11]. With respect to age, according to Baruzzi et al. [12], a higher proportion of cases of Jorge Lobo's disease are found in the 21–40 year age group considering the onset of the disease. The mean age above 40 years observed in the present study might be attributed to the fact that it corresponded to the mean age of the patients at sample collection.

Histopathological analysis of the slides showed the presence of an atrophic rectified epidermis in most cases. The opposite event, i.e., acanthosis and hyperkeratosis, was observed in a smaller number of patients and some cases exhibited a preserved epidermis. Similarly, Opromolla et al. [13] found a higher frequency of atrophic rectified epidermis in 40 patients with Jorge Lobo's disease. Another event observed was transepidermal elimination of

Fig. 3. Micrograph showing the cell infiltrate in the dermis during Jorge Lobo's disease. (A) rich in macrophages, the presence of finely granular macrophages (arrow), giant cells and lymphocytes infiltrate rare. (B) Giant cell containing asteroid bodies inside. (C) Presence of plasma cells. (D) Presence of eosinophils. Histological sections stained with hematoxylin-eosin. Magnification: (A) 630 $\times$ and (B), (C) and (D) 400 $\times$.

the fungus, a process by which the skin sheds inflammatory cells, tissue components, foreign body material, and microorganisms [14]. This process has been reported in other studies on lacaziosis [13,15].

A large number of fungi and macrophages, some lymphocytes and other cell types, which were present in smaller numbers or absent, were seen in the dermis of patients with Jorge Lobo's disease. Similar histopathological features have been described in other studies [12,13].

Immunohistochemistry revealed microvascular alterations in lesions of Jorge Lobo's disease. The presence of a hyperactive or insufficient vasculature has been reported for different dermatological disease, including many inflammatory disorders such as psoriasis rosacea and contact dermatitis [16]. Microvascular alterations have also been described in other infectious diseases such as dengue, malaria, and hemorrhagic fever [17e19].

Quantitative analysis of immunostaining for D2-40 and CD34 showed fewer blood and lymphatic vessels in lesions of patients with Jorge Lobo's disease when compared to the control group. Among fungal diseases, the event of vessel regression or inhibition of angiogenesis has been observed in invasive pulmonary aspergillosis [20]. That study demonstrated that secondary metabolites of the fungus *Aspergillus fumigatus* inhibited the differentiation and migration of endothelial cells, as well as the in vitro formation of capillary tubes. In addition, gliotoxin was found to exert a specific role in the antiangiogenic activity of *A. fumigatus* [20].

With respect to lymphatic vessels, although inflammation can be characterized as a mechanism that promotes lymphangiogenesis, this phenomenon is not observed in certain situations [21]. In the present study, a marked reduction in the density of lymphatic vessels was observed in Jorge Lobo's disease lesions. This reduction is also seen in ultraviolet light-induced skin damage [22]. In contrast, in psoriasis, the number of lymphatic vessels is

increased in the plaques [23]. This increase in lymphatic vessel density has also been described for arthritis and is intensified after standard therapy with infliximab [24].

Two hypotheses can be raised to explain the vascular regression in Jorge Lobo's disease: an immunological and a molecular hypothesis. Regarding the immunological hypothesis, immunohistochemical studies have shown intense staining for TGF- β in this disease [25,26]. At high concentrations, TGF- β can inhibit the proliferation and migration of endothelial cells, attenuating angiogenic stimuli and limiting vessel growth [27e29]. A probable molecular explanation for the regression of blood and lymphatic vessels in Jorge Lobo's disease is the loss of extracellular matrix and degradation of elastic fibers induced during the inflammatory process, leading to detachment of the endothelium [30,31].

Expanding the interpretation of the results obtained, the loss of blood vessels can cause tissue hypoxia that leads to fibrosis and, depending on its intensity, to necrosis, histopathological features characteristic of Jorge Lobo's disease [13]. Since *L. loboi* inhabits soil, the fungus is probably well adapted to low-oxygen environments

Table 2

Distribuição do infiltrado celular na lesão de pacientes com doença de Jorge Lobo. N = number of cases % = Percentage.

Cellular infiltrate	Absent		Rare		Moderate		Intense	
	N	%	N	%	N	%	N	%
Macrophages	e	e	e	e	e	e	24	100
Finely granular macrophages	3	12.5	9	37.5	8	33.33	4	16.66
Giant cells	e	e	10	41.66	7	29.16	7	29.16
Lymphocytes	3	12.5	10	41.66	11	45.66	e	e
Eosinophils	19	79.16	4	16.66	1	4.16	e	e
Plasma cells	20	83.33	1	4.16	2	8.33	1	4.16
Neutrophil	23	95.83	e	e	1	4.16	e	e

Fig. 4. Immunohistochemical staining of vessels in the microvasculature injury during Jorge Lobo's disease. (A) marking of CD34 (arrows), showing the vessel endothelium. (B) labeling the antigen podoplanin (D2-40) (arrows) of the lymphatic endothelial specific. Magnification: (A) 100 $\times$ (B) 400 $\times$.

[2,32]. The loss of lymphatic vessels results in the accumulation of interstitial fluid which, in turn, interferes with the influx of dendritic cells, the establishment of an adequate adaptive immune response, and the maturation and migratory capacity of Langerhans cells, inhibiting these cells because its migrate to the site of infection via blood vessels [33].

The reduced vessel density in lesions of Jorge Lobo's disease may explain why dissemination to other organs is rare despite the large number of fungi in the lesion. The only case of dissemination was reported in Costa Rica in a man with lesions on the left leg for more than 47 years, which were associated with lymphangitis and a testicular tumor [34].

The present study also evaluated skin microvasculature function based on the detection of adhesion molecules on endothelial cells. It is known that microvascular endothelial cells at the site of inflammation are active components of the inflammatory process. These activated cells express adhesion molecules that play an important role in cell recruitment [35]. In this respect, expression of the adhesion molecules ICAM-1, VCAM-1 and E-selectin was investigated by immunohistochemistry and the density of vessels expressing these molecules was determined. The results showed a significantly larger number of vessels expressing ICAM-1 and VCAM-1, but not E-selectin, in lesions when compared to control, demonstrating the participation of these molecules in the recruitment of leukocytes to the site of infection in Jorge Lobo's disease. This expression pattern of adhesion molecules differs from that of infections caused by other fungi. In this respect, *Candida albicans* stimulates the expression of ICAM-1, VCAM-1 and E-selectin [36]. In infections caused by *A. fumigatus* hyphae, endothelial cells are stimulated to express E-selectin and VCAM-1, but not ICAM-1 [37]. These differences may reflect a fine regulation between these

molecules and cytokines and chemokines produced during infection, interfering directly with cell recruitment and with the local immune response.

Evidence indicates that the cell-mediated immune response is compromised in Jorge Lobo's disease [38,39]. This fact might be explained by the low expression of E-selectin in the lesions. E-selectin is known to mediate the initial contact of Th1 and Th17, but not Th2 cells, with the activated endothelium and is expressed at high levels in the lumen of the plasma membrane of vascular endothelial cells at the site of inflammation [40e42]. Thus, if the cell-mediated response is compromised, the activity of E-selectin will be limited.

Fig. 5. Total vessel density in the lesion during Jorge Lobo's disease. * $p < 0.05$ compared with the control. JLD: group of cases of Jorge Lobo's disease. NC: control group.

Fig. 6. The density of blood and lymphatic vessels of the lesion in Jorge Lobo's disease. * $p < 0.05$ compared with the control. JLD: group of cases of Jorge Lobo's disease. NC: control group.

Fig. 7. Immunohistochemical staining of cell adhesion molecules in the lesion during Jorge Lobo's disease. (A) Endothelial cells expressing ICAM-1 (B) endothelial cells expressing VCAM-1. (C) endothelial cells expressing E-selectin. Magnification 400 $\times$.

ICAM-1 and VCAM-1 were significantly present in a larger number of vessels when compared to control, with the observation of a higher percent expression of ICAM-1. The binding of ICAM-1 and VCAM-1 to integrins present on the surface of leukocytes permits strong adhesion of these cells to the endothelial layer of vessels. These molecules are important modulatory components of cellular traffic to peripheral tissues and lymphoid organs, promoting an effector immune response [43].

The present results indicate ICAM-1 to be an important component for leukocyte recruitment in Jorge Lobo's disease. A study on experimental infection with *Paracoccidioides brasiliensis* demonstrated the participation of ICAM-1 in leukocyte recruitment, contributing to the initial inflammatory infiltrate which mainly consisted of neutrophils and macrophages [44]. Another study evaluating the recruitment of CD4 $^{+}$ and CD8 $^{+}$ T cells in the lungs of mice infected with *P. brasiliensis* showed that ICAM-1 is involved in the control of fungal replication [45].

As mentioned earlier, intense production of TGF- β occurs in lesions of Jorge Lobo's disease. In human endothelial cells, the elevated level of TGF- β has been shown to inhibit the expression of E-selectin, but does not interfere with the expression of ICAM-1 or VCAM-1 [45]. TGF- β seems to actively participate in the pathogenesis of Jorge Lobo's disease by interfering with microvascular density in the lesions and with the expression of E-selectin, thus highlighting the vast importance of TGF- β . Quaresma et al. [46] already called attention to the effect of TGF- β on fibrogenesis and local immunodeficiency.

Taken together, the results of the present study indicate the occurrence of microvascular dysfunction in lesions of Jorge Lobo's

Fig. 8. Analysis of the expression of cell adhesion molecules in the lesion during Jorge Lobo's disease. (A) vessels expressing ICAM-1. (B) vessels expressing VCAM-1. (C) vessels expressing E-selectin. * $p < 0.05$ compared with the control. JLD: group of cases of Jorge Lobo's disease. NC: control group.

disease, which reflects on the clinical progression of the disease. The damaged skin contains a reduced density of vessels that seems to limit the fungus to the site of infection and interferes with adequate recirculation of cells and with the establishment of an adequate adaptive immune response. On the other hand, endothelial activation consisting of the expression of ICAM-1 and inhibition of E-selectin permits maintenance of the infectious process.

Therefore, immunohistochemical results of TGF- β in Jorge Lobo lesions already described in the literature [26,46] suggest a possible inhibition of the expression of E-selectin and the influx of immune cells at the site of infection, creating an immunosuppressive microenvironment characterized by a deficient cell-mediated immune response. In addition, endothelial activation, which is mainly demonstrated by the expression of ICAM-1 associated with the production of chemokines, is responsible for the marked presence of macrophages. Together with this process, TGF- β induces fibrosis that interferes with microvascular density, causing the destruction

Fig. 9. Percentage of expression of cell adhesion molecules in the lesion during Jorge Lobo's disease. * $p < 0.05$ compared with the control. % Percentage.

of vessels, especially lymphatic vessels, edema formation, tissue hypoxia and cell retention, as well as maintaining the fungus restricted to the site of infection.

The present study expands the view on the immunopathogenic events involved in the chronicity of Jorge Lobo's disease and, at the same time, highlights the importance of more in-depth research on this topic. There is a need to better understand the process of vessel density reduction, to better characterize the production of cytokines and chemokines, and to identify possible positive and negative regulators of angiogenesis and lymphogenesis produced in the local microenvironment.

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Conflict of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Ethical aspects

The study was approved by the Ethics Committee for the Analysis of Research Projects (Comissão de Ética para Análise de Projetos de Pesquisa e CAPPesq) of the Clinical Board of the University Hospital, FM-USP (Protocol No. 191/12). The study was conducted in accordance with Resolutions 196/96 and 347/05 of the National Health Council regarding the guidelines and standards for research involving humans.

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Research Article

Langerhans Cells Express IL-17A in the Epidermis of Chromoblastomycosis Lesions

Aline Alves de Lima Silva^{a, b} Paulo Ricardo Criado^b Ricardo Spina Nunes^b
Luciane Kanashiro-Galo^a Maria Irma Seixas Duarte^a Mirian N. Sotto^{a, b}
Carla Pagliari^{a, b}

^aLaboratório da Disciplina de Patologia de Moléstias Transmissíveis/Departamento de Patologia-Faculdade de Medicina da Universidade de São Paulo, and ^bDepartamento de Dermatologia – Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil

What Is It about?

Chromoblastomycosis (CBM) is an important disease in tropical regions and is responsible for much biopsychosocial damage in patients. Our study presented for the first time the coexpression of IL-17 and Langerin in dendritic cells in the epidermis and dermis of lesions, which is not seen in healthy patients. Dendritic cells in CBM play an important role both as antigen-presenting cells and in the elimination of fungi. The immune response is also marked by the imbalance in Th17 response. We hope that our data can contribute to a better understanding of the disease, leading to more effective therapies for CBM.

Keywords

Chromoblastomycosis · Skin lesions · Langerhans cells · Langerin · Th17

Abstract

Chromoblastomycosis (CBM) is a chronic fungal infection that affects skin and subcutaneous tissue, and little is known about the immunological aspects of such lesions. We have previously described the high expression of IL-17 in this group. Understanding the innate immune response of patients with CBM would improve the knowledge of its immunopathogenesis and contribute to the most appropriate therapies. Nineteen biopsies of verrucous form were obtained from patients with clinical and histopathological diagnosis of CBM, without treatment. This was done with a double immunostaining with conventional immunohistochemistry and immunofluorescence technique as well as confocal microscopy to detect Langerin and IL-17 expression. All of the specimens that were analyzed showed expression of Langerin in the epidermis – the same as the control group. However, only the CBM group presented cells expressing CD207 in the dermis. Interestingly, the coexpression of IL-17 and Langerin was visualized along the epidermis and dermis in 100% of the lesion group. We demonstrated for the

Aline Alves de Lima Silva, BSc
Universidade de São Paulo – Faculdade de Medicina
Av Dr Arnaldo, 455, sala 1118
São Paulo 01246-903 (Brazil)
E-Mail alinalves@usp.br

first time in situ coexpression of IL-17 and Langerin (CD207) in epidermal cells of patients with CBM and speculated on their role as IL-17-producing cells or whether they could be a new subpopulation of dendritic cells distinct from Langerhans cells.

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Introduction

Chromoblastomycosis (CBM) is a chronic fungal infection present worldwide and with remarkable incidence in tropical and subtropical countries [1]. The disease is caused by dimorphic dematiaceous fungi. The lesions usually affect the skin and subcutaneous tissue of the lower limbs of rural workers [1, 2].

Langerhans cells (LCs) constitute a subclass of dendritic cells of the skin and mucosa. They are characterized by the presence of Birbeck granules and Langerin (CD207), a specifically expressed C-type lectin receptor [3]. The role of Langerin in fungal infections is not fully elucidated. LCs can phagocytose *Fonsecaea pedrosoi* sclerotic cells and inhibit fungi hyphae formation [4].

LCs, as well as macrophages and FXIIIa+ dermal dendrocytes, play a role in fungi antigen presentation and immunomodulation in CBM human lesions [5]. Thus, at least in the pathogenesis of CBM, LCs seem to be involved both in innate and dynamic adaptive responses.

When Th1 and Th2 patterns of cytokines are ineffective, another pattern of immune response seems to get established as an immune booster: the Th17 cells. These are mostly CD4+ cells characterized by the expression of proinflammatory cytokines, especially IL-17 and IL-23 [6, 7]. Th17 lymphocytes have been detected as protagonists in chronic inflammatory microenvironments and there are currently many studies about their development and functions [6].

Recently, a significant presence of IL-17+ cells in human lesions of CBM has been shown. These cells could represent an attempt to help the primary immune profile against the fungi. However, it is possible that the marked imbalance between the effectors and regulatory profiles contribute to the ineffective response against verrucous CBM [8].

Interestingly, dendritic IL-17+ cells have been observed in the epidermis and dermal infiltrate in human CBM verrucous lesions [8]. In order to verify whether these IL-17+-expressing dendritic cells are in fact LCs, we studied CD207 and IL-17 coexpression by double immunolabeling CBM lesions.

Materials and Methods

Biopsies

Nineteen biopsies of CBM lesions were retrieved from the files of the Dermatopathology Laboratory (Division of Clinical Dermatology, Hospital das Clinicas, Faculty of Medicine, University of São Paulo). The biopsies were taken from patients with CBM verrucous lesions, sclerotic bodies at histopathological exam, and *F. pedrosoi* in culture (84.3% males, mean age 62.5 years old). Ten normal skin fragments constituted the control group.

The Ethics Committee of Clinical Hospital, Faculty of Medicine of University of São Paulo approved the study (protocol No. 0317/11).

Histopathological Analysis

All the hematoxylin-eosin-stained biopsies were reviewed in order to confirm the CBM diagnosis.

Immunohistochemistry

4- μ m paraffin-embedded fragments of each biopsy were deparaffinized and hydrated in ethanol, and subjected to antigen retrieval in TRIS/EDTA buffer pH 9.0 for 20 min at 95°C. The primary antibodies anti-IL-17 (clone IL-17A) and CD207 (clone 12D6) were diluted in 1% bovine albumin solution and incubated overnight at 4°C. This was followed by the second antibody incubation and streptavidin-peroxidase complex. The reaction was developed with 3,3-diaminobenzidine tetrahydrochloride (DAB) chromogen and the slides were counter-stained lightly with hematoxylin. All reactions were performed with positive (normal skin and palatine tonsil fragments) and negative controls. The negative controls of the reaction were obtained by replacing the primary antibodies by isotypic nonreagent antibodies.

Double Immunostaining with Conventional Immunohistochemistry and Immunofluorescence Technique and Confocal Microscopy

The initial procedures were similar as described above. The detection of Langerin+ cells was performed with DAB as described above, followed by incubation with anti-IL-17 antibody and a streptavidin-biotin phosphatase developing system and permanent red chromogen.

Briefly, the immunofluorescence technique protocol for confocal microscopy was performed as follows: the antibodies anti-IL-17 (clone IL-17A) and anti-Langerin (clone 12D6) were diluted in 1% bovine albumin solution and incubated for 48 h at 4°C. After this, isotype-specific secondary conjugated antibodies (Alexa Fluor 488 and Alexa Fluor® 546, Invitrogen, Carlsbad, CA, USA) were applied and nuclear staining with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; Sigma-Aldrich, Steinheim, Germany) was done for 60 min. The slides were mounted in mounting medium (Hydromount; National Diagnostics, Atlanta, GA, USA) and on glass coverslips, and maintained protected from light at 4°C until analysis. Fluorescent images were acquired using UV/laser excitation on a LSM/Meta 510 Zeiss microscope and LSM Image Examiner software (Carl Zeiss, Standort Gottingen, Germany) at the Confocal "Rede Premium" Multiuser Facility of the Heart Institute of São Paulo University.

Quantitative Analysis

The Langerin+ cells visualized through single-staining procedure were quantified by counting the positive stained epidermal area fraction using a $\times 10$ ocular lens with a square grid and a $\times 40$ objective [9]. The IL-17+ cells were quantified by counting the number of immunolabeled cells in 9 randomized high-power fields for each specimen with a $\times 10$ ocular lens with a square grid area of 0.0625 mm².

Statistical Analysis

The expression of CD207 both in CBM and control epidermis was compared using Graph Pad Prism version 5.00 for Windows (Graph Pad Software, San Diego, CA, USA) to perform the Mann-Whitney test with the level for significance set at 95% ($p \leq 0.05$).

Results

Histopathological Analyses

All the CBM lesions displayed epidermal hyperplasia with hyperkeratosis, parakeratosis, and acanthosis with irregular epidermal elongations. The dermis exhibited granulomatous infiltrate with neutrophils exudate foci, mainly in the granuloma center. There was variable number of dematiaceous yeast cells phagocytosed by giant cells or in granuloma exudative foci.

Expression of Langerin (CD207) and IL-17

All of the CBM specimens studied displayed CD207+ dendritic cells in the epidermis and among the dermal inflammatory infiltrate. The positive cells were distributed throughout the epidermis and also the dermis (Fig. 1a, c). The frequency of CD207+ cells varied from mild to moderate intensity. The normal skin biopsies displayed CD207+ dendritic cells among keratinocytes in the epidermis. In the dermis, these cells were very scant or even absent (Fig. 1b, d).

IL-17+ cells were observed in all CBM specimens. These cells were distributed in the dermis among the inflammatory infiltrate. The epidermis displayed few IL-17+ dendritic and spindle cells (Fig. 1e, g). Biopsies from the control group displayed only a few IL-17-expressing cells in the dermis (Fig. 1f, h).

Coexpression of CD207 and IL-17 through Double-Labeling Immunohistochemistry and Immunofluorescence Technique

The exam of biopsies subjected to both techniques displayed coexpression of CD207 and IL-17 in some epidermal and dermal dendritic cells in CBM lesions (Fig. 2a–g). None of the normal skin group displayed double-labeling cells in the epidermis or dermis.

Discussion

The skin is the first barrier to pathogens, both physically and immunologically, and in CBM it is also the structure by which the fungus presents tropism [1].

The immunological cascade begins with the capture and presentation of antigens, a masterful activity of LCs [10]. Langerin (CD207) is a marker of mature LCs [3]. Nineteen skin biopsies of verrucous type were analyzed and diagnosed as CBM by positive culture of *F. pedrosoi* and clinical analysis.

In CBM the immune response is mediated mainly by Th2 cytokines [11]. Previously we demonstrated that in CBM lesions a predominance of Th17 cells over Treg cells occurs [8]. The imbalance between the regulator and effector profiles could be a major contributor to the inefficiency of the immune response established in this mycosis.

The epidermal CD207 expression in CBM lesions was lower when compared to normal skin ($p = 0.0001$). Otherwise, 68.42% of CBM specimens displayed CD207-expressing cells among the dermal inflammatory infiltrate, and these cells were almost absent in the dermis of the normal skin group.

The presence of CD207+ cells in the CBM dermis led us to speculate that it may be due to migration of LCs in the way to present fungi antigens at the lymph nodes. There is speculation whether CD207-expressing cells are in fact LCs, or another population of dendritic cells, mainly in the dermal compartment [12]. Langerin-expressing cells were frequently observed in the dermis of patients affected by paracoccidioidomycosis [13]. CD207-positive dermal cells may exert a role in repopulation, which does not seem to be played by the LCs; furthermore, dermal Langerin+ cells usually grow up in microenvironments where LCs are absent [14].

Fig. 1. Immunoexpression of Langerin (CD207) and IL-17 in the chromoblastomycosis (CBM) skin lesion and control groups. **a, c** Expression of CD207 in epidermal cells in CBM lesions. **b, d** expression of CD207 in specimens of normal skin. **e, g** Note numerous immunoreactive IL-17A+ cells in the epidermis. They are of similar morphology as Langerin+ cells (compare **a** and **b**). The dermis also contains IL-17A+ cells, presumably T cells. **f, h** Expression of IL-17A+ cells in normal skin; note the presence of these cells in the dermis, which probably are sentinels of skin immune system (SIS) (streptavidin-biotin peroxidase in $\times 400$).

(For figure see next page.)

Fig. 2. Double immunostaining (conventional and confocal) demonstrating coexpression of Langerin/IL-17. Peroxidase-labeled streptavidin-biotin detection of Langerin was evidenced in brown staining; alkaline phosphatase-labeled detection of IL-17 was evidenced in red. Note the presence of immunostaining cells in the dermis (**a** and **b**, magnifications $\times 400$, details on **c** and **d**). **e** Detection of Langerin was evidenced in orange staining (546). **f** Detection of IL-17 was evidenced in green (488). **g** Demonstration of merge with nuclear staining in blue (DAPI) (magnifications $\times 200$). **h** Comparative analysis of Langerin among the chromoblastomycosis (CBM) and normal skin groups: distribution of number of cells/epidermal area fraction expressing Langerin. Mann-Whitney test, $*p < 0.0001$.

In addition to our previous report that demonstrated positive IL-17 dermal cells in the inflammatory infiltrates, a more detailed analysis of IL-17 expression in CBM lesions allowed us herein to identify the positive immunoreactivity of dendritic cells in the epidermis. The double-staining immunohistochemistry technique and immunofluorescence with confocal microscopy have shown that CD207-expressing cells in the epidermis coexpress IL-17 in CBM lesions.

IL-17 expression by CD207-positive dendritic cells was previously described by Coury et al. [15] in skin and bone lesions of LC histiocytosis. Blood monocytes of LC histiocytosis patients also produce IL-17 [16].

Similar to the results of Bursch et al. [12], we previously demonstrated that CBM lesions display low expression of TGF- β [8], which could reinforce the hypothesis that CD207-positive dermal cells are a new subpopulation of distinct dendritic cells and that, at least in our study, they seem to cooperate with the cascade of the Th17 immune response [12, 14].

In order to avoid misunderstandings, the analysis of our data took place at 3 different times: analysis of both IL-17 and CD207 markers through simple immunohistochemistry followed by the double immunohistochemistry technique and validation of the results with immunofluorescence technique and confocal microscopy.

The authors feel that the data presented herein may contribute to the understanding of the role of LCs as expressing IL-17 in the immunopathogenesis of CBM. In addition, the cells expressing CD207 could constitute new subclass of this lineage of dendritic cells.

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Ethics Statement

The use of the material that constituted the casuistry was approved by the Ethics Committee of Clinical Hospital, Faculty of Medicine of University of São Paulo (No. 0317/11).

Disclosure Statement

The authors have no conflicts of interest.

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