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JANNYSON JOSÉ BRAZ JANDÚ

O USO DE pCRAMOLL COMO TRATAMENTO DA CRIPTOCOCOSE EM CAMUNDONGOS IMUNOCOMPETENTES E IMUNOSSURIMIDOS

Recife

2019

JANNYSON JOSÉ BRAZ JANDÚ

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Tese apresentada ao Curso de Pós Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como pré-requisito para a obtenção do título de Doutor em Ciências Biológicas.

Área de concentração: Microbiologia

Orientadora: Profª Drª Maria Tereza dos Santos Correia
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(Paulicéia Desvairada ANDRADE, Mario 1987).

RESUMO

Historicamente, as doenças infecciosas são as principais causas de mortalidade e morbidade e refletem em alto impacto social e econômico em todo o mundo. O fenômeno da resistência microbiana é um fator importante para a alta prevalência dessas infecções e reduz exponencialmente as estratégias de tratamento disponíveis. Nesse contexto, há uma necessidade urgente de buscar novas abordagens terapêuticas, sendo a imunoterapia um meio eficaz de combate a essas doenças. Neste sentido, este trabalho aborda o efeito de lectinas de plantas como possíveis ferramentas imunomoduladoras para melhorar a resposta imune do hospedeiro contra microrganismos patogênicos. *Cryptococcus gattii* é um dos principais agentes infecciosos da criptococose em indivíduos saudáveis, mas também afeta indivíduos imunossuprimidos. Para o tratamento da infecção, drogas antifúngicas isoladas ou em combinação são usadas, no entanto, devido a toxicidade dessas drogas e o aumento de cepas resistentes impulsiona essa busca por terapias que sejam eficientes. Na revisão da literatura, observou-se que in vitro as lectinas possuem ações antimicrobiana e imunorreguladora, bem como em modelos experimentais in vivo, aumentando a sobrevida de camundongos sob desafio microbiano, seja profilaticamente ou terapeuticamente. Desta forma, o efeito de pCramoll, uma lectina imunorreguladora extraída de sementes de Cratylia mollis Mart [planta endêmica de Caatinga (região semi-árida do Nordeste)], foi demonstrado em modelo de infecção intratraqueal por *Crytococcus gattii*. No desafio microbiano projetado, a infecção por *C. gattii* e os tratamentos propostos (pCramoll 1ug / dose isoladamente ou em associação com fluconazol 200 mg.kg-1), foram analisados parâmetros como carga fúngica nos órgãos-alvo e a quantificação nos níveis dos fatores inflamatórios envolvidos na doença. O tratamento com pCramoll aumentou *in vitro* a fagocitose de *C. gattii* pelos macrófagos medulares após 3h de incubação, associada à alta produção de espécies reativas de oxigênio e nitrogênio, e diminuiu a taxa de proliferação fúngica intracelular. *In vivo*, pCramoll isoladamente ou em combinação com fluconazol aumentou a sobrevida e melhorou a morbidade dos animais infectados, com benefícios em aspectos como (estado neuropsiquiátrico, comportamento motor, função autonômica, tônus e força muscular e função reflexa / sensorial), modulando os níveis de IFN-γ, IL-6, IL-10 e IL-17 e o infiltrado inflamatório durante o processo infeccioso. Diante desse potencial imunorregulatório apresentado pelo pCramoll na luta contra a criptococose, observou-se a influência da lectina no tratamento da infecção por *C. gattii* em camundongos após imunossupressão iatrogênica sob a combinação de Ciclofosfamida 200 mg.kg-1 e 5-Fluoroacil 150 mg. kg-1, o tratamento com pCramoll reduziu a carga fúngica nos pulmões e cérebros dos animais e, consequentemente, aumentou a taxa de sobrevivência (100% de

sobrevivência) dos animais imunossuprimidos em comparação com os imunossuprimidos, infectados e não tratados. Portanto, foi possível demonstrar que o uso de lectinas de plantas, como o pCramoll, são ferramentas promissoras no desenvolvimento de novas estratégias terapêuticas baseadas em imunomoduladores no combate a microrganismos infecciosos de difícil controle antimicrobiano, como os agentes da criptococose *C. gattii*.

PALAVRAS-CHAVE: *Cryptococcus gattii.* Lectinas. *p*Cramoll. Imunomodulação. Imunossupressão.

ABSTRACT

Historically, infectious diseases are the main causes of mortality and morbidity and reflect in high social and economic impact worldwide. The phenomenon of microbial resistance is an important factor for the high prevalence of these infections, and exponentially reduce the available treatment strategies. In this context, there is an urgent need to search new therapeutic approaches, and the immunotherapy is an effective way of to combat these diseases. In this sense, this work approaches the effect of plant lectins as possible immunomodulatory tools to improve host immune response against pathogenic microorganisms. *Cryptococcus gattii* is a major infectious agent of cryptococcosis in healthy individuals, but it also affects immunosuppressed individuals. For the treatment of infection, antifungal drugs alone or in combination are used, however, to the toxicity of these drugs and the increasing appearance of resistant strains drives this search for therapies that are efficient. In the literature review, it was observed that *in vitro* the lectins have antimicrobial and immunoregulatory actions, as well as in experimental models, *in vivo,* increasing the survival of mice under microbial challenge, either prophylactically or therapeutically. This way, the effect of pCramoll, an immunoregulatory lectin extracted from seeds of *Cratylia mollis* Mart [endemic plant from Caatinga (Northeastern Semi-arid region)], was demonstrated in a model of intratracheal infection by *Crytococcus gattii*. In the microbial challenge projected, the infection by *C. gattii* and the proposed treatments (pCramoll 1ug / dose alone or in association with fluconazole 200 mg.kg-1), were analysed parameters as fungal load in the target organs and quantification in the levels of the inflammatory factors involved in the disease. pCramoll treatment increased *in vitro* the phagocytosis of *C. gattii* by marrow macrophages after 3h of incubation, associated with high production of reactive oxygen and nitrogen species, and decreased intracellular fungal proliferation rate. In vivo, pCramoll alone or in combination with fluconazole increased survival and improved morbidity of infected animals, with benefits in aspects such as (neuropsychiatric state, motor behavior, autonomic function, tonus and muscle strength, and reflex / sensory function), modulated the levels of IFN-γ, IL-6, IL-10 and IL-17 and the inflammatory infiltrate during the infectious process. In view of this immunoregulatory potential presented by pCramoll in the fight against cryptococcosis, it was observed the influence of lectin in the treatment of *C. gattii* infection in mice after iatrogenic immunosuppression under Cyclophosphamide 200 mg.kg-1 and 5 -Fluoroacyl 150 mg.kg-1 combination, the pCramoll therapy reduced the fungal load on the lungs and brains of the animals, and consequently increased survival rate

(100% survival) of immunosuppressed animals to compared with immunosuppressed, infected and untreated. Therefore, it was possible to demonstrate that the use of plant lectins such as pCramoll are promising tools in the development of new therapeutic strategies based on immunomodulators in the fight against infectious microorganisms with difficult antimicrobial control such as cryptococcosis agents.

KEY WORDS: *Cryptococcus gattii.* Lectins. PCramoll. Immunomodulation. Immunossuppression.

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FUNDAMENTAÇÃO TEÓRICA

SUMÁRIO

1 INTRODUÇÃO

Cryptococcus gattii e *Cryptococcus neoformans* são os principais agentes da criptococose, uma infecção de fonte exógena, que ocorre pela inalação de esporos ou leveduras dessecadas, e cujas manifestações clínicas variam desde doença pulmonar (restrita aos pulmões), até meningoencefalite (acometimento do Sistema Nervoso Central) (CHEN et al., 2000). Anteriormente, a criptococose, principalmente causada por C. *gatti*, era considerada uma doença de ocorrência restrita a regiões tropicais e subtropicais, relacionadas a espécies vegetais onde os fungos eram encontrados (HEITMAN et al., 2011). Entretanto, ao passar dos anos, evidências mostram casos de criptococose na Ásia, Europa, África, e áreas da América do Norte, ou seja, em diferentes zonas climáticas e variadas vegetações (GALANIS et al., 2010).

A criptococose pode ocorrer como uma infecção primária em imunocompetentes ou como uma infecção oportunista em indivíduos imunossuprimidos, por exemplo, indivíduos HIV positivos ou que apresentam fatores que predispõem à infecção, como: terapias prolongadas com corticoesteróides, uso de imunossupressores em transplantados, câncer, lupus eritematoso sistêmico, diabetes mellitus ou processos infecciosos primários. Condições fundamentais para o estabelecimento da infecção criptocócica e progressão da doença (COSTA et al., 2016).

As principais terapias para o tratamento da criptococose são fluconazol (antifúngico azólico) e anfotericina B (antifúngico do tipo poliênico) (WORASILCHAI et al., 2017). O fluconazol é usado em casos de doença pulmonar com sintomas leves, enquanto nas infecções graves, a anfotericina B é recomendada (associada ou não com o 5-flucitosina), seguido de uma terapia prolongada com fluconazol. O itraconazol, voriconazol e outros azólicos são recomendados quando o uso do fluconazol está contraindicado ou é ineficaz (PERFECT et al., 2010). A resistência aos antifúngicos realça falhas clínicas, como prescrições inadequadas, o que aumenta a morbidade e mortalidade dos indivíduos infectados (GHANNOUM; RICE, 1999; ZHAI et al., 2013), além disso, os efeitos colaterais devido ao uso de anfotericina B incluem a nefrotoxicidade associada ao aumento da mortalidade, exigindo acompanhamento da função renal dos pacientes. Nesse contexto, a busca de novos produtos com ação antimicrobiana ou imunomoduladora são ferramentas alternativas no combate à infecção.

Vale-se compreender que lectinas são proteínas de origem não imune, ubiquamente encontradas na natureza, e que possuem a capacidade de se ligarem especificamente a

carboidratos e, em sua maioria, de aglutinar células (SHARON, 2008). *Cratylia mollis* Mart. é uma planta endêmica na Região do Semi-Árido pernambucano, e popularmente conhecida como feijão camaratuba ou camaratú. pCramoll corresponde a uma mistura deduas isoformas de lectinas purificadas de sementes de *C. mollis* por Correia e Coelho (1995), e que apresenta ações biológicas diversas, como imunomoduladora (MACIEL et al., 2004), antiparasitária (MELO et al., 2011a), e cicatrizante (MELO et al., 2011b). Adicionalmente, foi empregada na marcação de tecidos cancerígenos (LIMA, 2010), e no desenvolvimento de sensores para detecção de sorotipos do vírus da dengue (OLIVEIRA et al., 2011).

No Brasil, a criptococose (oportunista) por *C. neoformans* predomina nas regiões Sul, Sudeste e Centro-Oeste. Nas regiões Norte e Nordeste, a infecção primária por *C. gattii* é predominante em indivíduos saudáveis (CHANG et al., 2004). Contudo, dos indivíduos com criptococose e diagnosticados no nordeste brasileiro, 53% dos imunocompetentes e 87,5% dos HIV positivos foram acometidos por *C. gattii* (NISHIKAWA et al., 2003). Portanto, há uma necessidade de melhor entendimento acerca dos processos envolvidos na criptococose, como também, de incentivar o uso de novos produtos como terapias alternativas que diminuam essa alta frequência e os altos níveis de mortalidade devido à infecção (NISHIKAWA et al., 2003). Nesse sentido, este trabalho objetiva avaliar o uso de uma lectina de planta (pCramoll) como uma nova estratégia terapêutica frente a criptococose em animais imunocompetentes como imunossuprimidos, uma vez que, produtos naturais como as lectinas apresentarem ação imunomoduladora, e são possíveis ferramentas terapêuticas frente a doença.

Assim, a pesquisa desenvolvida para responder os questionamentos e objetivos propostos resultaram em três artigos apresentados nessa tese na forma de anexos e apêndice. O primeiro Artigo, intitulado Targeting the immune system with plant lectins to combat microbial infection (Anexo A) foi publicado na revista Fronties in Pharmacology, o segundo artigo, intitulado Treatment with pCramoll alone and in combination with fluconazole provides therapeutic benefits in *C. gattii* infected mice (ANEXO B) foi publicado na revista Fronties in Cellular and Infection in Microbiology, por fim, o terceiro artigo será submetido a publicação em uma revista da área sendo previamente intitulado pCramoll therapy decrease mortality and fungal burden in imunosuppressed mice infected with *Cryptococcus gattiii* (APÊNCIDE), salienta-se ainda que outros trabalhos acadêmicos foram desenvolvidos durante o doutoramento, .porém não fizeram parte dessa tese.

1.1 OBJETIVOS

1.1.1 Objetivo Geral

Avaliar o efeito de pCramoll frente criptococose em animais imunocompetentes e imunossuprimidos.

1.1.2 Objetivos Específicos

- Realizar revisão bibliográfica acerca do uso de lectinas de plantas frente infecções microbianas, principalmente fúngicas;
- Avaliar a influência da pCramoll na fagocitose de *C. gattii* por macrófagos murinos sob diferentes concentrações da lectina.
- Analisar parâmetros como análise morfométrica de macrófagos infectados por *C. gattii*, a produção de espécies reativas de oxigênio/nitrogênio resultantes da interação fungo-hospedeiro e quando estimuladas com pCramoll;
- Avaliar a progressão da criptococose e determinar a concentração/dose da pCramoll para o tratamento da infecção;
- Observar aspectos comportamentais na progressão da criptococose em camundongos e tratados com pCramoll, e associada ao fluconazol;
- Determinar carga fúngica nos órgãos alvos, como pulmão e cérebro, e fatores inflamatórios envolvidos na doença e após tratamento com pCramoll, e associada ao fluconazol em animais imunocompetentes.
- Avaliar a influência da pCramoll na criptococose em animais imunossuprimidos através da determinação da taxa de subrevida;
- Determinar a da carga fúngica no pulmão e no cérebro de animais imunossuprimidos infectados com *C. gattii*, e tratados compCramoll;

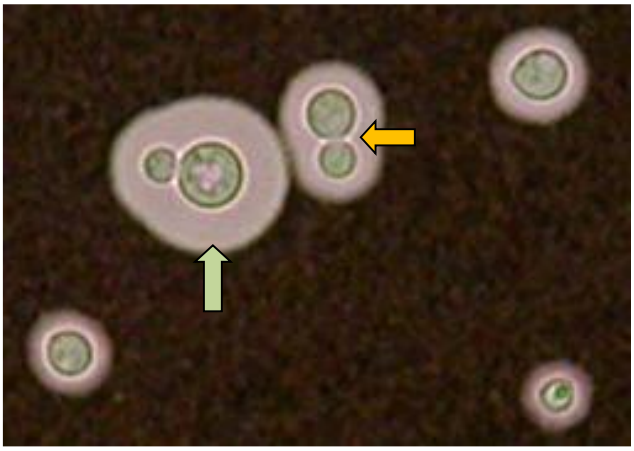
1 REFERENCIAL TEÓRICO

2.1 *CRYPTOCOCCUS GATTII*

O gênero *Cryptococcus* pertence ao Filo Basidiomicota, e compreende mais de 70 espécies distintas. Duas espécies, *C. gattii* e *C. neoformans*, são importantes patógenos responsáveis por causar a maioria dos casos de criptococose em humanos, levando à doença pulmonar e meningoencefalite, com substancial índice de mortalidade (MC MULLAN et al., 2013).

A levedura *C. gattii* realiza reprodução predominantemente assexuada (anaforma), caracterizada por apresentar células com brotamento único ou múltiplo, ovais ou cilíndricas, geralmente envolvidas por uma cápsula polissacarídica **(Figura 1).** Esse patógeno é considerado emergente primário, uma vez que acomete principalmente indivíduos saudáveis, embora também possa infectar indivíduos imunocomprometidos (CHATURVEDI; CHATURVEDI, 2011; SHARON et al., 2012).

Figura 1 - Células de *Cryptococcus gattii* isoladas e unibrotantes capsuladas contrastadas com tinta nanquim.



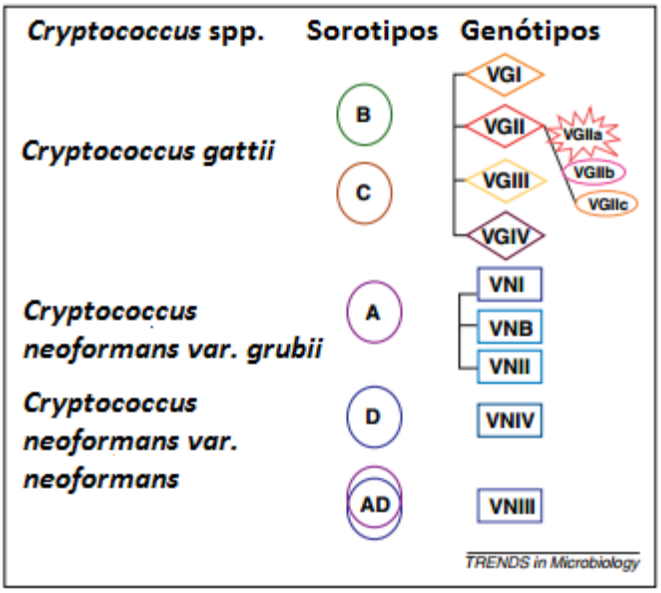
Fonte: MAGALHÃES; CARNEIRO, 2015.
Seta Verde – Cápsula polissacarídica, Seta Amarela – Reprodução Assexuada – brotamento

Inicialmente, *C. gattii* foi considerado uma variação do complexo *C. neoformans*, sendo essa descrita como uma espécie única com alguns sorotipos variantes (KWON-CHUNG et al., 2006), de modo que, quando descrito, baseando-se em análises morfológicas a partir de uma cepa isolada de um paciente com leucemia, na África em 1970, o isolado de *Cryptococcus* sp. apresentou forma leveduriforme elipticamente distinta do *C. neoformans*, o que introduziu as variações taxonômicas do complexo *C. neoformans* (SHARON et al., 2012). Assim, foram descritas três variantes,

C. neoformans var. grubii (sorotipo A), *C. neoformans var. neoformans* (sorotipo D) e *C. neofornas var. gattii* (sorotipo B e C). Posteriormente, por estudos fenotípicos com o uso de anticorpos monoclonais para antígenos da cápsula e técnicas moleculares para avaliações filogenéticas, *C. gattii* foi separado do complexo *C. neoformans*, sendo considerada outra espécie do gênero *Cryptococcus* (KWON-CHUNG et al., 2006; KWONG-CHUNG et al., 2002).

A partir de comparações de fragmentos de sequências de múltiplos genes, amplificação de segmentos de polimorfismos e outras técnicas modernas de tipagem gênica (NGAMSKULRUNGROJ et al., 2012), foram identificados quatro tipos moleculares de *C. gattii*, VGI, VGII, VGIII e VGIV **(Figura 2),** que divergem evolutivamente em até 12,5 milhões de anos (BOVERS et al., 2007). Esses genótipos apresentam subtipos como: VGIIa, VGIIb e VGIIc, que foram diferenciadas métodos de caracterização gênica, dados de prevalência, condições ambientais e fatores de virulência, apresentando também distintas ocorrências geográficas (SIDRIM et al., 2010; MAYER et al., 2011; COGLIATI et al., 2013).

Figura 2 - Diferentes sorotipos e genótipos de *Cryptococcus gatti, C. neoformans var. grubii* e *C. neoformans var. neoformans.*



Fonte: Adaptado de CHATURVEDI; CHATURVEDI, 2011.
As estirpes de *C. gattii* são serotipo B ou C, as estirpes do serótipo BC foi raramente relatado. As estirpes de *C. neoformans* da var. *grubii* são sorotipo A, enquanto var. *C. neoformans* linhagens neoformans são sorotipo D. A taxonônomia das estirpes de sorotipo AD não são bem esclarecidas. Os genótipos de *C. gattii* e C. neoformans são baseados após digitação de sequência multilocus de vários fragmentos de genes.

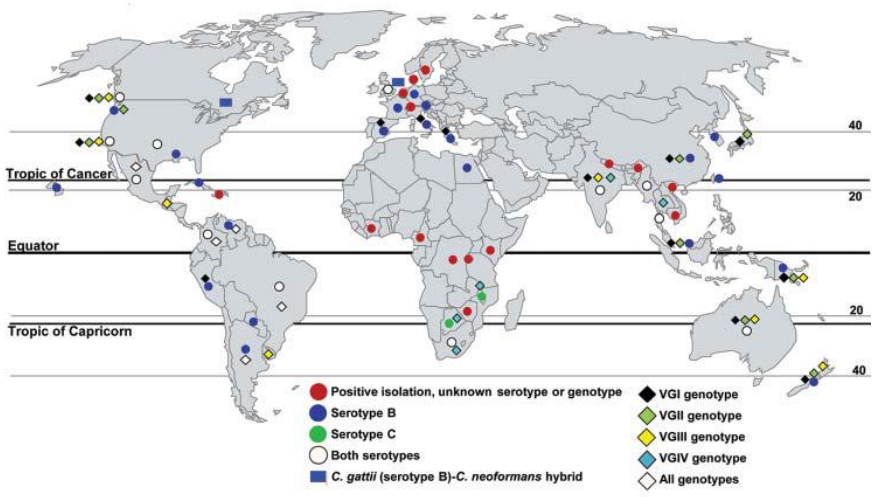
2.1.1 Epidemiologia

O primeiro isolamento ambiental de *C. gattii* sorotipo C foi em 1988, em amendoeiras; sendo o sorotipo B identificado pela primeira vez em 1990, por Ellis e Pfeiffer, de árvores de Eucalipto na Colômbia (CHEN et al., 2014), sendo esse sorotipo o mais prevalente em amostras clínicas e ambientais no mundo hoje.

A ocorrência de criptococose por *C. gattii*, inicialmente, era restrita a regiões tropicais e subtropicais, sendo posteriormente observados genótipos da espécie na América do Norte, a partir de um surto de criptococose na ilha de Vancouver, Canadá, em 1999 (CANNON et al., 2009; VOELZ et al., 2010) que se disseminou para a região Nordeste do Canadá, como também, para regiões dos Estados Unidos (BARTLETT et al., 2013; BILLMYRE et al., 2014). Além disso, infecções por *C. gattii* também foram identificadas em países com variados climas como França, Itália, Espanha e Grécia (NGAMSKULRUNGROJ et al., 2012). Ademais, espécies de *C. gattii* têm sido causa de criptococose também na Ásia, África e Oceania, tornando-se um fungo de ocorrência global (CHATURVEDI et al., 2011; CHEN et al., 2014).

Essa distribuição global do patógeno a partir de fontes clínicas, isolamento de *C. gattii* ambiental, e amostras biológicas mostram um agente infecioso emergente **(Figura 3)**, pois, evidencia-se que, por exemplo, na Austrália, a infecção é endêmica e a incidência anual estimada é de 610.000 pessoas, ao ano, acometidos por *C. gattii*, sendo o VGI o tipo molecular mais comum (COGLIATI, 2013).

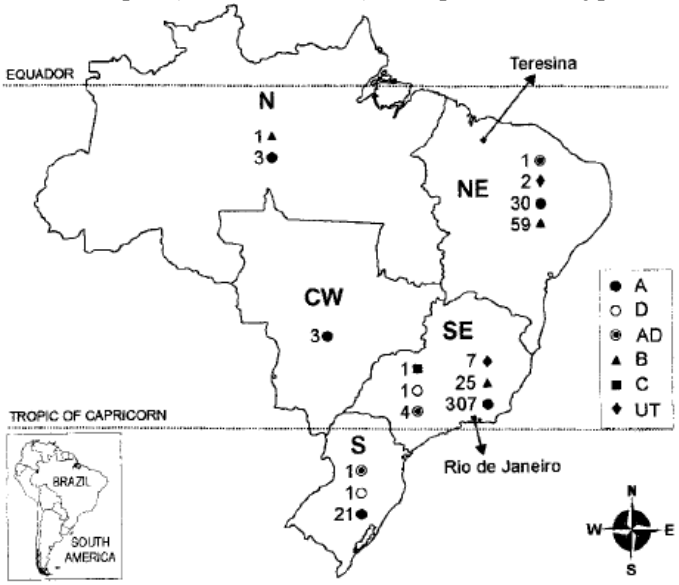
Figura 3 - Distribuição global e epidemiológica dos sorotipos e genótipos de *Cryptococcus gattii*.



Fonte: Adaptado de SPRINGER; CHATURVEDI, 2010.

Em um total de 223.100 casos de meningite criptocócica que foram estimados como tendo ocorrido globalmente em HIV positivos em 2014, o terceiro maior número de casos no mundo foi na América Latina, com uma incidência estimada de 5.300 casos por ano. Destes, Brasil e Colombia foram os países com maior incidência, entre 1.001 a 2.500, seguidos pela Argentina e México com incidência de 501 a 1.000 casos (RAJASINHGAM et al., 2017). Assim, no Brasil, tem sido reportada a incidência de ambos os sorotipos, sendo o VGII o predominante em pacientes HIV negativos; na Argentina há uma maior prevalência do sorotipo VGI; e na Colômbia já foram isolados todos os sorotipos. Na América do Norte, isolados do solo e ambientais são, na maioria, VGIIa e VGIIb, tendo esses tipos alta frequência, também, entre os isolados clínicos. Na Europa, África, e Ásia, a presença de *C. gattii* também foi reportada, e todos os sorotipos evidenciados sem haver predominância sob a frequência dos tipos moleculares encontrados (Mc MULLAN, 2013). Ainda no Brasil, espécies do gênero *Cryptococcus* foram isoladas em regiões geográficas distintas, tais como Bahia, São Paulo, Mato Grosso do Sul, Goiás, Rio de Janeiro e Rio Grande do Sul (LEITE et al., 2012; CHEN et al., 2014), sendo o Norte e Nordeste brasileiro regiões endêmicas. De acordo com um estudo realizado no Estado do Pará no período de 2003 a 2007, dos 56 isolados de *Cryptococcus* spp. que causaram criptococose disseminada, 50% eram *C. gattii*, dentre esses, 44,65% pertencem ao sorotipo VGII e 5,26% ao VGI (SANTOS et al., 2008).

Figura 4 - Distribuição de sorotipos (A, B, C, D, AD) de espécies de *Cryptococcus* no Brasil.



Fonte: Adaptado de NISHIKAWA et al., 2003.

Ademais, Nishikawa e coladoradores, em 2003, através da sorotipagem de 467 isolados ambientais e clínicos mostraram o perfil de incidência de espécies de *Cryptococcus* no Brasil, e dos isolados de *C. gattii*, o sorotipo B foi predominantemente incidente no Nordeste brasileiro **(Figura 4)**.

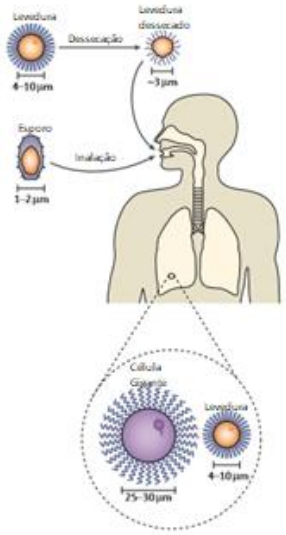
Segundo o manual de vigilância da criptococose 2012 pelo Ministério da Saúde, a criptococose ocorre como principal manifestação fúngica oportunista em cerca de 4,4% dos casos de AIDS no Brasil, com uma taxa de letalidade cerca de 35 a 40%. Nas regiões Sul e Sudeste há um predomínio da doença associada a AIDS em indivíduos do sexo masculino, causada principalmente pelo *C. neoformans*, e estima-se a prevalência da criptococose associada a AIDS ente 8 a 12% nos centros de referência da região sudeste, sendo os casos por *C gattii* de ocorrência esporádica nessa região. Por outro lado, nas regiões Norte e Nordeste do Brasil predominam casos de criptococose em indivíduos sem evidencia de imunossupressão, tanto no sexo masculino quanto o feminino, HIV-negativos, causados pelo *C. gattii*, com elevada morbidade e letalidade, cerca de 37 a 49% dos casos, sendo uma infecção endêmica nessa região (Ministério da Saúde, 2012).

Portanto, sendo uma doença de maior prevalências nas regiões tropicais, recentemente foi demonstrado que as variações climáticas no Brasil influenciam na quatidade de hospitalizações por infecções fúngicas, tendo maior número de casos em concomitância com o fenômeno La niña, a criptococose é uma das quatro micoses sistêmicas de maior impacto no país, juntamente à coccidioidomicose, histoplasmose e paracoccidioidomicose (SILVA et al., 2019).

2.2 CRIPTOCOCOSE

A criptococose é adquirida pela inalação de basidiósporos produzidos durante o ciclo sexuado (teleoformo) do fungo, ou leveduras dessecadas que se depositam nos alvéolos, acometendo inicialmente o pulmão, onde o fungo encontra fatores favoráveis para proliferação, e via corrente sanguínea dissemina-se para outros órgãos (DATTA et al., 2009).

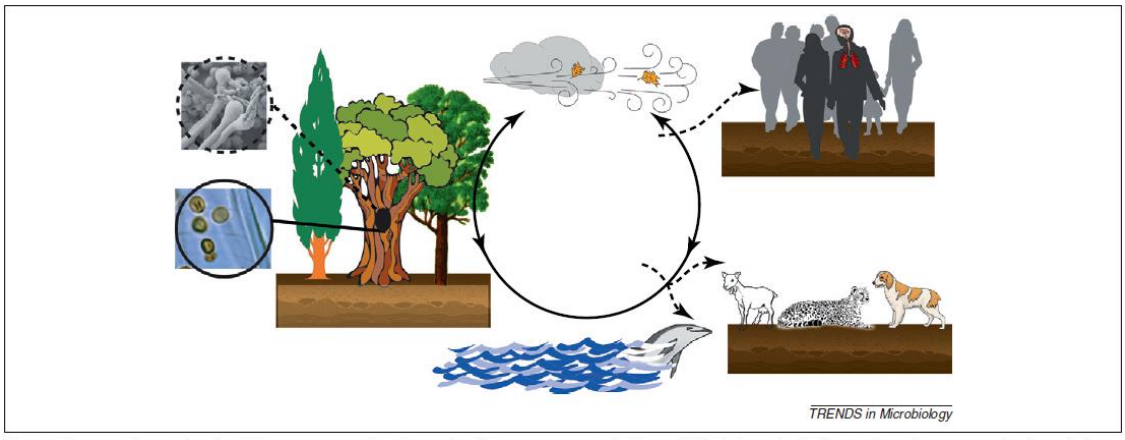
Figura 5 - Tipos de células morfologicamente distintas contribuem para a virulência do *Cryptococcus* ssp. e influenciam o processo de patogenicidade.



Fonte: Adaptado de KRONSTAD et al., 2011.

Os esporos e as leveduras dessecadas, presentes no ambiente, são propágulos altamente infecciosos por serem pequenos (1-3 µm), principalmente, em relação àquelas crescendo ativamente em ambientes favoráveis (4-10 µm), grandes demais para invadir os alvéolos, mas fundamentais no estabelecimento da infecção e no desenvolvimento de estruturas de resistência como as células gigantes (25-30 µm) **(Figura 5)**, favorecendo o processo infeccioso (KRONSTAD et al., 2011). Desse modo, como demostrado pelo ciclo reprodutivo **(Figura 6)**, os fungos que naturalmente habitam árvores, principalmente eucaliptos, podem se propagar pelo ambiente sem qualquer hospedeiro intermediário e infectar diversos animais (domésticos ou silvestres) e humanos.

Figura 6 - Inalação de estruturas fúngicas para acometimento pulmonar e desenvolvimento de Criptococose.



Fonte: CHATURVEDI;CHATURVEDI, 2011.
O ciclo natural do *Cryptococcus gattii* indica que o patógeno pode estar presente em plantas, solo, ar e água sem hospedeiros vivos intermediários (linha sólida com setas). Levedura de *C. gattii* também foram repetidamente isoladas de animais (domésticos e animais selvagens) e humanos que entram em contato com propágulos fúngicos (células de leveduras ou esporos sexuais) - (linhas pontilhadas com setas) muitas vezes assintomáticos, mas uma pequeno número de humanos e animais infectados desenvolve infecções graves nos pulmões e no cérebro.

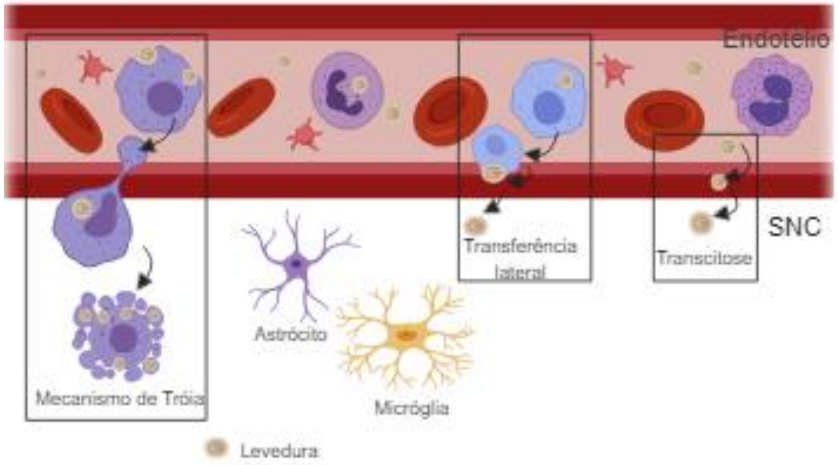
Além desses aspectos morfoambientas, os fatores de virulência também auxiliam a invasão, o parasitismo e o posterior estabelecimento da criptococose, sendo esses, a formação da cápsula polissacarídica a 37 ºC, produção de melanina, superexpressão de enzimas como fosfolipase B e urease, que na interação patógeno-hospedeiro são fundamentais na determinação do curso da doença (KRONSTAD et al., 2011). O sistema imune busca defender-se frente esses fatores, levando à formação de um complexo linfonodal pulmonar primário, que na maioria dos casos, produz uma infecção pulmonar assintomática autolimitada. As leveduras, deste modo, permanecem latentes dentro desse complexo granulomatoso ou são eliminadas, já que na ocorrência de imunossupressão podem ser reativadas e desenvolver a doença (CHIKUMOTO et al., 2019).

Nos casos em que os indivíduos apresentam baixa imunidade, a inalação de uma grande quantidade de fungo, ou o acometimento por determinados sorotipos de *C. gattii* altamente virulentos, como o VGII, podem apresentar sintomas pulmonares característicos, conduzindo para quadros de pneumonia grave e o estabelecimento da infecção (CHATURVEDI; CHATURVEDI, 2011; NEGRONI, 2012). Com a progressão da doença, para que alcance o sistema nervoso central (SNC), as leveduras precisam passar pela barreira hematoencefálica (BHE), levando aos casos de meningite ou meningoencefalite, que são as formas mais graves da doença. Os principais sinais e sintomas nessa fase da doença são: cefaleia, febre, neuropatia craniana (danos nos nervos cranianos), alteração da consciência, letargia (perda temporária ou completa da sensibilidade do movimento), perda de memória, sinais de irritação meníngea e coma (NADROUS et al., 2003).

O mecanismo pelo qual as leveduras penetram a BHE não é totalmente entendido, embora várias possibilidades são propostas, que incluem: a passagem direta entre células endoteliais próximas (entrada paracelular), a migração para o SNC dentro de fagócitos infectados (modelo cavalo de tróia), ou por absorção e travessia através de

células endoteliais que revestem os vasos sanguíneos no cérebro (transcitose) **(Figura 7)** (CHANG et al., 2004; CHALIER et al., 2009). Diversos fatores do patógeno modulam a interação *Cryptococcus*-BHE e estão envolvidos nessa transmigração para o SNC como urease, lacase, cápsula e ácido hialurônico (EISENMAN; CASADEVALL; MCCLELLAND, 2009; HUANG et al., 2011).

Figura 7 - Estratégias utilizadas por *Cryptococcus* para atravessar a barreira hematoencefálica: Cavalo de Tró
ia; Transferência Lateral; Transcitose



Fonte: O autor.

Esse tropismo para o SNC está relacionado à alta concentração de substratos assimiláveis pelo fungo no líquor como tiamina, ácido glutâmico, carboidratos e minerais, além da presença abundante de catecolaminas, que são fundamentais para síntese de melanina pelo fungo. Sendo essa preferência pelo SNC, ainda, um "refúgio" contra a resposta imune do hospedeiro (KIM, 2008).

2.2.1 Interação *Criptococcus*-Hospedeiro

A importância clínica dos patógenos oportunistas tem aumentado cada vez mais nas últimas décadas, principalmente após endemia da AIDS. *Cryptococcus* é um agente comum nas infecções fatais em indivíduos imunodeprimidos, entretanto, casos de criptococose em indivíduos hígidos são recorrentes, como o surto que ocorreu em Vancouver-Canadá (CHATURVEDI et al, 2011). Esse curso epidemiológico da criptococose revela uma expressiva influência do sistema imune frente ao patógeno e no desenvolvimento da doença.

Há vários fatores da resposta imune inata que interferem no estabelecimento de infecções, por exemplo, barreiras físicas (pele e mucosa), fatores séricos (sistema complemento) e moléculas (peptídeos) com ação antifúngica que são estratégias primárias fundamentais nos mecanismos de respostas inespecíficas frente aos agentes infecciosos de modo geral (VOELZ et al., 2010).

É válido pontuar que o sistema complemento é uma cascata antipatógenos, que uma vez ativada, por via clássica, de lectinas ou alternativa, converge na formação da C3 convertase. Essa enzima quando clivada auxilia na opsonização de patógenos, facilitando a fagocitose e aumentando a resposta inflamatória, estimulando a quimiotaxia de mais fagócitos contra diversos patógenos, incluindo os fungos. Diante disso, estudos em modelos animais *Knockout*, por exemplo, mostram que a ausência de fatores do sistema complemento diminui a sobrevida e prejudica a eliminação das cepas de espécies de *Cryptococcus*. Ademais, mecanismos de resistência desenvolvidos por esses fungos, como a formação da cápsula polissacarídica, também impedem sua fagocitose e inibem a via clássica de ativação do sistema complemento (VOELZ et al., 2010), sendo assim necessário, o orquestramento desses fatores para o desenvolvimento de uma resposta mais efetiva no combate a esses agentes.

Observa-se, também, que a fagocitose é o principal mecanismo efetor para inativação ou eliminação das leveduras, que, ao encontrarem macrófagos alveolares ou células dendríticas desencadeiam uma resposta imune. Assim, culmina com esterilização ou, mais frequentemente, na restrição da infecção dentro de granulomas inflamatórios a partir da reação tecidual, levando à formação de estruturas compostas principalmente por fagócitos mononucleares, histiócitos e células gigantes multinucleadas que envolvem as células fúngicas (SHIBUYA et al., 2005).

Dentre esses mecanismos de defesa, os macrófagos e as células dendríticas reconhecem Padrões Moleculares Associados aos Patógenos (PAMPs) por meio de seus receptores, e possuem papel relevante e paradoxal no decorrer da infecção, atuando para o controle e permanência da doença (MCQUISTON; WILLIAMSON, 2009). Estudos revelaram que a depleção dos macrófagos diminui a sobrevivência de animais com criptococose, como em outras micoses sistêmicas (OSTERHOLZER et al 2009). Contudo, foi demonstrado, também, que os macrófagos são importantes para que o *Cryptococcus*, especialmente, se dissemine pelo organismo, principalmente para o SNC, pois esse fungo tem a capacidade de sobreviver e reproduzir-se dentro dos fagócitos, o

que torna seu papel paradoxal diante da infecção por *Cryptococcus* spp. (KECHICHIAN, 2007). O *C. neoformans,* por exemplo, não inibe a formação de fagolisossomas, sendo capaz de replicar-se no seu interior; dentro desse compartimento, a levedura é exposta a baixo pH, EROs, RNS e carência nutricional, tendo que vencer todos esses obstáculos (SHOHAM; LEVITZ, 2005). Para isso, *Cryptococcus* spp. aumenta a expressão de genes de resposta à carência nutricional e de estresse oxidativo, entre outros mecanismos de sobrevivência (DERENGOWSKI et al., 2013).

Existem quatro famílias de Receptores de Reconhecimento de Patógenos (PRRs) que desencadeiam a ativação do sistema imunológico: os receptores do tipo Toll (TLR), os receptores de Domínios de Ligação e Oligomerização de Nucleotídeos (NOD - NLR), os receptores do tipo Ácido Retinóico Induzível do Gene 1 (RIG-I RLR) e os receptores de lectina tipo C (CLR).

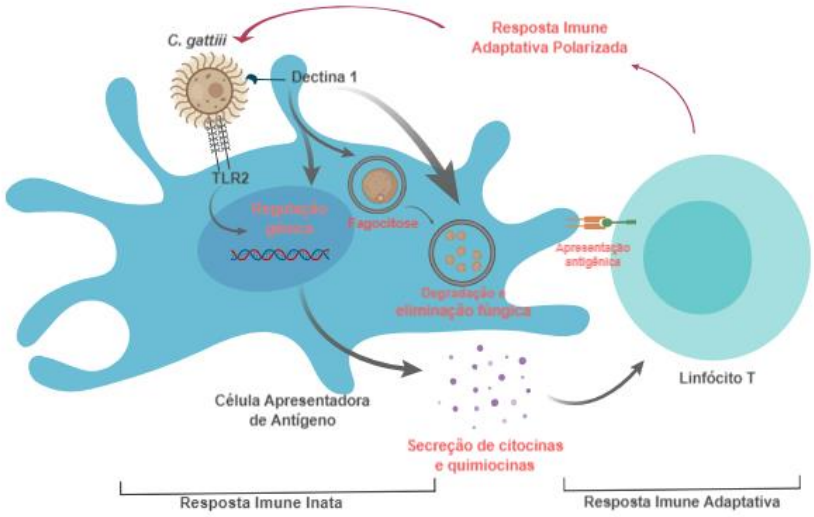
Inicialmente, os NLRs e os RLRs não são muito importantes para reconhecimento fúngico, pois estão presentes no citoplasma dos fagócitos e ligam-se, principalmente, a PAMPs bacterianos e RNA viral, respectivamente (HARDISON; BROWN, 2012). Em contrapartida, os TLRs estão fortemente relacionados ao reconhecimento de fungos por intermédio das classes TLR1, TLR2, TLR4, TLR6, TLR7 e TLR9 (BOURGEOIS et al., 2011; BROWN; DENNING; LEVITZ, 2011; HARDISON; BROWN, 2012). Porém, em relação à criptococose, os TLRs 2 e 9 mostraram ser mais importantes, uma vez que animais *Knockout* para esses receptores são mais sensíveis às infecções por espécies de *Cryptococcus* (COELHO; BOCCA; CASADEVALL, 2014).

Os CLRs, assim como os TLRs, são bastante importantes para o reconhecimento fúngico e para a resposta antimicótica **(Figura 8)**. Esses receptores fazem parte de uma superfamília heterogênea de moléculas solúveis ou transmembranas e possuem em comum um domínio lectina do tipo C, que se liga, de modo geral, a todos os fungos patogênicos para humanos. Esses receptores interagem com carboidratos presentes na parede celular fúngica, como β-glucana e a manana (HARDISON; BROWN, 2012).

Dentre os CLRs, as dectinas 1 e 2, ao se ligarem e reconhecerem a célula fúngica, ativam cascatas de sinalização que culminam na ativação de fagócitos pela produção de espécies reativas de oxigênio (EROs) e espécies reativas de nitrogênio (RNS) que são importantes fatores microbicidas. Além disso, agem na ativação do inflamossoma, e do fator nuclear kappa B (NF-κB) que, via TLR, induz a produção de

citocinas pró-inflamatórias como interleucinas (IL)-1β, IL-18, IL-6 e IL-12, as quais atuam no recrutamento e ativação de células imunes, assim como, na diferenciação de outras células, como por exemplo, os linfócitos (COELHO, BOCCA; CASADEVALL, 2014).

Figura 8 - Funções de TLR2 e Dectina-1 na resposta antifúngica.



Fonte: O autor.

Ainda nesse contexto, moléculas solúveis como as citocinas, associadas à respostas Th1 e Th17, são essenciais para o controle dos agentes infecciosos, com a expressão, por exemplo, de TNFα, IFNγ e IL-17. Os receptores de manana, presentes nas células dendríticas, reconhecem o antígeno fúngico e por endocitose e posterior processamento antigênico, apresentam o antígeno às células T, promovendo uma resposta antifúngica (REIBER et al., 2015). Müller e colaboradores (2007) demonstraram que o significante aumento de IL-17 associada à resposta Th17 modula a sobrevivência de camundongos infectados com *C. neoformans*.

Embora muitos fenômenos imunológicos envolvidos na infecção sejam descritos, ainda não estão elucidados os mecanismos que permitem *C. gattii* infectar pessoas imunocompetentes mas, *C. neoformans* não, apesar que, algumas hipóteses sugeridas propõem que o *C. gattii* afeta indivíduos hígidos por ser capaz de impedir a migração neutrofílica (CHATURVEDI; CHATURVEDI, 2011), o que não se observa com *C. neoformans* (DONG; MURPHY, 1995). Algumas pesquisas demonstraram que determinadas moléculas produzidas por *C. gattii*, como acetoína e diidroxiacetona,

estimulam menos a resposta pró-inflamatória do que metabólitos produzidos por *C. neoformans*, o que resultaria em maior permanência e sobrevivência de *C. gattii* no hospedeiro (WRIGHT et al., 2002). Acredita-se, também, que *C. gattii* poderia estimular menos o sistema imunológico por conter uma cápsula maior do que *C. neoformans* (CHATURVEDI; CHATURVEDI, 2011).

Portanto, o eficiente controle da infecção criptocóccica requer um balanço nas respostas pró e anti-inflamatórias, sendo a primeira mais importante para a contenção da doença. Isso tem sido comprovado, em estudos que mostram a inibição ou silenciamento gênico de citocinas pró-inflamatórias acarreta no desenvolvimento de uma infecção mais invasiva e a sobrevivência dos animais infectados por *Cryptococcus spp.* diminui. (DECKEN et al, 1998; ZARAGOZA et al., 2007). Já quando se realiza o mesmo procedimento com fatores da resposta anti-inflamatória, observa-se aumento na sobrevida dos animais (MÜLLER et al., 2007).

2.2.2 Criptococose em Imunossupressão

As infecções são uma das principais causas de hospitalização e morte em pacientes imunodeprimidos, sendo a maioria devido a infecções bacterianas, entretanto, a prevalência de infecçoes fúngicas está aumentando nessas populações (VENTURA et al., 2013). Isso, porque o principal suporte da defesa do hospedeiro contra fungos patogênicos é o sistema imune inato (imunodeficiências primárias) ou imunodeficiências secundárias (GUPTA et al., 2004), o que explica sua alta incidência entre pacientes imunossuprimidos.

Três estudos da Austrália e Nova Zelândia que indicam a ocorrência da infecção por *C. gattii* predominantemente em pacientes imunocompetentes e por *C neoformans* em imunodeprimidos (CHEN et al., 2000), e analisando cuidadosamente a criptococose nos três maiores subgrupos com base no estado imune do hospedeiro, observa-se aqueles com HIV, os receptores de transplante de órgãos sólidos e os indivíduos HIV negativos não transplantados (NHNT). Esses subgrupos apresentam diferentes riscos para a infecção, demonstrando características clínicas variadas, diferentes taxas de mortalidade e variadas abordagens terapêuticas. (GEORGE et al., 2017).

Na maioria desses casos, a criptococose é frequentemente associada à infecção por HIV/AIDS, mas um número crescente ocorre entre os receptores de transplante de

órgãos sólidos e outros que recebem imunossupressão exógena, como pacientes que estão recebendo agentes imunossupressores, glicocorticosteróides, quimioterapia citotóxica, inibidores de TNF e outros agentes; além de um grupo heterogêneo de indivíduos com distúrbios subjacentes, como síndromes de falência orgânica, problemas imunológicos inatos e distúrbios hematológicos (DELLIERE et al., 2018; SATO et al., 2019; SHOURIN et al., 2019).

Em um estudo dirigido pela Universidade do Alabama, em Birmingham, entre 1996 e 2010, com individuos com criptococose, aproximadamente 40% dos indivíduos foram considerados imunodeprimidos, e identificou-se que dos 302 pacientes diagnosticados com a doença 36% eram HIV positivos, 28% eram receptores de transplante de órgãos (OTRs) e 36% eram pacientes NHNT, dentre esses, 39 indivíduos normais (BRIZENDINE et al., 2013).

Outros estudos revelam, também, que aproximadamente 20 a 60% da criptococose em pacientes HIV negativos ocorre em receptores de transplante de órgãos sólidos (VILCHEZ et al., 2002), tendo os receptores de transplante de rim, por exemplo, uma taxa de mortalidade em torno de 73% dos casos (PAPPAS et al., 2010). Uma observação importante é que *C. gattii* causa uma doença pulmonar isolada, que leva ao comprometimento do SNC tanto em pacientes imunocompetentes quanto imunossuprimidos. Isso foi visto, também, nos casos do surto ocorrido em British Columbia no Canadá e em pacientes do Norte do Pacífico dos Estados Unidos (GALANIS et al.,2010).

Por tais fatos, a vigilância ativa para casos de *C. gattii* está sendo realizada para melhor compreender a prevalência de *C. gattii* nos Estados Unidos e elucidar importantes questões relacionadas à patogênese e virulência que deferem da infecção por *C. Neoformans,* além da adequação dos tratamentos a serem propostos (PAPPAS, 2013). Comparados aos pacientes com infecções por *C. neoformans*, os pacientes com infecções por *C. gattii* são mais propensos a terem meningoencefalite e criptococomas pulmonares, e assim, serem submetidos a procedimentos cirúrgicos para o tratamento dessa complicação (YANG et al., 2019).

Os pacientes NHNT são aqueles que apresentam imunodeficiências subclínicas inatas ou adquiridas, e as complicações geradas pela criptococose nesse subgrupo incluem sequelas neurológicas permanentes como acidente vascular cerebral, cegueira, surdez e outras anormalidades no nervo craniano, além dos casos dos

individuos clinicamente "normais" que compreendem de 17% a 22% da população total dos individuos NHNT (PAPPAS et al., 2010).

Outro estudo, englobando 2041 casos de criptococose no Hospital de doenças infecciosas em Buenos Aires – Argentina, durante 30 anos, em um total de 2000 casos (98%) eram pacientes HIV positivos, e desses, 90% apresentaram comprometimento meníngeo, além de comprometimento neurológico em 71% dos indivíduos. Dos 2% HIV negativos (41 pacientes), 46,3% apresentaram comprometimento neurológico e 29,3% respiratório, sendo 25 pacientes acometidos por *C. neoformans* e 4 por *C. gattii*, sendo nos demais casos o agente infeccioso não identificado (ARECHAVALA et al., 2017).

Outro recente estudo, com 3.728 pacientes atendidos em hospitais da Califórnia e Flórida – EUA, mostrou que 44% dos pacientes com criptococose eram HIV negativos, sendo 4,5% OTRs (60% composto por pacientes receptores de rim) e 39,4% NHNT. Dentre os pacientes NHNT, 22,5% tinham alguma doença renal e 14,4% apresentavam câncer como doença base, tendo destaque os pacientes com leucemias. Ademais, OTRs e NHNT apresentaram taxas de sobrevida menores que pacientes HIV positivos (GEORGE et al., 2017).

Os pacientes HIV negativos com criptococose, no cenário das doenças nefrológicas, representam um grupo susceptível a essa infecção, e caracterizam-se por anos de hemodiálise e falência do órgão, levando, consequentemente, ao transplante, fazendo-se necessário o uso de imunossupressores, como reportado em 3 casos no Hospital Central do Porto -Portugal. Estudo no qual 2 pacientes com doenças renais crônicas, submetidos a anos de hemodiálise e uso de imunossupressores como ciclosporina ou ciclofosfamida, foram posteriormente transplantados, e devido ao controle da rejeição orgânica seguiram terapias imunossupressoras, levando ao acometimento por *C. neoformans.* E um terceiro caso de criptococose ocorreu após tratamento imunossupressor com ciclofosfamida no tratamento de uma síndrome nefrótica em consequência do Lupus Eritematoso Sistêmico, assim, para ambos os casos, tratamentos convencionais não foram resolutivos, sendo necessário o uso de anfotericina B lipossomal associada a altas doses de fluconazol, além das terapias adequadas Às doenças base; entretanto, os indivíduos transplantados foram a óbito (VENTURA et al., 2014).

No Brasil, a criptococose tanto em pacientes HIV positivo como outros imunodeprimidos ainda é negligenciada, não estando compreendida nas doenças de notificação compulsória, que associada ao difícil diagnostico apresenta uma prevalência subestimada, contudo, recentemente um estudo com cinquenta isolados de pacientes HIV positivo obtidos do Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto – USP, sendo identificadas cepas de *C. neoformans* (maioria tipo VNI) e *C. gattii* (VGII predominantemente) (FIGUEIREDO et al., 2016).

Portanto, as infecções devido às espécies de *Cryptococcus* ocorrem globalmente, e em uma grande variedade de hospedeiros, que vão desde aqueles que são severamente imunossuprimidos para aqueles que possuem sistemas imunológicos fenotipicamente normais.

2.2.3 Tratamento da Criptococose

É importante salientar que a escolha do tratamento para criptococose está ligada a fatores como toxicidade, biodisponibilidade da droga, sítio de infecção, estado imunológico e principalmente ao quadro clínico do paciente (PERFECT et al., 2010). Em casos de manifestações clínicas leves a moderada, a Sociedade Americana de Doenças Infecciosas preconiza o tratamento da criptococose causada por *C. gattii* por meio da terapia com fluconazol ou itraconazol (entre outros azólicos) por 6 a 12 meses. Contudo, para casos mais graves como meningoencefalite, o intraconazol não é recomendado por não ter boa penetração no sistema nervoso central (SUBRAMANIAN & MATHAI, 2005), sendo assim, os antifúngicos comumente utilizados são a anfotericina B combinada com fluconazol e/ou 5-flucitosina (NOONEY; MATTHEWS; BURNIE, 2005), já que a monoterapia pode se mostrar pouco eficiente por causa da resistência desenvolvida pelos organismos (REVANKAR et al., 2004; SUBRAMANIAN; MATHAI, 2005).

Além dessa crescente resistência aos antimicrobianos, determinantes empíricos de cada droga, como forma de administração e toxicidade, limitam o seu uso. A anfotericina B, por exemplo, apresenta ação antifúngica frente a vários patógenos, sendo que apresenta elevada nefrotoxicidade e não consegue atuar em todos os sítios do organismo (NOONEY; MATTHEWS; BURNIE, 2005; SUBRAMANIAN; MATHAI, 2005). O fluconazol apresenta alta eficácia, baixa toxicidade, tolerada administração parenteral e oral, rápida absorção e ação fungistática, embora o aumento das taxas de

falha clínica, em decorrência da resistência, expressa grandes implicações na morbidade e mortalidade (GHANNOUM; RICE, 1999).

Sendo representante da classe dos triazólicos, o fluconazol [2-(2,4-difluorofenil)-1,3-*bis*(1H-1, 2,4-triazol-1-il)propan-2-ol] é constituído por dois anéis com 3 atómos de nitrogênio e um anel benzênico com dois átomos de flúor, e está inserido no grupo de agentes antifúngicos que atuam como inibidores da biossíntese do ergosterol, que é o esterol predominante da membrana plasmática dos fungos, importante para a integridade e para a atividade de muitas enzimas ligadas a este envoltório, inibindo, por esse alvo, o crescimento do fungo (CASALINUOVO; DI FRANCESCO; GARACI, 2004; SANTOS et al., 2012).

Ademais, o fluconazol apresenta uma penetração adequada mesmo por via oral, podendo alcançar concentrações plasmáticas maiores ou iguais a 90% quando comparadas a uma dose endovenosa, e concentrações no sistema nervoso central de aproximadamente 70% da concentração plasmática, sendo assim, a escolha para o tratamento das diversas etapas clínicas de infecções fúngicas invasivas como a criptococose (CASALINUOVO; DI FRANCESCO; GARACI, 2004; SANTOS et al., 2006). O fluconazol também pode ser administrado em apenas uma dose diária pois tem um prolongado tempo de meia-vida, além disso, devido à sua polaridade e simetria, é altamente hidrossolúvel e predominantemente (aproximadamente 80%) eliminado via excreção renal na sua forma inalterada (CASALINUOVO; DI FRANCESCO; GARACI, 2004; LEWIS, 2005, SANTOS et al., 2012).

Em muitos locais, principalmente os que possuem poucos recursos como os países da África, a monoterapia com o fluconazol é a única opção de tratamento para a meningoencefatite criptocócica (SUDAN et al., 2013). Embora os perfis farmacocinéticos e farmacodinâmicos do fluconazol sejam favoráveis (CHEN et al., 2010), a sua recomendação para o tratamento da criptococose tem sido limitada devido ao aumento no número de casos de cepas de *C. gattii* resistentes aos azólicos (PFALLER et al., 2007). Dessa forma, o interesse na busca por tratamentos menos tóxicos ao hospedeiro e mais eficientes do que as monoterapias convencionais são fundamentais, principalmente em associação com essas drogas, diminuindo efeitos colaterais e aumentando a qualidade de vida dos indivíduos em tratamento. Os produtos naturais, por exemplo, são alvos terapêuticos importantes, pois, como adjuvantes podem

auxiliar no combate a infecções fúngicas, sendo as lectinas exemplo de ferramentas estudadas para tal função.

2.3 LECTINAS

2.3.1 Breve Histórico

Ao longo da evolução, as plantas desenvolveram um sofisticado e elaborado sistema de defesa diante do grande número de patógenos herbívoros e predadores potenciais que as cercam como fungos, insetos, bactérias, vírus, ácaros, nematóides e mamíferos. Esse mecanismo de defesa é mediado por reações complexas, muitas dessas, sendo ativadas em resposta as agressões (BRAGA, 2009). Essas respostas dispõem de um vasto número de peptídeos e proteínas, dentre outras substâncias, capazes de protegê-las contra o ataque de pragas e patógenos (VAN DEN BERGH et al., 2002, OLIVEIRA et al., 2004; BRAGA, 2009). Dentre essas, as lectinas, são importantes como proteínas de defesa por sua vasta atuação contra herbívoros e patógenos. Tal proteção ocorre devido à sua capacidade de ligação a glicoconjugados e, consequentemente, desestabilização do metabolismo do agressor, exercendo, assim, efeitos deletérios (BANDYOPADHYAY et al., 2001).

Há anos, sabe-se que animais, plantas e vários produtos de diversos organismos são tóxicos aos seres humanos (SINGH; SARATHI, 2012). Sementes de leguminosas, cruas ou mal cozidas, quando ingeridos, em alguns casos, levam a sintomas intestinais agudos e podem estar relacionados à presença de substâncias com alta estabilidade térmica, e resistentes a enzimas digestivas e ácidos, características que são comuns às lectinas (KUMAR et al., 2012).

Embora as lectinas apresentem certa toxicidade *in natura*, muitos estudos mostram a diminuição desse efeito quando essas proteínas são modificadas por meio de várias técnicas, como o uso de radiação ionizante (VAZ et al., 2012; VAZ et al., 2013). Por outro lado, seus benefícios são também documentados, na literatura, como ferramentas de interesse terapêutico para diversas pesquisas, como no tratamento do câncer, imunologia, ação antibacteriana, antiparasitária, entre outras (SINGH; SARATHI, 2012).

Em 1888, Stillmark foi o primeiro a experimentalmente mostrar que a fração proteica do feijão castor (*Ricinus communis)*, chamado “Ricina” era capaz de aglutinar células vermelhas, sendo as proteínas presentes denominandas “fitohemaglutininas”

(OLSNES; KOZLOV, 2001). Em 1936, Landsteiner afirmou que a atividade hemaglutinante de vários extratos de sementes era diferente quando testadas frente a eritrócitos de diferentes animais, e, essa especificidade para determinados eritrócitos foi, posteriormente, melhor investigada por Boyd e Shapleigh, os quais cunharam, em 1654, o termo “lectinas”, sendo derivado do latim “legere”, que significa escolher, selecionar. A partir da década de 60, as lectinas começaram a tomar evidência como potentes ferramentas na biologia e medicina (NICOLSON; SINGER 1971), sendo até hoje denominadas como proteínas ubiquamente distribuídas na natureza, podendo ser encontradas em microrganismos, plantas, animais, etc, geralmente oligoméricas, e de variadas estruturas, apresentando ao menos um domínio não catalítico e de ligação específica a carboidratos (DA SILVA, 2014). Originalmente, comparadas com anticorpos devido a similaridades na especificidade de suas ligações, em 1972 o termo lectina foi generalizado por Sharon & Lis para todas proteínas de origem não imunológica com capacidade de ligação a carboidratos, com atividade hemaglutinante ou não (SHARON; LIS 2004).

A ligação lectina-carboidrato ocorre por ligações não-covalentes como pontes de hidrogênio, interações de van der Walls e hidrofóbicas, estando o(s) domínio(s) de ligação a carboidratos dispostos preferencialmente na superfície protéica (VAN DAMME, 1998; SHARON, 2008). Outra característica das lectinas é a não modificação bioquímica dos carboidratos aos quais se ligam, essa propriedade as diferenciam das glicosiltransferases, glicosidases e enzimas que introduzem um substituinte (grupo sulfatos, por exemplo) em um carboidrato. Essa distinção é necessária porque algumas glicosidases podem se ligar aos carboidratos e, sob algumas condições, como a baixa temperatura, podem aglutinar células ao se ligarem a carboidratos na superfície celular (BRECHTEL et al., 2001). Em contrapartida, algumas lectinas de plantas e animais podem apresentar atividade enzimática, porém, essa atividade está relacionada a um domínio estrutural distinto do sítio de ligação ao carboidrato (TRAINOTTI et al., 2001; RAO et al., 2012).

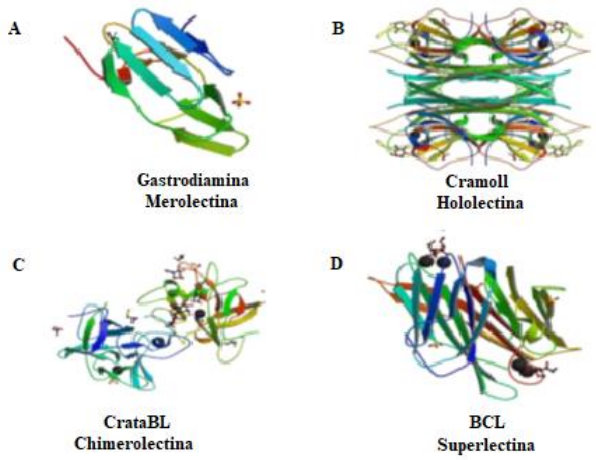
Assim, as lectinas medeiam processos biológicos como reconhecimento célula-célula, interações com a matriz extracelular, fertilização gamética, desenvolvimento embrionário, crescimento celular, diferenciação celular, sinalização celular, adesão e migração celular, apoptose, imunomodulação, inflamação, interação parasita-hospedeiro, enovelamento e direcionamento de glicoproteínas, indução mitogênica e

hemostasia (SHARON; LIS, 2004; GHAZARIAN et al., 2011).

2.3.2 Classificação das Lectinas

Com base na arquitetura global das lectinas de plantas, quatro grupos principais podem ser distinguidos: lectinas com apenas um domínio de ligação a carboidrato (merolectinas), dois ou mais domínios de ligação idênticos (hololectinas), no mínimo dois domínios de ligação a carboidratos diferentes (superlectinas), um ou mais domínios de ligação a carboidratos e um domínio que exerce atividade biológica independente do domínio de ligação a carboidratos (quimerolectinas) **(Figura 9)**. Todos esses grupos possuem a capacidade de aglutinar células, exceto as merolectinas, por possuírem apenas um domínio de ligação a carboidrato (VAN DAMME et al., 2008; VANDENBORRE et al., 2009).

Figura 9 - Estruturas de representantes das Merolectinas, Hololectinas, Quimerolectinas e Superlectinas.



Fonte: DA SILVA et al., 2014.
Legenda: (A) Gratrodianina de *Gastrodia elata* (PDB: 1XD6); (B) Cramoll de *Cratylia mollis* (1MVQ); (C) CrataBL de *Crataeva tapia* (4IHZ); (D) *Burkholderia cenocepacia* lectin A (2XR4);

Hoje, existe outra importante classificação, considerando sequências disponíveis nos bancos de dados, através de análises de genoma/transcriptoma, onde há doze famílias de lectinas de plantas relacionadas por seus domínios de ligação a carboidratos, (VAN DAMME et al., 2008, VANDENBORRE et al, 2009), que incluem (1) Domínios típicos de aglutininas homólogas de *Agaricus bisporus*, (2) Amarantina, (3)

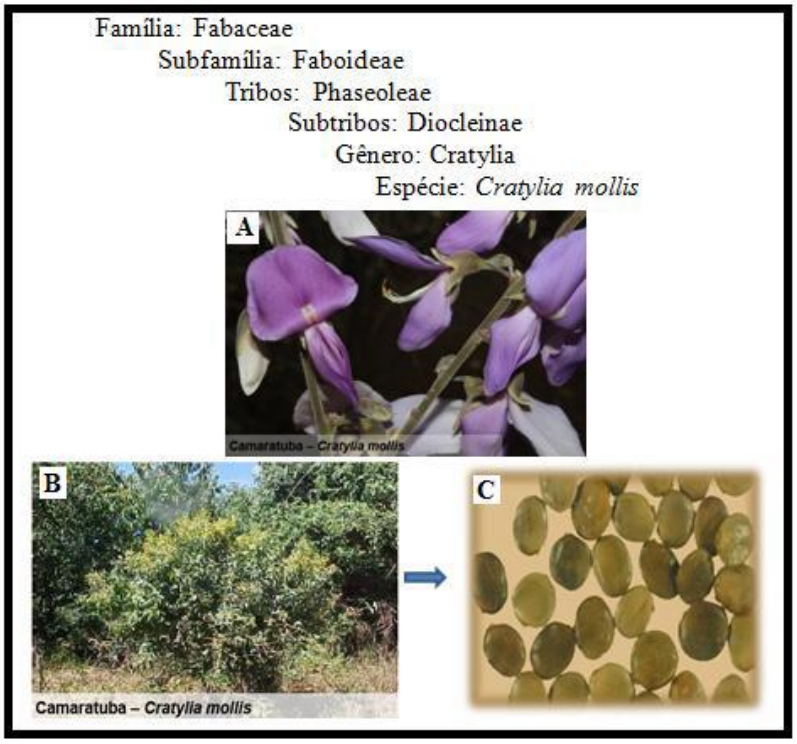
Homólogos de Classe V de quitinases com atividade de lectina, (4) Família *Nicotiana tabacum*, (5) Família Cyanovirin, (6) Família EEA - *Eonymus europaeus*, (7) Família GNA - *Galanthus nivalis*, (8) Jacalinas, (9) Domínios de LysM, (10) Proteínas com domínio heveínico, (11) Proteínas com domínios de lectinas de leguminosas, e (12) Família Ricin-B.

2.3.3 *Cratylia mollis* e Isolectinas

A família das leguminosas ou Fabaceae é a terceira maior família de angiospermas, compreendendo cerca de 727 gêneros e 19.325 espécies (LEWIS et al., 2005), apresentando uma grande importância ecológica, medicinal e econômica. Dentre suas tribos, a Phaseoleae compreende um vasto número de gêneros, nessa tribo, encontra-se a subtribo Diocleinae que apresenta treze gêneros reconhecidos, compreendendo, entre tantos outros, *Canavalia* e *Cratylia* (VARELA et al., 2004).

Cratylia mollis, popularmente conhecida como feijão Camaratu ou Camaratuba, é uma espécie do semi-árido do nordeste do Brasil (LIMA-RIBEIRO et al., 2012). *Cratylia* é uma planta arbustiva, tolerante à seca e a solos ácidos, e tem potencial para adubação verde e forrageamento **(Figura 10)** (ARAÚJO et al, 2011).

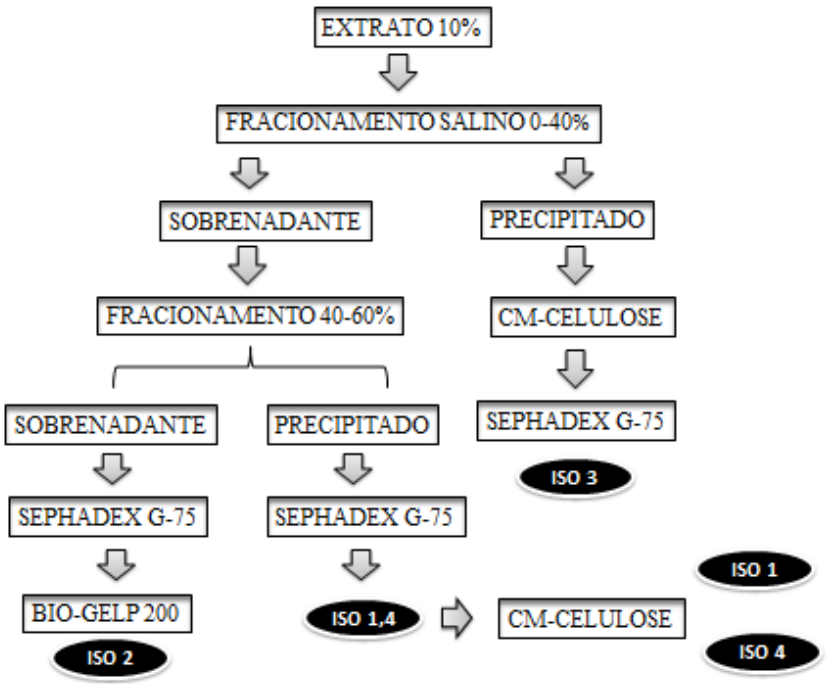
Figura 10 - Posição taxonômica de *Cratylia mollis.* A. Flores, B. Arbusto, C. Sementes.



Fonte: ARAÙJO et al., 2010.

Das sementes de *C. mollis* foram purificadas quatro isoformas de lectinas, (Cramoll 1, 2, 3, 4), classificadas de acordo com a migração eletroforética em gel para proteínas básicas, sendo a isoforma 1 mais básica e de maior migração. Essas isoformas, foram separadas pelos diferentes estágios durante processo de fracionamento salino com sulfato de amônio. Em 1992, as isoformas 2 e 3 foram purificados por Paiva e Coelho, e, em 1995 Correia e Coelho purificaram as isoformas 1 e 4. Essas 2 últimas foram purificadas por cromatografia de afinidade em sephadex G-75, chamada fração ISO1,4, hoje, também chamada de pCramoll. As isoformas 1 e 4 podem ser separadas, posteriormente, através de mais uma etapa cromatográfica com CM-celulose, gerando um rendimento de 94% para a isoforma 1 **(Figura 11)**.

Figura 11 - Etapas principais para extração e purificação das isoformas de lectinas de *Cratylia mollis*.



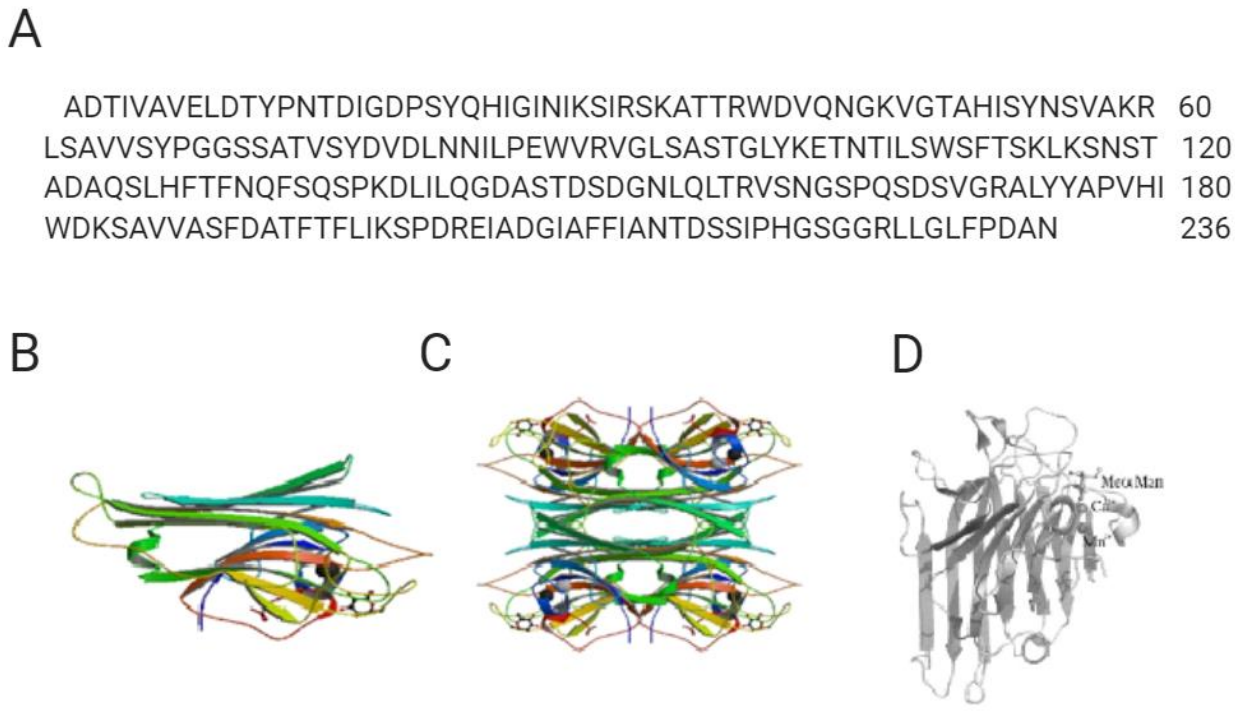
Fonte: O autor.

As isoformas 1 (31 kDa), 2 (60 kDa) e 4 (31 kDa), não são glicosiladas, e ligam-se a resíduos de glicose/manose, enquanto que a isoforma 3 (31kDa), que é uma glicoproteína se liga a galactose. As isoformas 1, 2, e 3 apresentam diferenças de migração eletroforética: Cramoll 1 (pI 8.5-8.6), Cramoll 2 (pI 4.15-6.7) e Cramoll 3 (pI

5.25-5.8) (PAIVA; COELHO, 1992). As isolectinas 1 e 3 de sementes de *C. mollis*, como várias lectinas de leguminosas, ocorrem na matriz amorfa dos corpos protéicos e nas paredes celulares dos eixos embrionários das sementes. A co-localização dessas isolectinas, com perfis de glicosilação e especificidade de reconhecimento a carboidrato diferentes, sugerem diferentes funções, seja na defesa contra predadores, na ação mitogênica nos processos embrionários, além de reserva proteica (SANTOS et al., 2004, DA SILVA, 2014).

A Cramoll 1 é a lectina majoritária da preparação ISO1,4 (pCramoll) e consiste em 236 resíduos de aminoácidos **(Figura 12A)**, dentre os quais quatro são triptofanos. A estrutura terciária de Cramoll 1 foi determinada por cristalografia de raio-X a 1.77Å e revelou três β-folhas conectadas por loops, estrutura conhecida como domínio jellyroll **(Figura 12)**, essencialmente semelhante à Concanavalina A (SOUZA et al., 2003; VAREJÃO et al., 2010).

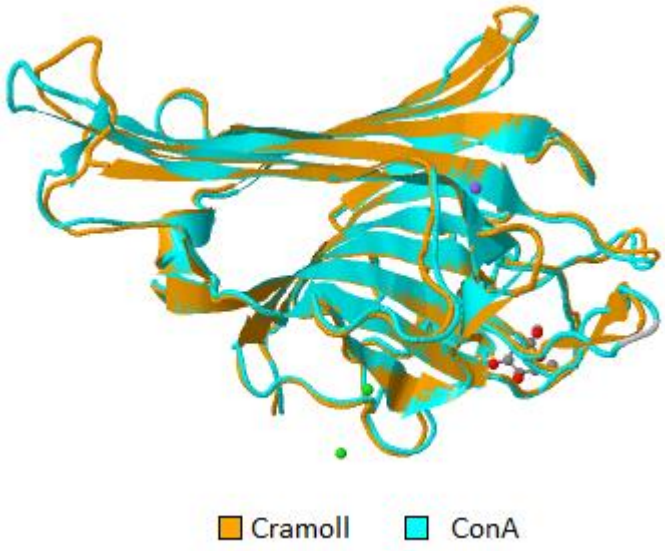
Figura 12 - Estrutura Terciária de Cramoll 1



Fonte: adaptado de DA SILVA, 2014.
PDB ID: 1MVQ (**A**) Monômero, (**B**) Tetrâmero e (**C**) Sítio de ligação a monossacarídio e aos íons Ca2+ e Mn2+.

Essa similaridade corresponde a 82% de identidade com ConA **(Figura 14)**, apresentando um sítio de ligação idêntico para metil-α-manopiranosídio, Mn^{2+} e $Ca^{2+,}$ entretanto, são observadas diferenças na ligação a oligossacarídeos e glicoproteínas pelas duas lectinas. Os dois sítios que se ligam aos metais são encontrados na proximidade do sítio de ligação ao monossacarídeo e são indispensáveis para ligação do carboidrato. Os metais interagem diretamente com a cadeia polipeptídica, como também, na interação com moléculas de água (SOUZA et al., 2003).

Figura 13 - Alinhamento estrutural de Cramoll isoforma 1 e Concanavalina A



Fonte: DA SILVA et al., 2011.

2.4 pCRAMOLL E ATIVIDADES BIOLÓGICAS

O potencial de pCramoll tem sido mostrado tanto para diagnóstico quanto na terapêutica em diversos âmbitos de pesquisa. O uso de pCramoll foi avaliado, inicialmente, na análise do perfil diferencial de marcação tecidual, e foi possível observar uma maior marcação dos tecidos neoplásicos em relação aos sadios, mais do que a Concanavalin A (ConA), lectina comercial reconhecida com ação antitumoral (BELTRÃO et al, 1998). Também foi visto o perfil de glicosilação diferencial de tecidos normal, hiperplásico e carcinoma de próstata, o padrão de coloração foi mais intenso no tecido hiperplásico quando comparado ao tecido normal (LIMA et al., 2010). Tal marcação diferencial no perfil de glicosilação poderá também ser observado a partir

da associação de pCramoll com quantum dots, previamente testados em *Candida albicans* (DA CUNHA et al., 2017), como uma proposta promissora no diagnóstico diferencial de neoplasias Na ação anticâncer, foi visto que tanto pCramoll como a sua forma recombinante (rCramoll) promoveram a morte das células PC-3, de adenocarcinoma próstatico, através da sinalização de cálcio, levando ao colapso mitocondrial (DE OLIVEIRA FIGUERÔA et al., 2017).

Ademais, a ação anticâncer de pCramoll encapsulada em lipossomas foi investigada contra Sarcoma 180 em camundongos Swiss, na qual pCramoll apresentou inibição de 30% superior a sua forma livre (ANDRADE et al., 2004). Essa atividade também foiposteriormente apresentada por sua recombinante (rCramoll), encapsulada em lipossomas furtivos, frente a animais com Sarcoma 180 (DA CUNHA et al., 2016).

A ação antiparasitária de pCramoll foi demonstrada contra *Trypanosoma cruzi* e *Schistosoma mansoni*. pCramoll apresentou atividade tripanocida contra as formas epimastigotas de *T. cruzi* com potencial de aglutinação, inibição da proliferação e morte celular por necrose. Este mecanismo molecular de morte envolve permeabilização da membrana plasmática, seguido por influxo e acúmulo de Ca^{+2} mitocondrial, estimulando a geração de espécies reativas do oxigênio mitocondrial com diminuição do potencial de membrana, dificultando a fosforilação do ADP (FERNANDES et al., 2010). O efeito frente a *S. mansoni* foi elucidado em modelo de infecção em camundongos Swiss. Esses foram infectados via subcutânea e caudal e foram tratados via intraperitoneal. Os tratamentos propostos foram eficientes, com redução da oviposição e recuperação das formas adultas, e formação de granuloma (MELO et al., 2011a).

O efeito cicatrizante de pCramoll foi primeiro avaliado no tratamento das lesões cutâneas experimentais em camundongos imunocompetentes e imunocomprometidos, mostrando alto poder de reparação tecidual e grande potencial como futuro composto cicatrizante (MELO et al., 2011b). Ainda, em um estudo posterior, tratamento regular utilizando hidrogel contendo pCramoll, em queimaduras de segundo grau em camundongos, acelerou a granulação, o processo de reepitelização e retração da ferida em comparação ao hidrogel sozinho (LIMA-RIBEIRO et al., 2012). A ação cicatrizante de pCramoll também foi observada a partir da sua associação com uma matriz polissacarídica de galactomanana, sendo capaz de favorecer a repitelização e retração da ferida mais brevemente comparado (ALBUQUERQUE et al., 2017). Recentemente, o

uso terapêutico de um gel com pCramoll na cicatrização de queimaduras de segundo grau em animais imunodeprimidos mostrou menor infiltrado inflamatório, precoce proliferação celular, presença de fibroblastos e neorepitelização em camparação a animais tratados com hidrogel sem a lectina, assim reforçando o potencial terapêutico da lectina, que pode ser utilizado com outros bioprodutos, como um filme curativo, para o tratamento desse problema (ALBUQUERQUE et al., 2017; PEREIRA, 2019).

Alguns estudos com viés biotecnológico foram feitos com pCramoll, quando imobilizada em matriz de Sepharose CL4B e preparada uma coluna de afinidade, sendo possível o isolamento e caracterização de glicoproteínas do plasma humano (LIMA et al., 1997). O mesmo sistema pCramoll-Sepharose possibilitou isolar proteínas com ação anticoagulante (SILVA et al., 2011), e pela difusão em gel de proteínas, foi possível identificar as que apresentam glicose/manose na sua estrutura (DA SILVA et al., 2014). Além disso, como alternativa para acelerar a osseointegração em nanotubos de dióxido de titânio, a pCramoll foi imobilizada e esse sistema e apresentou um aumento na adesão de células semelhantes a osteoblastos quando comparado com o uso das superfícies de dióxido de titânio (OLIVEIRA et al., 2018).

Para fim diagnóstico, pCramoll foi imobilizada em nanopartículas de ouro adsorvidas em eletrodos e esse sistema foi usado na construção de um biossensor sensível a glicoproteínas ou glicoconjugados em solução (OLIVEIRA et al., 2008). Em 2011, um novo sensor utilizando pCramoll foi desenvolvido com alta sensilibidade e seletiva discriminação para lipossacarídio bacteriano, sendo demonstrada a capacidade de pCramoll de ligação com LPS de vários microrganismos a exemplo de *Serratia marcescens*, *Escherichia coli*, *Salmonella enterica* e *Klebsiela pneumoniae* (OLIVEIRA et al., 2011). pCramoll também mostrou ter um grande potencial na detecção de padrões anormais de glicoproteínas no soro de pacientes com dengue, apresentando padrões diferentes para os sorotipos virais 1, 2 e 3, com forte reação para o sorotipo 2 (OLIVEIRA et al., 2011), além disso, recentemente, nanoeletrodos com pCramoll foramusados para diagnóstico diferencial entre câncer prostático e displasia benigna prostática (SILVA et al., 2017).

Dessa forma, as lectinas de *C. mollis* têm mostrado diversas atividades biológicas podendo ser utilizada como fonte para diversas aplicações biomédicas e médicas.

2.4.1 Atividade Imunomoduladora de pCramoll

A primeira referência que pCramoll apresentou propriedade imunomoduladora foi na indução mitogênica de linfócitos T, similar efeito também visto com ConA. Essa habilidade em células T foi inibida por metil-D-manose, indicando o envolvimento do sítio de ligação a carboidratos da lectina com a resposta imune desenvolvida. Ainda foi observada a ação apenas da isoforma 1 e não foi visto diferença significativa na ação quando usada em combinação com a 4 (pCramoll) (MACIEL et al., 2004). Essa propriedade mitogenica também foi demonstrada em esplenócitos de camundongos, obtidos 72 horas após administração intraperitoneal de 100 µg/mL da lectina, além da produção de altos níveis de IL-2, IL-6, INF-γ e óxido nítrico.

pCramoll também não induziu apoptose e estimulou um número significante de células na fase S do ciclo celular, mostrando que essa lectina pode ser usada como agente mitogênico nos ensaios de imunoestimulação (MELO et al., 2010).

Posteriormente, num estudo experimentalmente similar usando uma dose de 235 µg/mL, após sete dias, a análise dos linfócitos retirados do baço indicou um aumento de resposta Th2, expressando IL-6, IL-10 e IL-1β e aumentando EROs mitocondrial e citosólico e Ca^{+2} (MELO et al., 2011c). A atividade imunomodulatória nos linfócitos do baço e na produção de IFN-γ e supressão óxido nítrico foi observada com uso de pCramoll (MELO et al., 2010c). Recentemente, também foi avaliada a habilidade da lectina na indução da via Th17, expressando IL-6, IL-17A, IL-22, e IL-23 melhor que no estímulo com ConA, além de gerar memória imunológica por reestimulação de linfócitos (OLIVEIRA et al., 2013).

2.5 LECTINAS E INFECÇÕES FÚNGICAS

Apesar dos agentes antifúngicos usados, infecções fúngicas invasivas são responsáveis por altas taxas da mortalidade. A exemplo, a criptococose, é responsável por 1 milhão de casos de meningoencefalite, principalmente em indivíduos HIV positivo, com 624.000 mortes, anualmente (MCMULLAN et al., 2013). Doenças imunossupressoras, em geral, associados ao crescente fenômeno de resistência antifúngica (GULLO et al., 2013; CHOWDHARY et al., 2014), tornam o tratamento de grande parte dessas infecções oportunistas ineficaz e impulsiona estudos *in vitro* e e, modelos experimentais no desenvolvimento de imunoterapias. Citocinas, anticorpos,

opsoninas, terapia celular, gênica e imunomodulatória são alternativas descritas na regulação do sistema imunológico (AMSTRONG-JAMES; HARISON, 2012), combinados ou não aos antifúngicos em infecções como criptococose (ANTACHOPOULOS et al., 2012), candidíase (SAFDAR et al., 2005) e aspergilose (STUEHLER et al., 2011). Vários estudos mostram efeitos imunorregulatórios por lectinas, particularmente, na migração e ativação de células mononucleares e polimorfonucleares, desenvolvimento de resposta Th1, Th2, Th17 e estímulo da fagocitose (COELHO et al., 2017).

Modelos experimentais e análises *in vitro* que mostram aumento da resposta antifúngica por estratégias imunorregulatórias por lectinas são reportadas. Em estudo com modelo de infecção por *Candida albicans*, usando como terapêutica Con A (Concanavalina A) observou-se a ativação de respostas antifúngicas por aumento da fagocitose por macrófagos (MORESCO et al., 2002; LOYOLA et al., 2012a).

A lectina Artin M, presente em sementes de *Artocarpus integrifolia*, apresentou atividade imunorregulatória estimulando a migração de neutrófilos por haptotaxia. Essa lectina tem habilidade de ligação a glicanos que apresentam manose (GANIKO et al., 1998), frequentes em componentes da matriz extracelular (como laminina) auxiliando a migração celular para tecidos injuriados (GANIKO et al., 2005), importante fenômeno na resposta inflamatória frente infecções. Também em modelo frente *C. albicans*, Artin M demonstrou efeito protetor por aumento de resposta Th1 e Th17. Em combinação com Jacalina (lectina galactose específica) também presente no extrato das sementes de *A. integrifolia*, estimula a produção de IL-12 por macrófagos intraperitoneais, e aumentou TNFα mediado por receptor Dectin-1, e maior estímulo de IFN-γ e IL-17 (LOYOLA et al., 2012b). Esses mecanismos favorecem a fagocitose e ação anti *C. albicans* (CUSTODIO et al., 2011).

Coltri e colaboradores (2008) demonstraram o efeito protetor de Artin M (nativa e recombinante) com administração terapêutica em camundongos acometidos por *Paracoccidioides brasiliensis.* Este efeito ocorreu com produção de IL-12 (via TLR-2), mecanismo fundamental na resistência frente à paracoccidioidomicose (TRICHIERI et al., 2003).

Em outros estudos, foi demonstrado que a paracoccina (lectina de ligação a N-acetilglicosamina) presente na parede celular do *Paracoccidiodes brasiliensis* induz a produção de TNF-α e NO por macrófagos, tem ação N-acetilglicosaminidase (DOS

REIS ALMEIDA et al., 2010) e influencia na morfogênese e crescimento do próprio patógeno (DOS REIS ALMEIDA et al., 2011). Tanto a paracoccina nativa (PCN) quanto recombinante (rPCN), apresentam propriedades imunorregulatórias que auxiliam na resposta contra o próprio fungo, levando a diminuição da carga fúngica, formação de granulomas menores e menos densos, associado com aumento dos níveis de IL-12 e IFN-γ (ALEGRE et al., 2014a). Esta ação é resultante da interação da rPCN com receptores TLRs, observado em células HEK293T transfectadas com plasmídeos TLR 2/1, TLR 2/6 e TLR 4, e detecção da expressão de IL-8. A ativação por TLR2 é dependente do reconhecimento do carboidrato e independente da heterodimerização com TLR1 e TLR6 e presença dos co-receptores CD14 ou CD36 (ALEGRE et al., 2014b).

Como outras lectinas, a paracoccina apresenta efeito imunomodulador por interagir com glicanos celulares do sistema inato e adaptativo do hospedeiro, e reforçam o combate a doenças infecciosas (UNITT; HOMIGOLD, 2011; SOUZA et al., 2013; DA SILVA; CORREIA, 2014). Esses estudos imunoterapêuticos implicam num enorme conhecimento da interação patógeno-sistema imune, como também, na descoberta de agentes que atuem no aumento da eficácia antifúngica.

2 CONCLUSÕES

Diante dos objetivos propostos por esse trabalho foi possível depreender que o uso de lectinas de plantas frente infecções microbianas (bactérias, fungos e parasitas) é uma alternativa viável para o tratamento dessas infecções, sendo ferramentas terapêuticas importantes na estimulação de agentes imunes. Além disso, o uso de lectinas de *Cratylia mollis* foi pela primeira vez descrito como agente terapêutico frente a criptococose, sendo a pCramoll sozinha, ou combinada ao fluconazol, melhorando a sobrevida e aspectos comportamentais em camundongos imunocompetentes infectados por *C. gattii*. Também, sozinha, ou combinada ao fluconazol, capaz de diminuir a carga fúngica de órgãos alvos, como pulmão e cérebro; atuando na modulação dos fatores inflamatórios envolvidos na doença, assim, levando a uma resposta mais efetiva no combate ao agente infeccioso, como no aumento da fagocitose de *C. gattii* e produção de espécies reativas de oxigênio por macrófagos murinos.

A imunossupressão iatrogênica com ciclofosfamida e 5-fluoruacilo foi efetiva no modelo animal de criptococose, uma vez que diminui significativamente o efetivo celular imunológico para combater a infecção, resultando numa diminuição da sobrevida dos animais com criptococose, acarretando em uma maior carga fúngica nos órgãos alvos envolvidos na doença. Nesse cenário, pCramoll foi capaz de aumentar a sobrevida de animais imunossuprimidos e infectados por *C. gattii*, diminuindo a carga fúngica no pulmão e no cérebro de animais imunossuprimidos e infectados por *C. gattii*.

Portanto, como base nos dados gerados por esse trabalho e outros presentes na literatura depreende-se o importante papel das lectinas de plantas como possíveis ferramentas terapêuticas diante de alvos do sistema imune diante de doenças infecciosas, o que impulsiona o desenvolvimento de pesquisas futuras a revelar quais mecanismos moleculares estão envolvidos nesse padrão de ativação e resolução desses eventos imunológicos.

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APÊNDICE A - pCRAMOLL THERAPY DECREASE MORTALITY AND FUNGAL BURDEN IN IMMUNOSUPPRESSED MICE INFECTED WITH *CRYPTOCOCCUS GATTII*

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Abstract

Cryptococcosis is an infectious disease caused mainly by two species *Cryptococcus neoformans* and *C. gattii*, and presents insidious clinical onset and courses with headaches, fevers and mental status changes. The infection occurs mainly in immunosuppressed individuals, with high prevalence with the HIV pandemic, and is the third most common invasive fungal infection in SOT recipients. The main treatment actuality consolidate is lipid amphotericin B plus 5-flucytosine is used as moderate-to-severe pulmonary infection and disseminated infection, followed by fluconazole. Fluconazole is used for mild-to-moderate pulmonary infection, but the toxicity and resistance phenomena improve the search of news antimicrobials and immunorregulatory agents. The propose this word is to use pCramoll, an immmunmodulatory lectin obtained from seeds of *Cratylia mollis* Mart [an endemic plant of Caatinga (Northeastern Brazil semi-arid area)] as therapy against the cryptococcosis in infected mice under iatrogenic immunosuppression by 200 mg/Kg Cyclophosphamide and 150 mg/Kg of 5-fluorouracil association. This way, was

observed that the immunosuppression promote susceptibility to *C. gatttii* infection, occurring tissue damage of lung and brain and rates survival decreased. With pCramoll treatment, the immunosuppressed mice exhibit loss fungal burden on the target organs decreasing tissue damage and promote increase 100% rate survival. This work shows for the first time the use of plant lectins as possible immunoregulatory tools in immunosuppressed animals infected with *C. gattii*.

Introduction

Infectious diseases are the major cause of hospitalization and death in immunocompromised individuals, though most are due to bacterial commitment, the prevalence of invasive fungal infection is increasing worldwide [1]. Cryptococcosis is one of the most common opportunistic infections in immunocompromised hosts and the *Cryptococcus neoformans* and *C. gattii* is responsible for 80% and 20% of cases of human disease, respectively [2]. Recent data show that the incidence of disease has increased with the HIV pandemic has been responsible for the development of cryptococcal meningitis (accounting for 80–90% of cases) [3].

Moreover, the iatrogenic immunosuppression is also one of the most important risk factors for *Cryptococcus* spp. infection in solid organ transplant recipients (that receive potent immunosuppressive agents) [4], patients with underlying disorders (such as Primary Immune Deficiency) and individuals with hematologic- or rheumatologic-related pathologies present predisposing factors for cryptococcosis involvement in non-HIV infected patients [5, 6].

The infectious course occur after inhalation of spores and subsequent isolate accometiment pulmonary (most frequent in immunocompetent patients), or on the progression of disease (in immunocompromised patients or microbial resistance cases) may the cryptococcosis disseminate to the central nervous system and cause meningitis or meningoencephalitis. Clinical manifestations features of human cryptococcosis are variable and can affect essentially every organ of the body, including nodular or ulcerative skin lesions until eye lesions [7].

Tradicionally, several studies suggest that *Cryptococcus* spp. may have a different host preference based on the immunological status of the patient. Epidemiologic data have shown that *C. neoformans* tends to infect

immunocompromised patients, while *C. gattii* (serotypes B and C) generally infects apparently immunocompetent people [8, 9], with occurrences in tropical regions [10] and temperate climates [11, 12], for example *Cryptococcus* spp. is one of four most important endemic systemic mycoses in Brazil [13]. However, a growing number of cases have *C. gattii* as etiologic agent of infections in both immunosuppressed and immunocompetent individuals [14, 15].

In this scenario, it is crucial to understand the relationships between the immunological status of the host and susceptibility to systemic disease caused by *C. gattii*. In turn, this knowledge facilitates the development of new effective therapies to fight against this infection. Previously, pCramoll a lectin obtained from seeds of *Cratylia mollis* Mart [an endemic plant of Caatinga (Northeastern Brazil semi-arid area)] was highlighted due its therapeutic effects against cryptococcosis induced by *C. gattii*. pCramoll also increased the effects of fluconazole [16].

The action of this protein in cryptococcosis is related to the improvement of host immune system since it did not show any effect on yeast viability. In fact, the immunomodulatory effects of pCramoll is a well-known characterized in several *in vivo* and *in vitro* studies, including in experimental infections models [17]. In this sense, this paper aims to evaluate the effects of pCramoll treatment in immunosuppressed mice infected with *C. gattii*.

Materials and methods

Strains and Inocula

For this study was used the *Cryptococcus gattii* L2701 strain, (VGII molecular type) a clinical isolate from the Mycology Laboratory of Universidade Federal de Minas Gerais, Brazil. This strain was previously used in others works [18, 19]. It was subcultured in 3 mL of Sabouraud dextrose agar (SDA) at 35 ºC for 48 h. The viability cells were determined and the cells were suspended in sterile PBS 1X for inoculation.

Animals

Six-week-old male Swiss mice (weighing approximately 25 g) were obtained from Laboratory Keizo-Asami (LIKA) of the Universidade Federal de Pernambuco (UFPE). All animals were housed in groups of 5 mice per cage and provided sterilized

food *ad libitum* and water (with 5 mg/mL of Chloramphenicol for the immunosuppressed groups). All experiments performed with the approval of Ethical Committee of UFPE with number protocol 23076.030660/2016-11.

Immunosuppression

Swiss mice were immunosuppressed by administering 200 mg/Kg Cyclophosphamide (Genuxal) and 150 mg/Kg of 5-fluorouracil (Flusan) through intraperitoneal route (i.p.). The immunosuppression occurred 1 day before the infection and once per week after infection.

Evaluation of Immunosuppression

The immunosuppression was confirmed by evaluation of white blood cells (WBC) counts and Cellularity. For this, two uninfected groups of 5 swiss mice per group were established to determine the immunosuppression level. The first group was immunosuppressed as described above; the second group served as control and received no immunosuppressive treatment (treated only with PBS). After immunosuppression treatment, the mice were sacrificed and samples of blood (0.5 mL) were obtained from the puncture cardiac on 0, 7, 14 and 28 days. The total white blood cells were determined by Cell-Dyn Ruby Hematology Analyzer and the differential cell counts were determined microscopically. On these days, the thymus, liver, spleen and kidneys were removed, weighed and the cellularity of the immune organs (only thymus and spleen) were analysed. The thymocytes and splenocytes were obtained by mechanical breakup of lysing buffer. The homogenate solution was passed across a 40 nm nylon mesh and wash 3X with phosphate-buffered saline (PBS) plus 0.1% BSA. Posteriorly, the WBC separated with Ficoll solution of the homogenate, the pellet was obtained and the cells count observed in Newbauer camera.

Mortality and Fungal burden studies

Male swiss mice (6–8 weeks old) were anaesthetized by intraperitoneal (i.p.) injection with ketamine (80 mg/kg) and xylazine (10 mg/kg) in sterile saline, and then inoculated by intratracheal (i.t.) injection with 30 μL strain L27/01 suspension (1×10^4 CFU/animal) or PBS [non-infected] one day after immunosuppression. Further, mice were separated into two major groups immunocompetent and immunocompromised

mice, such group was divided as follows (n -6/group): immunocompetent, infected and non-treated (CINT), immunocompetent, infected and pCramoll treated (CIT), immunosuppressed, infected and non-treated (INT) and immunosuppressed, infected and pCramoll treated mice (IIT). The mice treated with pCramoll received 1 µg/dose initiated at 24 h after infection and administered by i.p. injection, once daily. Moreover, two groups control were established: immunocompetent non-infected (CNI) and immunosuppressed non-infected mice (INI). Following this protocol, the mice were monitored daily for survival until they succumbed. After analysis of the survival curve, other groups of mice were infected by i.t. injection, and treated on the same way, following infection, the animals were killed at 2, 7, 14- and 28-days post infection (d.p.i.) to collect the lungs and brain. The organ homogenates were plated on to SDA for 48 h at 35°C to determine the fungal burden, expressed as CFU per gram of tissue. Before succumbed, the blood was collected for WBC counts analysis.

Statistics

Statistical analysis of all data was performed using GraphPad Prism version 5.0 with $p < 0.05$ considered significant. The survival curve was plotted by Kaplan-Meier analysis and the results were analysed using the log rank test. Also, the results of organs weight, cells count, and CFU were analysed by analysis of variance (ANOVA) followed by Student's t-test.

Results

Cellular profile of the mice submitted to the Immunosuppression

At the second day after the iatrogenic immunosuppression by cyclophosphamide and 5-fluorouracil, it was possible to observe a significant decrease of organs weight such spleen and thymus ($p<0.005$) in relation to control animals. The immunosuppressed mice showed 3.6- and 2.6-folds reductions on the weight of spleen and thymus, respectively when compared to control group (FIGURES 1A and 1B). The significant decreases in spleen and thymus weights were also observed on 7 day (3.8- and 2.5-folds reduction), 14 (4.1- and 3.0-folds reduction) and 28 (4.0- and 2.5-folds reduction) after

onset of immunosuppression, respectively. Although, no significant differences were observed in the kidneys weights among the experimental groups (data not shown).

Associated with this decrease in organ weight, a significant reduction in the cellular content of these lymphoid organs was observed ($p < 0.005$). The control group (immunocompetent) presented a mean of the cellular content, during the full period analyzed, around 39,386 ± 8,207 x 10^6 cells / mL for the spleen, and 28.47 ± 2.151 x 10^6 cells / mL for the thymus, not had significant statistic (data not show). On the other hand, for immunosuppressed group the spleen present 2.6-, 2.7-, 2,5- and 2,5-folds reduction on days 2, 7, 14 and 28 (FIGURE 1C); while and the thymus 2.3-, 3.0-, 2.6- and 2.9-folds reduction , respectively, on the same times.

This depletion of the immune system reflect decreasing the total blood leukocyte levels (TABLE 1), with 3.2-folds reduction in second unit fourteenth days post immunosuppressed injection, and 3.7 folds reduction in twenty-eighth days. For the immunocompetent group, the blood cell content not had the significant difference in full period observed with the medium value of 2.17 ± 0.103 x 10^3cells/mm^3 of total leukocytes. The lymphocyte and monocyte levels showed in 2 (3.8- and 1.7-folds reduction), 7 (2.8- and 1.38-folds reduction), 14 (2.2- and 2.8-folds reduction), and 28 (2.3- and 2.8-folds reduction) days, respectively. However, only on 14 and 28 days the amount of neutrophils decrease to 0.08 ± 0.05 x 10^3 cells/mm^3 (7.5-fold reduction) and 0.07 ± 0.05 x 10^3 cells/mm^3 (8.5-fold reduction), respectively, for these immunosuppressed animals. The animals without immunosuppression present the count of 1.04 ± 0197 x 10^3cells/mm^3 of lymphocytes, 0.34 ± 0.028 x 10^3cells/mm^3 of monocytes and 0.6 ± 0.113 x 10^3cells/mm^3 of neutrophils, respectively, and also did not presented significant difference between the periods analysed.

Survival Curve of immunosuppressed and immunocompetent mice and fungal burden in the immune organs with pCramoll treatment

The treatment with pCramoll significantly increase the survival of immunosuppressed mice, in relation to untreated animals ($p<0.001$). The CINT group had an average survival of 29.5 days, while the pCramoll-treated mice (CIT) survived for 34 days.

However, the IINT animals had an average survival of 7 days (approximately 40% survival on this day), having a reduction of 76.27 % compared to the CINT group. The immunosuppressed animals treated with pCramoll (IIT group) showed an average survival of 14 days, having an increase of 100% survival (80% survival on 7 day) (FIGURE 2A). The non-infected groups did not show mortality during the experimental study.

In regards to fungal burden in the lungs, the IINT group presented the higher levels in the second ($0.43 \pm 0.09 \times 10^5$ CFU/g) and seventh days of infection ($1.81 \pm 0.22 \times 10^5$ CFU/g). In these two periods, the treatment with pCramoll efficiently reduced the yeast load in the lungs ($p<0.05$) with values of $0.29 \pm 0.016 \times 10^5$ CFU/g and $1.06 \pm 0.15 \times 10^5$ at 2 d.p.i and 7 d.p.i. At 14 d.p.i., pCramoll-group showed a high fungal burden ($3.35 \pm 0.19 \times 10^5$). In the animals without immunosuppression only was possible to detected yeast in the lungs at 7 d.p.i. These mice showed lower levels of fungal burden in relation to immunosuppressed groups. The non-immunosuppressed animals treated pCramoll exhibited lower levels of fungal burden than untreated animals (CINT). Untreated animals (CINT) showed fungal loads of $1.1 \pm 0.08 \times 10^5$ CFU/g, $2.15 \pm 0.19 \times 10^5$ CFU/g and $1.28 \pm 0.39 \times 10^5$ CFU/g of tissue at the 7, 14 and 28 d.p.i., respectively. These values were reduced by pCramoll treatment ($0.65 \pm 0.011 \times 10^5$ CFU/g, $1.51 \pm 0.17 \times 10^5$ CFU/g and $0.095 \pm 0.037 \times 10^5$ CFU/g) at the 7, 14 and 28 d.p.i., respectively.

In addition, in the IINT mice were observed higher levels of fungal burden in the lungs in relation to all experimental groups.

Similarly, in the second day post infection not was detected fungal growth in the animals brain (FIGURE 2C). In seventh days post infection the IINT group presented higher levels of fungal in the tissue brain with value of $1.9 \pm 0.15 \times 10^4$ CFU/g. In this same period, the pCramoll treatment (IIT group) reduced significantly the yeast load in the brain ($p <0.05$) with values of $1.0 \pm 0.05 \times 10^4$ CFU/g, in the 14 d.p.i the this group presented $0.65 \pm 0,22 \times 10^4$ CFU/g. The animals without immunosuppression only was possible to detected yeast in the brain at 14 d.p.i with fungal loads of $1.28 \pm 0.3 \times 10^4$ CFU/g, and following the curve infection a value of $2.1 \pm 0.7 \times 10^4$ CFU/g at 28 d.p.i.

These non-immunosuppressed mice with pCramoll treatment reduced the fungal burden with loads of $0.51 \pm 0.13 \times 10^4$ CFU/g and $0.6 \pm 0.2 \times 10^4$ CFU/g.
In addition, in the spleen and liver were observed fungal growth after 14 d.p.i only in the immunosuppressed groups (data not shown).

Discussion

At this work, was observed the higher cryptococcosis predisposition after CY 200 mg/mL and 5-phuoracyl 150mg/kg administration, this association decrease the thymus and spleen cellularity, change the peripheral blood cells profile like lymphocytes, monocytes and neutrophils. CY is a drug used for the treatment of a broad spectrum of malignancies and cause humoral immunity depletion, and also can produce a cellular impairment such as neutropenia state, alone or together with other immunosuppresses depleted the immune system causing a direct toxicity for myeloid precursors [20]. The reduction of polymorphonuclear neutrophils (PMNs) count by immunosuppression treatment predisposes the host to severe infections diseases [21], the CY use, speciality, course with various respiratory infections. Anteriorly study showed that single 200 mg/kg dose of CY readily induced neutropenia without significant mortality in mice [22, 23], making them more susceptible to virus, bacteria, and fungal infections [24, 25, 26].At this work, the immunosuppression established decrease 76.26% of mice survival infected with *C. gattii* when compared to competent mice infected. Moreover, associated to reduction of survival, was observed the increase of fungal burden on organs such as lungs and brain of immunosuppressed animals. As proved that the use of glucocorticoids and higher mortality rates in cryptococcosis cases after SOT [4], decreasing substantially the survival [27], ratifying CY and 5-FLU use like extensive tissue injury and more mortality. Additionally, this low immune profile and the higher fungal burden on target organs of infection result to more morbidity and the occurrence of severe symptoms and sequels [28].

Faced this problematic, news immunomodulatory therapies are developed for immune enhancement in immunosuppressive states and to combat infection diseases. pCramoll lection it has shown immunomodulatory abilities (cellular proliferation, Th2 and Th17 cytokines modulation, phagocytosis induction) included in wound healing model in immunocompromised mice [29, 30], and experimental infections with *Schistosoma mansoni* [31], and in the combat to *C. gattii* infection in competent mice [15]. Several works also shown the plant lectins (ArtiM, ConA) with

immunoregulatory action against infections [, 32, 33, 34], and assuming be biological tolls against microbial injuries.

Thus, is possible to know the therapeutic enforce of pCramoll in decrease fungal burden on lungs of immunosuppressed mice infected with *C. gattii* and the influence in to increase of 100% of survival rate in immunosuppression state (FIGURE 3). Several immunoregulatory mechanism induced by pCramoll influences the inflammatory response in the combat to cryptococcosis. Once observed previously, pCramoll induce the phagocytosis mechanisms and production of NO and ROS against this pathogenic agent [35]. Moreover, pCramoll increases the expression of Il-17 cytokine in an experimental cryptococcosis infection model, this regulatory pathway is fundamental for the increase of the cellular immunity and recruitment of PMN in the fight against the infectious agent [36]. So far, pCramoll is a first lectin of plant discrete with potential biotechnological tool in Th17 pathway studies, inducing immunologic memory regulation [37] essential pathway during cryptococcosis depletion. Them, now known that pCramoll is one possible therapeutic toll against this infection disease, being a possible weapon in the therapy before phenomena that depress the immune system of the host.

Conclusion

It was possible to observe that the use of the cyclophosphamide and 5-fluoroacil association were able to alter the cellular immunity profile of the animals, leaving them more susceptible to cryptococcosis. As well, this work shows for the first time the use of plant lectins as possible immunoregulatory tools in immunosuppressed animals and infected with *C. gattii*. Thus, the beginning for understanding and discovery of the immunoregulatory mechanisms involved, and as pCramoll and others plant lectins be possible biological tools.

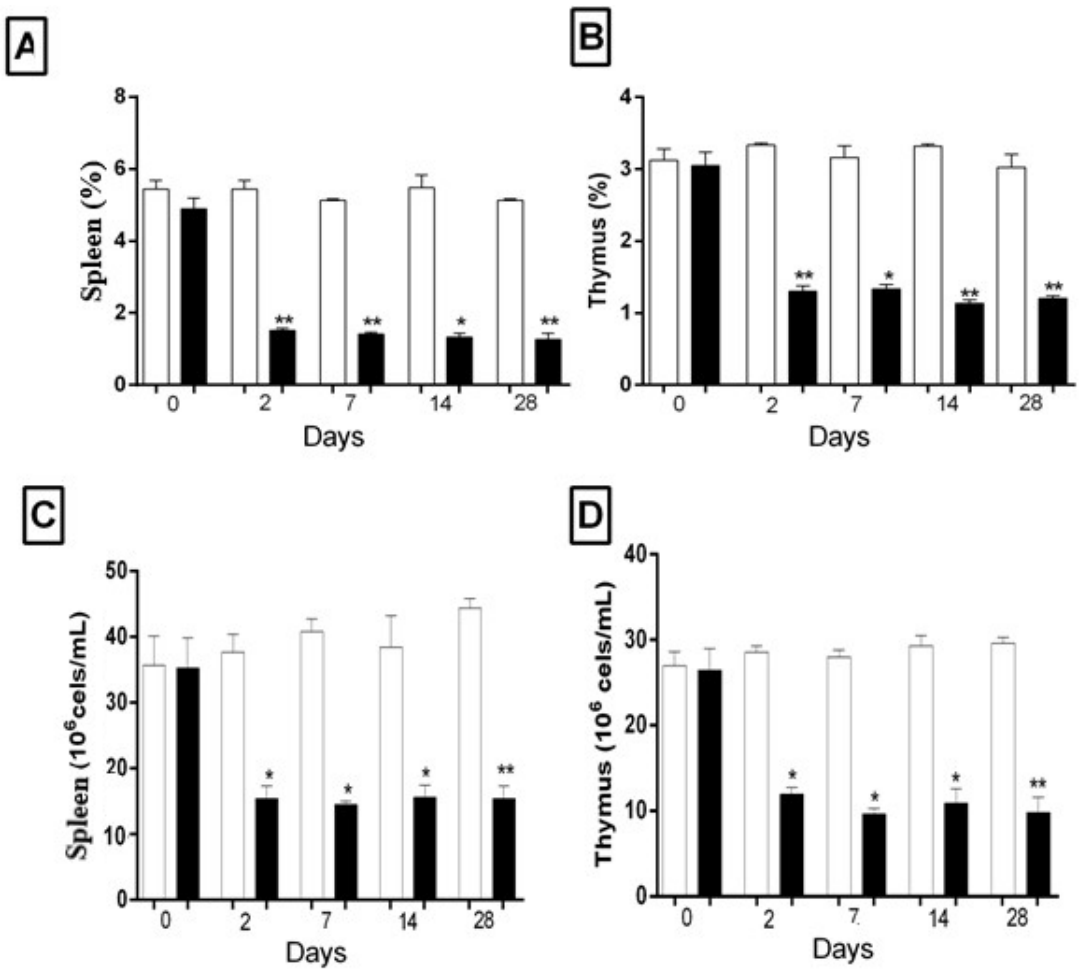

FIGURE 1: Relative variation weight and cellularity of immune organs (spleen and thymus) of immunocompetent (white column) and immunosuppressed (black column) mice. **A)** Spleen relative variation weight**, B)** Thymmus relative variation weight, **C)** Cellularity of spleen, **D)** Cellularity of thymmus. Five mice per group (control and animals treat with Cyclophosphamide and 5-fluorouracil intraperitoneally (i.p.) on 0 day and once per week after) were scarified and the spleen and thymus were retired, weight and their cellularity content analysed *$p < 0.05$ and **$p < 0.01$(compared to control).

Hematimetrics Parameters 10^3 Cells/mm^3	Controll (0 Day)	CY/5-FLU (2 Day)	(7 Day)	(14 Day)	(28 Day)
Total Leucocytes	2,17±0,103	0,67±0,026**	0,652±0,053**	0,672±0,07**	0,58±0,02**
Neutrophils	0,6±0,113	0,17±0,005	0,17±0,02	0,08±0,05*	0,07±0,005*
Limphocytes	1,04±0,197	0,27±0,010*	0,37±0,023*	0,48±0,010*	0,45±0,01*
Monocytes	0,34±0,028	0,2±0,015*	0,25±0,010*	0,12±0,010*	0,12±0,015*
Eosinophils	0,18±0,028	0,04±0,015	0,00	0,00	0,00
Basophils	0,01±0,014	0,00	0,00	0,00	0,00

TABLE 1: Hematimetrics parameters of immunosuppressed and immunocompetent mice. 5 mice per group (control and animals treat with Cyclophosphamide and 5-fluorouracil intraperitoneally (i.p.) on 0 day and once per week) the blood was collected and the hematimetrics parameters observed (total leucocytes, neutrophils, limphocytes, monocytes, eosinophils and basophils). *$p < 0.05$ and **$p < 0.01$(compared to control).

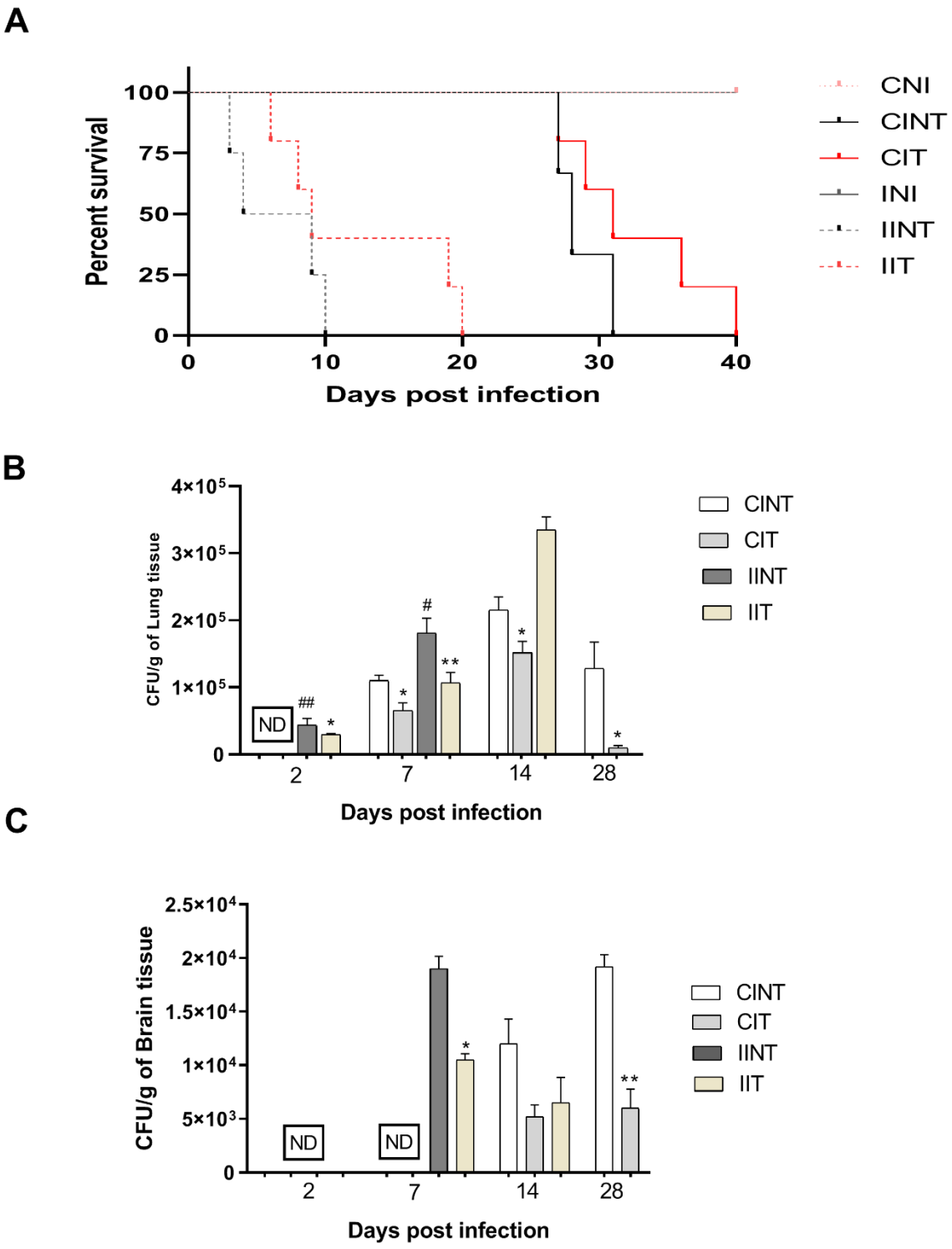

FIGURE 2: Curve survival and burden fungal in organs of immunosuppressed and immunocompetent mice infected with *C. gattii* strain L27/01. **(A)** Six mice per

group were immunosuppressed by 200 mg. Kg-1 of Cyclophosphamide and 150 mg. Kg-1 of 5-fluorouracil (i.p.) 1 day before of inoculation 10^4 cells of L27/01 strain and the curve survival was analysed. The immunosuppressed group had an average decrease of 73.68% in survival compared to immunocompetent group (control) ***p < 0.001. **(B, C)** Colony-forming Units (CFU) recovered from lungs and brain. ND (not recovered CFU). *p < 0.05 and **p < 0.01 (difference between treated with pCramoll and non-treated group), $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ (difference between immunosuppressed group and immunocompetent group).

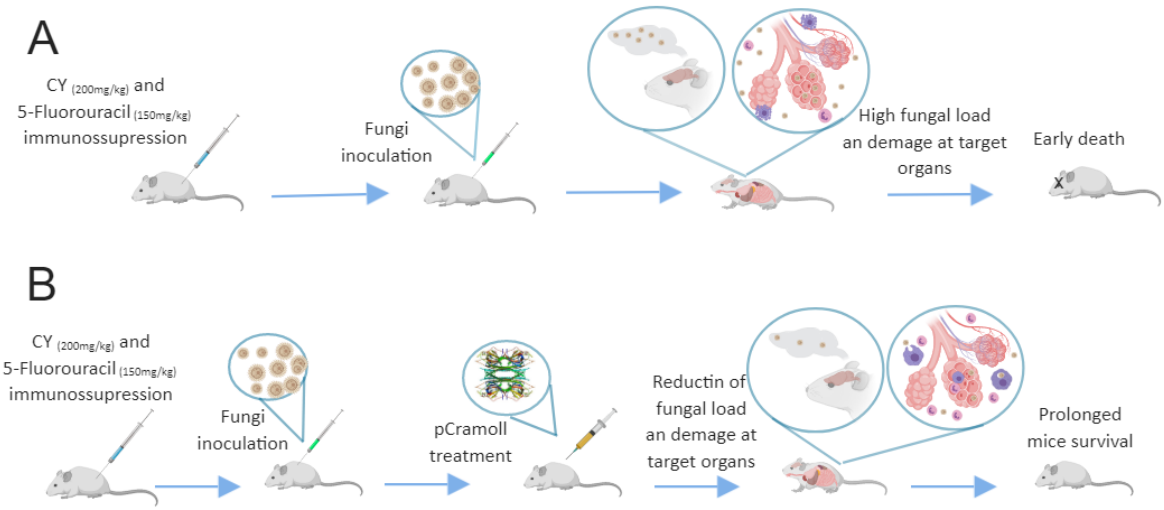

FIGURE 3. An overview of the pCramoll effect in immunosuppressed mice infected with *C. gattii*. **A)** The immunosuppression iatrogenic by cyclophosphamide and 5-fluoroacil association promote susceptibility to *C. gatttii* infection, occurring tissue damage of lung and brain and rates survival decreased. B) Whith pCramoll treatment the immunosuppressed mice exhibit loss fungal burden on the target organs decreasing tissue damage and high rates survival.

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ANEXO A - TARGETING THE IMMUNE SYSTEM WITH PLANT LECTINS TO COMBAT MICROBIAL INFECTIONS

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Running title: Plant lectins to combat microbial infections

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Abstract

The arsenal of drugs available to treat infections caused by eukaryotic and prokaryotic microbes has been declining exponentially due to antimicrobial resistance phenomenon, leading to an urgent need to develop new therapeutic strategies. Host-directed immunotherapy has been reported as an attractive option to treat microbial infections. It consists in the improvement of host defenses by increasing the expression of inflammatory mediators and/or controlling of inflammation-induced tissue injury. Although the *in vitro* antimicrobial and immunomodulatory activities of lectins have been extensively demonstrated, few studies have evaluated their *in vivo* effects on experimental models of infections. This review aims to highlight the experimental use of immunomodulatory plant lectins to improve the host immune response against microbial infections. Lectins have been used *in vivo* both prophylactically and therapeutically resulting in the increased survival of mice under microbial challenge. Other studies successfully demonstrated that lectins could be used in combination with parasite antigens in order to induce a more efficient immunization. Therefore, these plant lectins represent new candidates for management of microbial infections. Furthermore, immunotherapeutic studies have improved our knowledge about the mechanisms involved in host-pathogen interactions, and may also help in the discovery of new drug targets.

Keywords: immunomodulatory lectins, host-parasite interaction, immunization, adjuvants, new treatments.

1. Introduction

Through centuries, microbial infectious diseases continue to be among the leading causes of mortality and morbidity worldwide (Morens and Fauci, 2013;Sands et al., 2016;Rogalski et al., 2017). No doubt, the antibiotics discovery in the 1930's has revolutionized medicine and changed the treatment of infectious diseases, resulting in a dramatic increase in life expectancy and quality (Aminov, 2016). However, ever since these drugs were introduced, microbial resistance has evolved and spread very rapidly (Davies and Davies, 2010). Indeed, eukaryotic and prokaryotic microbes can acquire drug resistance by several mechanisms [for review see (Van Acker et al., 2014;Blair et al., 2015;Fairlamb et al., 2016;Goncalves et al., 2016;Hall and Mah, 2017)]. This fact critically reduces the shelf life of antibiotics that are not efficient to combat the emerging multidrug resistant strains (Holmes et al., 2016;Laxminarayan et al., 2016). In addition, pathogens have developed several mechanisms to evade and suppress the host defenses and/or to induce exacerbated inflammation (which may cause host tissue injury) (Chaves et al., 2016;Gomes et al., 2016;Ko, 2016;Malachowski et al., 2016;Sha et al., 2017). This scenario encourages the development of new approaches to treat microbial infection, such as those based on the modulation of the host immune system. The immunomodulatory therapies are based in the stimulation of specialized and specific host immune responses against microbes rather than target microbe viability or virulence (Hancock et al., 2012;Czaplewski et al., 2016;Fura et al., 2017).

Among the natural products, plant lectins are known as potent immunomodulatory agents, able to act in both innate and adaptive immune system. They modulate the production of cytokines and other mediators of immune response (such as reactive oxygen and nitrogen species), and, thus, improve the defenses against microbes (Souza et al., 2013;da Silva and Correia, 2014;Coelho et al., 2017). Plant lectins comprise one heterogeneous class of proteins with at least one non-catalytic carbohydrate-binding domain (Coelho et al., 2017). The lectin-carbohydrate interactions has been associated with several biotechnology applications (Komath et al., 2006;de Oliveira Figueiroa et al., 2017). In several cases, the immunomodulatory activity of plant lectins was associated with their interaction with glycan moieties present on the surface of immune cells. Such interaction can result in signal transduction which triggers the effector mechanisms involved in the response against microbial infections (Souza et al., 2013).

Plant lectins play a crucial role in the protection against microbial phytopathogens (Hwang and Hwang, 2011;Kim et al., 2015). Based on this, the antimicrobial and antivirulence actions of several lectins have been demonstrated *in vitro* against different bacteria of medical importance, as reviewed by several authors (Islam and Khan, 2012;Dias Rde et al., 2015;Coelho et al., 2017;Palharini et al., 2017). Other works have demonstrated the antibacterial effects of lectins using *in vitro* cell-based assays. For example, a lectin isolated from *Aegle marmelos* fruit inhibited the adherence and invasion of *Shigella dysenteriae* to human colonic epithelial cells (HT29 cells), protecting these cells against cell death (induced through apoptosis) (Raja et al., 2011). Similarly, the chitin-binding lectin isolated from the juicy sarcotesta of *Punica granatum* (named PgTel) showed to have broad-spectrum antibacterial action (inhibiting Gram-positive and Gram-negative bacteria). PgTel was also able to inhibit the invasion of some bacteria to HeLa cells (human epithelioid cervix carcinoma) (Silva et al., 2016b).

The immunomodulatory effects of plant lectins on different immune cells have been also addressed by several authors (Unitt and Hornigold, 2011;Pereira-da-Silva et al., 2012;Souza et al., 2013;da Silva and Correia, 2014;Coelho et al., 2017). Based on this, some works have demonstrated that some well-known immunomodulatory lectins are able to enhance the phagocytic ability of immune cells and their cytokine production in the presence of bacteria (da Silva et al., 2015b;Batista et al., 2017). This review aims to highlight the use of immunomodulatory plant lectins in contending infection provoked by bacterial, fungal and protozoan pathogens. The lectins selected for this review did not exhibit direct inhibition of microbial growth (using *in vitro* assays), thus their *in vivo* actions are related to their ability to target the immune system. Initially, these *in vivo* studies were primarily focused on mouse models of infection and are summarized in Table 1.

2. **Plant Lectins to combat bacterial infections**

As mentioned before several papers have investigated the potential of plant lectins direct inhibit bacterial growth (Souza et al., 2013;da Silva and Correia, 2014;Coelho et al., 2017), however, only few experimental studies are available about their *in vivo* effects (as illustrated in Figure 1). These lectins are well known due their ability to modulate the host immune system).

2.1. Benefits of prophylactic and therapeutic treatments with Concanavalin A in *Klebsiella pneumoniae* infection

The most studied plant lectin is Concanavalin A (ConA) which is isolated from *Canavalia ensiformis*. ConA is able to stimulate the proliferation of immune cells and enhance the expression of toll-like receptors (Sodhi et al., 2007;da Silva and Correia, 2014). In order to evaluate whether ConA immunodulatory properties could inhibit a bacterial infection, a murine model based on intragastrical inoculation of *Klebsiella pneumoniae* was employed (Kuo et al., 2007). *K. pneumoniae* is a pathogen commonly associated with nosocomial infections that can invade tissues provoking damage on essential organs (such as liver necrosis) and sepsis (Wu et al., 2017). Although ConA had no effect on the *in vitro* bacterial growth, beneficial effects were observed when infected mice were submitted either to prophylactic or therapeutic treatment with this lectin. The pretreatment (2 h before infection) with single doses of ConA (1 mg/Kg or 2 mg/Kg) enhanced the mice survival to 55% after 9 days of infection (the survival rate for untreated infected mice was 10%). When ConA (2 mg/Kg) was administered in consecutives doses (2h before infection followed by other doses at 48h and 96h) the animal survival rate was 83% (the best effect observed in this study). The last treatment was based on the administration of two successive doses at 24 h or 72 h after infection, resulting in a 50% of mice survival for ConA at 2 mg/mL. The effects of ConA were further demonstrated by the inhibition of liver necrosis induced by *K. pneumoniae*, reduced levels of aspartate aminotransferase and alanine aminotransferase and bacterial survival in blood and liver (Kuo et al., 2007).

2.2. ConBr and CFL have prophylactic effects on *Salmonella enterica* infection

Recently, the prophylactic effects of the lectins isolated from *Canavalia brasiliensis* (ConBr) and *Cratylia argentea* (CFL) were evaluated in an experimental model of *Salmonella enterica* serovar Typhimurium infection (Silva et al., 2016a). Both lectins were previously reported as immunodulatory agents (de Oliveira Silva et al., 2011). Firstly, the authors showed that these lectins did not have anti-*S. enterica* activity in concentrations ranging from 0.019 μg/mL to 10,000 μg/mL. Afterwards, each lectin was inoculated into Swiss mice (intraperitoneal route; i.p.) at different concentrations (1 mg/kg, 5 mg/kg and 10 mg/kg) one day prior bacterial infection (i.p.). Both lectins

showed a 70% to 80% dose-dependent survival rate increase effect after 7 days of treatment, for ConBr and CFL, respectively. When the lectins were administered (at 10 mg/kg) daily for three days prior to bacterial infection (i.p. route), the survival ratios were 90% for CFL and 100% for ConBr. The authors also showed reduction of bacterial growth into the peritoneal cavity, bloodstreams, spleen and the liver of lectins pre-treated animals. Furthermore, both lectins reduced the amounts of TNF-α and IL-10 cytokines in the peritoneal fluid, but IL-1 was only reduced using ConBr (Silva et al., 2016a). In a later paper, these lectins were shown to inhibit the colonization of Swiss mice peritoneal macrophage by *Salmonella*, through modulation of the expression of TLR and inflammatory mediators (cytokines and nitric oxide) (Batista et al., 2017).

3. Plant Lectins for treatment of fungal infections

Despite the use of antifungal agents, the invasive fungal infections are responsible for high rates of morbidity and mortality (Badiee and Hashemizadeh, 2014;Camplesi et al., 2017;Enoch et al., 2017). For example, cryptococcosis is responsible for 1 million cases of meningoencephalitis, especially in HIV positive individuals, with 624,000 death per year (McMullan et al., 2013). Yeasts from *Candida* genus, particularly *Candida albicans*, are commonly found as etiological agent of neonatal bloodstream infections (Fu et al., 2017;Vaezi et al., 2017).

Other example is the neglected disease paracoccidioidomycosis, the most important systemic mycosis in Latin America (mainly in Brazil) with high mortality rates (de Macedo et al., 2016). In fact, paracoccidioidomycosis is the eighth most important cause of mortality among chronic infectious diseases, reaching rates of 1.65 deaths per 106 inhabitants (de Oliveira et al., 2015). Collectively, these invasive fungal infections are more prevalent in immunocompromised patients (Woyciechowsky et al., 2011;Kaur et al., 2017;Sungkanuparph et al., 2017). In addition, they are in general also associated with antifungal resistance, making their treatment ineffective for most cases (Gullo et al., 2013;Chowdhary et al., 2014;Scorzoni et al., 2017).

Taken together, these factors point out the need of studies, using both *in vitro* and *in vivo* models, for the development of new therapeutic alternatives to treat fungal infections. In this scenario, lectins with immunomodulatory compounds have been emerging as promising options (Armstrong-James and Harrison, 2012;Datta and Hamad, 2015). Cytokines, antibodies, opsonins, and immunomodulatory compounds

(combined or not with antifungals drugs) are therapeutic alternatives for the treatment of fungal infections (Armstrong-James and Harrison, 2012;Datta and Hamad, 2015;Posch et al., 2017;Scorzoni et al., 2017), such as cryptococcosis (Antachopoulos and Walsh, 2012), invasive *Candida* infections (Safdar et al., 2004) and aspergillosis (Stuehler et al., 2011). In fungal infection models, large number of lectins have been applied *in vitro* and *in vivo* in order to develop new antifungal strategies (Islam and Khan, 2012;Coelho et al., 2017). Some examples of plant lectins able to modulate fungal infections are provided below.

3.1. Con A pretreatment protects mice from *Candida albicans* infection

The effects of ConA in an experimental model of *Candida albicans* infection have been associated with activation of antifungal responses by increasing of phagocytosis and killing of yeast cells by macrophages and neutrophils (Loyola et al., 2002;Moresco et al., 2002). However the literature does not describe any direct effects of ConA on *C. albicans* viability or virulence. In the first paper, by Loyola et al (2002), ConA was intraperitoneally administrated and, after 6 h, the collection of neutrophils and macrophages from peritoneal exudate was performed. ConA administration increased the number of peritoneal cells and their *in vitro* ability to kill *C. albicans* (in both yeast and germ tube forms) and increased the expression of mannose receptors. Furthermore, ConA pre-treatment also increased the survival of animals challenged with *C. albicans* (6 h after the lectin inoculation) (Loyola et al., 2002). These data were confirmed by a similar work where ConA efficiently promoted the antifungal action of peritoneal macrophages from suckling and adult mice by increasing the phagocytosis and killing of *C. albicans*. This paper also showed that ConA protected suckling mice against intraperitoneal infection with *C. albicans* (Moresco et al., 2002).

3.2. Artin M has prophylactic and therapeutic effects on fungal infections.

The mannose-specific lectin present in *Artocarpus integrifolia* (Moraceae) seeds, nominated Artin M, is a well-known immunomodulatory protein able to stimulate neutrophils migration by haptotaxis (Ganiko et al., 2005;Souza et al., 2013). This capacity is due to its interaction with mannose residues, commonly found at extracellular matrix components (such as laminin), helping the cell migration into injured tissues. This is an important phenomenon in the inflammatory response against

infections (Ganiko et al., 2005;Souza et al., 2013). *A. integrifolia* seeds are also sources of jacalin, a galactose-binding lectin with the characteristic beta-prism-I fold (Raval et al., 2004). This domain consists in four-stranded beta-sheets and the lectins with this domain are assembled in a family called Jacalin-related lectins (JRL) (Esch and Schaffrath, 2017).

The prophylactic inoculation (3 days before infection) of the crude extract of *A. integrifolia* seeds (containing Artin M and jacalin) resulted in the enhanced survival and reduced liver injury of Swiss mice infected with *C. albicans*. These effects were not observed when mice were treated with jacalin alone. Using the same protocol, the authors showed that the Artin M alone or in combination with jacalin induced a Th1 and Th17 response mediated by dectin-1 and mannose receptors, resulting in a significant increase of TNF-α production, phagocytic and candidacidal activities (Custodio et al., 2011). Similarly, it was demonstrated that Artin M increased the TNF-α production and phagocytic activity of *C. albicans* by mice macrophages. These actions of Artin M were mediated by dectin-1 and mannose receptors (Loyola et al., 2012).

In addition, Artin M (in both native and recombinant form) also showed efficacy against the infection caused by *Paracoccidioides brasiliensis* (Coltri et al., 2008). The authors performed an elegant work where they first determined that the best treatment schedule consisted in the subcutaneous administration of Artin M in single dose (0.5 µg of KM in 50 µL of PBS) and 10 days after infection with *Paracoccidioides brasiliensis*. Mice treated with Artin M displayed reduced levels of yeasts on their lungs and consequently less pulmonary lesions. These effects were induced through production of IL-12 by a TLR-2 dependent mechanism (Coltri et al., 2008).

3.3. Cramoll has therapeutic benefits in mice infected with *Cryptococcus gatti*.

A recent paper reported the use of the lectin purified from seeds of *Cratylia mollis* (pCramoll or Cramoll 1,4) for the treatment of mice infected with *Cryptococcus gatti*. *C. mollis* is an endemic plant of Caatinga (Brazil semi-arid area), a plant from the Brazilian exclusive biome. pCramoll is a mannose specific lectin and it has shown several biotechnological applications, including induction of cell proliferation (Maciel et al., 2004;da Silva et al., 2015a), *in vitro* immunomodulation (de Melo et al., 2010;da Silva et al., 2015b), wound healing (Albuquerque et al., 2017) and anticancer properties (da Cunha et al., 2016). Particularly, the immunomodulatory ability of pCramoll has

been demonstrated in an *in vivo* model of wound healing in immunocompromised mice (de Oliveira Silva et al., 2011) and in experimental infection with *Schistosoma mansoni* (de Oliveira Silva et al., 2011). Cramoll, however, did not show antimicrobial activity *in vitro*.

Initially, pCramoll was administered in different concentrations (1 µg, 250 µg and 500 µg) 1 day before the intratracheal infection with *C. gattii*. Afterwards, every 10 days after infection a new dose of lectin was given to the mice. pCramoll enhanced the mice survival equally in all tested concentrations. When combined with fluconazole (azole antifungal drug), the best results were found for this lectin at 1 µg. pCramoll alone or in combination with fluconazole decreased pulmonary fungal burden of mice. These effects were associated with an increase of inflammatory infiltrate on the lungs, and modulatory action on cytokines levels (down-regulation of IFNγ, IL-6, IL-10 and up-regulation of IL-17A). The combined treatment of pCramoll and fluconazole also significantly decrease the fungal load in the brain, reducing the morbidity and behavior changes caused by the infection (i.e. neuropsychiatric state, motor behavior, autonomic function, tone and muscle strength and reflex/sensory function). Moreover, *in vitro* analysis revealed that bone marrow-derived macrophages treated with pCramoll were more able to phagocyte *C. gattii*, with higher production of reactive oxygen species, and decreased the intracellular fungal proliferation (Jandú et al., 2017). These findings are summarized in figure 2.

4. Plant Lectins and protozoan infections

Some plant lectins (Jacalin, Artin M, ScLL) have been also successfully applied against infections caused by protozoan. These lectins were used in infections caused by *Trypanosoma cruzi* (Albuquerque et al., 1999), *Leishmania* spp. (Panunto-Castelo et al., 2001), *Neospora caninum* (Cardoso et al., 2011;Cardoso et al., 2012) and *Toxoplasma gondii* (Leandro Peixoto Ferreira de et al., 2016). In these studies, the lectins were used also as adjuvants in combination to parasite antigens in order to induce a more efficient immunization.

4.1. Jacalin as adjuvant in *Trypanosoma cruzi* infection

The effects of jacalin in the humoral immune response towards *T. cruzi* infection were evaluated using Balb/c mice (Albuquerque et al., 1999). Jacalin is able to modulate

cellular and humoral immunity, which makes it a potential candidate for use as an adjuvant compound (Miyamoto et al., 2012;Danella Polli et al., 2016). Initially, the animals were inoculated with *T. cruzi* antigens in the presence or not of Jacalin. The mice submitted to immunization with *T. cruzi* antigens plus Jacalin produced more antibodies (and faster) than animals immunized only with parasite antigens. The efficiency of immunization using *T. cruzi* antigens plus Jacalin was also demonstrated by challenging one-month-old immunized mice with trypomastigotes. These animals showed reduced levels of parasitemia when compared to non-immunized mice. Similarly, mice immunized with viable *T. cruzi* epimastigotes (at 1.0 X 10^5 or 1.0 X 10^6) plus Jacalin produced more antibodies than mice inoculated with parasites alone. However, the combined inoculation of 1.0 X 10^5 *T. cruzi* epimastigotes plus jacalin resulted in lower levels of parasite after nine days of infection than animals immunized only with epimastigotes. Jacalin alone did not protected the animals from infection (Albuquerque et al., 1999) (Figure 3).

4.2. Artin M is a potent adjuvant in leishmaniasis model

The evidence that Artin M could be useful to treat protozoan infections was obtained from the ability of this lectin to induce the expression of IL-12p40, which could drive the production of Th1 cytokines instead of the Th2 pattern, typical of unresponsive parasite infections (Figure 4). The authors performed a combined administration of Artin M and soluble leishmanial antigen (SLA) into the footpad of BALB/c mice. SLA injected animals showed higher levels of IL-4 than the group treated with SLA+Artin M, while the IFN-γ concentration was higher in SLA+Artin M group. The animals treated with Artin M alone or SLA+Artin M were also more resistant to *Leishmania major* infection, and these mice showed smaller lesions than those groups treated with SLA alone or untreated animals (Panunto-Castelo et al., 2001).

Recently, it was shown that Artin M improved the *in vitro* killing of *L. major* by neutrophils through modulation of effector mechanisms, such as enhanced excretion of inflammatory cytokines, reactive oxygen species and neutrophil elastase and myeloperoxidase. In addition, the infected-neutrophils treated with Artin M did not form neutrophil extracellular traps and showed shorter life span than untreated infected

cells, both characteristics that may favor the maintenance of host tissue integrity (Ricci-Azevedo et al., 2016).

Artin M was also effective as an adjuvant of SLA in immunization against *Leishmania amazonensis* (Teixeira et al., 2006). Artin M + SLA administration reduced the parasite amounts in the footpad of mice infected with *L. amazonensis* 15 days after immunization, although the lesion size was not reduced. Mice treated only with Artin M showed smaller lesion and decreased parasite load in relation to the untreated group (but the levels of parasites were not smaller than Artin M+ SLA group). Other pro-inflammatory plant lectins (ConBr and PAA purified from *Pisum arvense*) were not able to inhibit the lesion size in mice infected with *L. amazonensis*, even when inoculated in combination with SLA. However, the association of ConBr and SLA resulted in smaller number of parasites in the footpad of immunized animals when compared to the controls (Teixeira et al., 2006).

4.3. ScLL and prophylactic treatment of *L. amazonensis*

The lectin obtained from leaves latex of *Synadenium carinatum* (ScLL) has also shown protective effects in a murine model of leishmaniasis induced by *L. amazonensis*. In this study, BALB/c mice received three doses of ScLL (10, 50 or 100 µg/animal) in the presence or not of SLA (25 µg/animal) with intervals of 15 days. Three days after this immunization, the animals were infected with *L. amazonensis* promastigotes in their left footpad. When administrated alone at 100 µg/animal, ScLL were more effective than when associated with SLA (as shown by reduction of lesion size and parasite load). Thus, this lectin showed a better potential as prophylactic agent than as adjuvant. The mice treated with SLA also showed higher levels of IgG2a and Th1 cytokine expression (IFN-γ, IL-12, and TNF-α) (Afonso-Cardoso et al., 2007). *In vitro* cell based analysis showed that ScLL reduced the association of macrophages and *L. amazonensis*, inducing the production of pro-inflammatory cytokines (IL-1, IL-12, and TNF-α) in a nitric oxide independent pathway (Afonso-Cardoso et al., 2011).

4.4. Adjuvant properties of Artin M, Jacalin and ScLL in experimental neosporosis

Neospora caninum (Apicomplexa), etiologic agent of neosporosis, is a prevalent intracellular parasite associated with cases of abortion in cattle and neuromuscular disease in dogs (Donahoe et al., 2015). The potential adjuvant actions of Artin M,

Jacalin and ScLL have been evaluated in murine models of neosporosis. In the first report, C57BL/6 mice received (subcutaneously) three doses with two week-intervals of *N. caninum* lysate antigen (NLA; 25 µg/animal) associated with Artin M (1 µg/animal) or Jacalin (100 µg/animal). Animals immunized with Artin M+NLA showed higher levels of specific antibodies against *N. caninum* (IgG, IgG1 and IgG2a) than all others groups in all times evaluated (15, 30 and 45 days after immunization). The association of Jacalin and NLA also enhanced the levels of total IgG in relation to animals immunized with NLA alone in all times, however the levels of IgG1 were only higher until 30 days after immunization. Animals immunized with Jacalin and NLA showed similar levels of IgG2 than NLA group. The mice were then infected with lethal doses of *N. caninum* tachyzoites 60 days after immunization. Artin M + NLA combination resulted in 86% of protection, while the other immunized groups (NLA + JAC, NLA, Artin M or JAC) were partially protected. In addition, Artin M + NLA reduced the number of parasites in the brain and induced a more robust inflammatory profile. The results highlights that Artin M has more potential to be used as adjuvant for neosporosis than Jacalin (Cardoso et al., 2011).

Cardoso et al. (2012) demonstrated the adjuvant and immunomodulatory effects of ScLL in a similar work. The authors showed that C57BL/6 mice dendritic cells produced inflammatory cytokines when treated with NLA + ScLL or ScLL alone. NLA (25 µg/animal) associated with ScLL (1 µg/animal) were inoculated in C57BL/6 mice three time for 45 days. The animals that received NLA + ScLL produced higher levels of IgG and IgG1 than the NLA immunized mice. The NLA + ScLL and ScLL groups were also more resistant to infection by *N. caninum* tachyzoites (which occurred 60 days after the last immunization) (Cardoso et al., 2012).

4.5. Artin M and ScLL for therapy of acute toxoplasmosis

Recently, the therapeutic properties of both Artin M and ScLL were studied in a model of murine toxoplasmosis. For this, C57BL/6 mice were orally infected with cysts of *T. gondii*, and treated intraperitoneally for 6 days with ScLL (50 µg), Artin M (1 µg) or ScLL (50 µg) plus Artin M (1 µg). The treatment with ScLL was more efficient, resulting in 100% survival, while 60% of the Artin M + ScLL treated animals and only 40% of Artin M treated group survived. The best results obtained with ScLL alone are related to its capacity to induce the production of Th1 cytokines (IL-2, IFN-γ, and IL-6)

resulting in reduced levels of parasite in the brain (Leandro Peixoto Ferreira de et al., 2016). Other study showed the action of eutirucallin on the *in vitro* infection of Human Foreskin Fibroblasts (HFF) by *T. gondii* (Palharini et al., 2017). Eutirucallin is a RIP-2 type lectin obtained from the latex of *Euphorbia tirucalli*, which also displays immunostimulatory action (increasing neutrophils migration and release of NO, IL12p40 and TNF-α by peritoneal macrophages) (Santana et al., 2014). Eutirucallin inhibited infection and intracellular replication of *T. gondii* with IC50 of 173.2 µg/mL and 133.3 µg/mL, respectively (Palharini et al., 2017).

5. Conclusions and perspectives

The high immunomodulatory abilities of plant lectins was proven efficient to combat microbial infections in different experimental models. Depending on the type of lectin and the kind of infection, the lectin showed better prophylactic or therapeutic behavior. As inductors of Th1 response, some lectins were used as adjuvant agents. These immunotherapeutic studies have also improved our knowledge about the pathogen-immunity relationship, and could be helpful to provide insights for the development of new therapeutic strategies. All these successful examples of plant lectins encourage the study of others lectins with immunomodulatory capabilities for the treatment of infectious diseases. However, it is always important to remind that the possible adverse effects (for example TNF-α-mediated hepatitis, renal and intestinal injury) should be evaluated before the clinical application of these plant lectins. The side effects depend on type of lectin, dose and administration route. To the best of our knowledge, the anti-infective effects of plant lectins have not been clinically evaluated in humans (although some lectins have been tested in clinical trials for cancer therapy).

Another challenge is the purification yields, that for some lectins could not be suitable for large scale production. The advances in protein engineering (recombinant protein production, structure-function improvement) and drug delivery technologies (liposomes, microcapsules, etc.) may improve the protein production, stability and their pharmacokinetics properties (delivery, bioavailability, controlled release and targetability). These actions may result in enhanced therapeutic index and may reduce likely side effects. Furthermore, the combination of *in silico* approaches and analytical tools for protein have provided more insight in lectin and ligand interactions. In

summary, these stimulating research data pave the way for the future use of plant lectin as immunomodulatory agents to combat microbial infections.

Conflict of interest

The authors have no conflict of interest to declare.

Author Contributions

All authors contributed to conception, design, and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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Tables and Figures

Table 1: Application of immunomodulatory lectins in *in vivo* experimental models of microbial infections.

Lectin	Plant Specie	Sugar specificity	Pathogen	Type of use	Reference
			Candida albicans	Prophylactic	Custodio et al. (2011)
Artin M	*Artocarpus integrifolia*	Mannose	*Paracoccidioides brasiliensis*	Therapeutic	Coltri et al. (2008)
			Leishimania sp.	Adjuvant	Panunto-Castelo et al. (2001) Teixeira et al. (2006)

			Toxoplasma gondii	Therapeutic	Santana et al. (2014)
			Neospora caninum	Adjuvant	Cardoso et al. (2011)
CFL	*Cratylia argentea*	Glucose/mannose	*Salmonella entérica*	Prophylactic	Silva et al. (2016a)
Con A	*Canavalia ensiformis*	Mannose	*Klebsiella pneumoniae*	Prophylactic and therapeutic	Kuo et al. (2007)

			Candida albicans	Prophylactic	Loyola et al. (2002); Moresco et al. (2002)
Con Br	*Canavalia brasiliensis*	Glucose/mannose	*Salmonella entérica*	Prophylactic	Silva et al. (2016a)
Cra moll	*Cratylia mollis*	Mannose	*Cryptococcus gatti*	Therapeutic	Jandú et al., (2017)
Jacal in	*Artocarpus integrifolia*	Galactose	*Trypanosoma cruzi*	Adjuvant	Albuquerque et al. (1999)

			Neospora caninum	Adjuvant	Cardoso et al. (2011)
			Leishimania amazonenses	Prophylactic	Afonso-Cardoso et al. (2011)
ScL	*Synadeniu*	Galactose	*Neospora*		Cardoso et
L	*m carinatum*		*caninum*	Adjuvant	al. (2012)
			Toxoplasma gondii	Therapeutic	Santana et al. (2014)

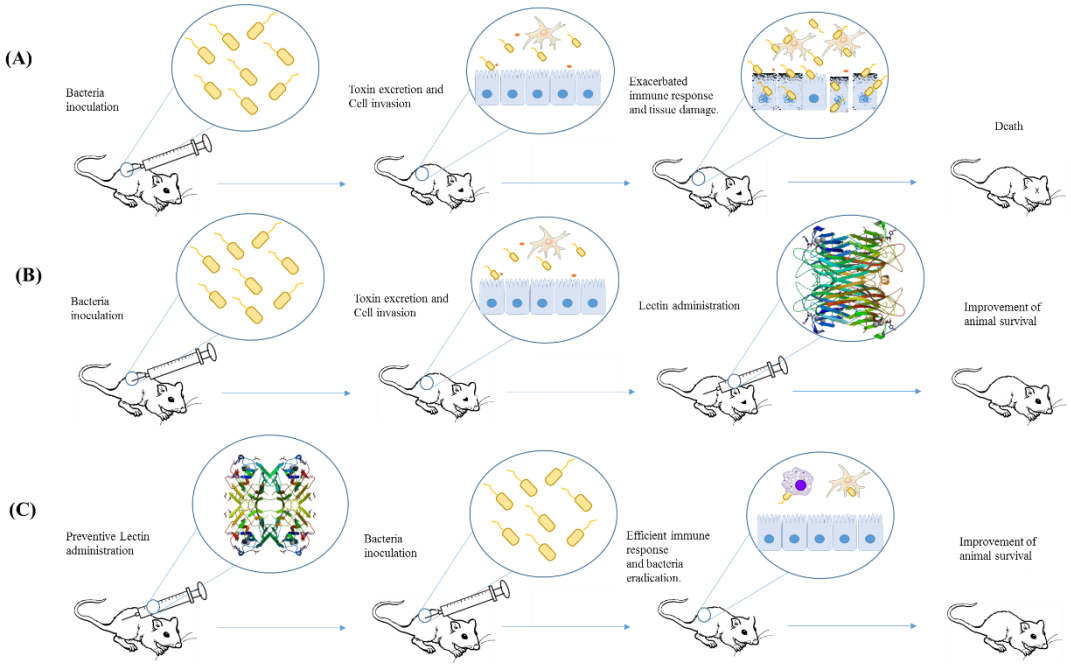

Figure 1: Schematic representation of studies employing plant lectins in experimental bacterial infection. (A) After inoculation, bacteria can invade cell and provoke damage by releasing different virulence factors and inducing an exacerbated immune response. The final effect is organ dysfunction and animal death. (B) Administration of a lectin (for example ConA) after infection could improve the mice response against bacterial infection and increase the animal survival. (C) Pre-treatment of animal with lectin (such as ConBr) induce an immune response able to protect against bacterial virulence resulting in the improvement of animal survival. The proteins structures were obtained from Protein Data Bank, the ID codes are 4PF5 and 4P14 for ConA and ConBr, respectively.

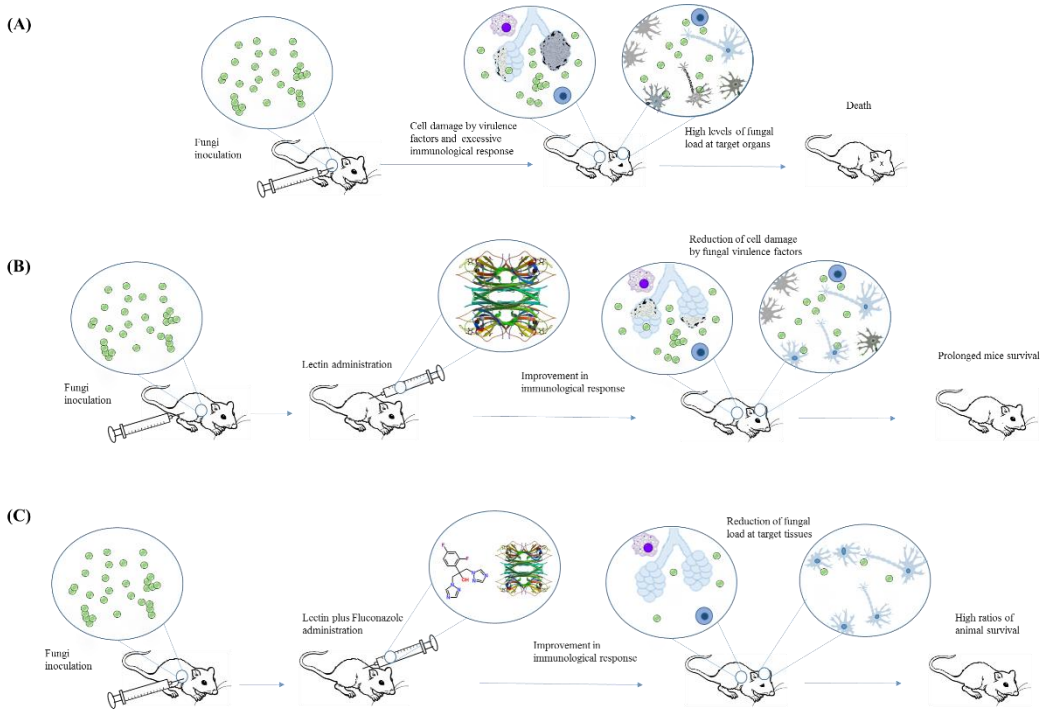

Figure 2: An overview of the effects of Cramoll alone or in combination with fluconazole in an experimental cryptococcosis model. (A) *Cryptococcus gattii* provokes tissue damage and organ dysfunction by releasing different virulence factors and inducing an exacerbated immune response. (B) When Cramoll was administrated alone (B) or in combination with fluconazole (C), infected mice exhibited increased ratios of survival and reduced levels of morbidity and behavior alteration. The PDB code for Cramoll structure is 1MVQ.

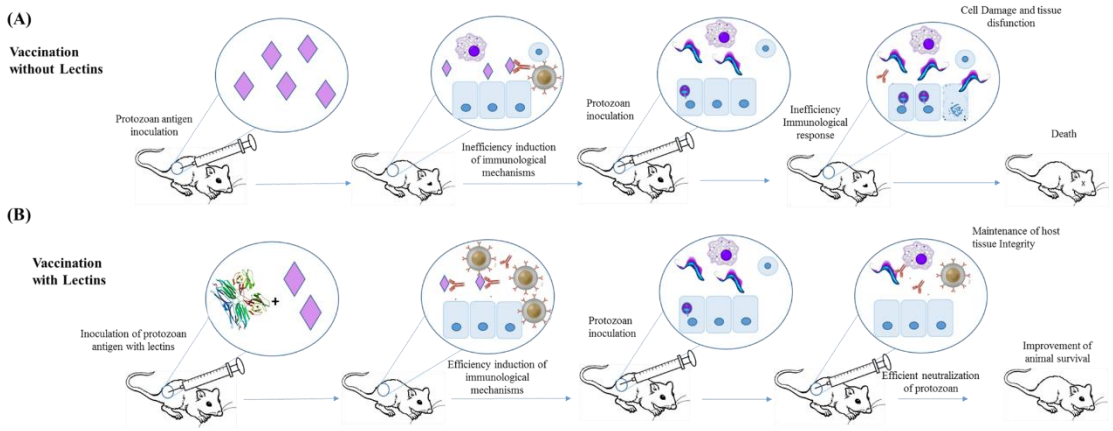

Figure 3: Application of Jacalin as adjuvant for immunization against *T. cruzi* (A) Mice were immunized with *T. cruzi* antigens and after one month infected with *T. cruzi*. The antigens failed in inducing an efficient protective humoral response. (B) Animal subjected to immunization with jacalin (PDB ID 1JAC) plus *T. cruzi* antigens showed higher antibodies titers and lower parasitemia levels than mice that received only *T. cruzi* antigens.

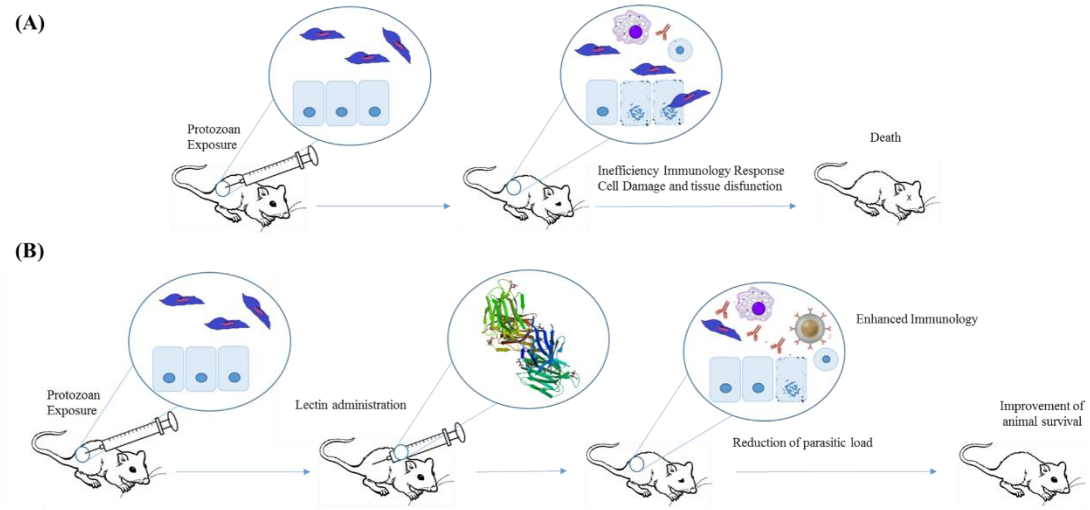

Figure 4: Therapeutic effects of plant lectins in protozoan infections. Experimental exposure of mice to protozoan results in cell damage, tissue destruction and consequently animal death (A). Lectins can improve protozoan-infected animal survival by increasing production of Th1 cytokines. The induction of pro-inflammatory response leads to a reduction in parasite levels and organ dysfunction (B). The structure of Artin M was obtained from PDB (ID: 1J4U).

ANEXO B - TREATMENT WITH PCRAMOL ALONE AND IN COMBINATION WITH FLUCIONAZOLE PROVIDES THERAPEUTIC BENEFITS IN *C. GATTI* INFECTED MICE

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Abstract

Cryptococcus gattii is one of the main causative agents of cryptococcosis in immunocompetent individuals. Treatment of the infection is based on the use of antimycotics, however, the toxicity of these drugs and the increase of drug-resistant strains have driven the search for more effective and less toxic therapies for cryptococcosis. pCramoll are isolectins purified from seeds of *Cratylia mollis*, a native forage plant from Brazil, which has become a versatile tool for biomedical application. We evaluated the effect of pCramoll alone and in combination with fluconazole for the treatment of mice infected with *C. gatti*. pCramoll alone or in combination with fluconazole increased the survival, reduced the morbidity and improved mice behavior i.e. neuropsychiatric state, motor behavior, autonomic function, muscle tone and strength and reflex/sensory function. These results were associated with i) decreased pulmonary and cerebral fungal burden and ii) increased inflammatory infiltrate and modulatory of IFNγ, IL-6, IL-10 and IL-17A cytokines in mice treated with pCramoll. Indeed, bone marrow-derived macrophages pulsed with pCramoll had increased ability to engulf *C. gattii*, with an enhanced production of reactive oxygen species and decrease of intracellular fungal proliferation. These findings point towards the use of pCramoll in combination with fluconazole as a viable, alternative therapy for cryptococcosis management.

KEYWORDS: Cryptococcosis, Immunomodulation, Lectins, *Cratylia mollis* lectin, Fluconazole, Survival

1. Introduction

Cryptococcus gattii is a pathogenic fungus that affects mainly immunocompetent individuals. The desiccated yeasts and spores are inhaled and enter the body via the respiratory system, finally infecting the central nervous system and causing meningo-encephalitis (Thompson et al. 2012), which frequently has a poor prognosis. Meningitis and meningo-encephalitis are typical in HIV/AIDS and transplant-recipient patients, but also in apparently healthy individuals (Sharon et al. 2012). The main therapies for cryptococcosis treatment caused by *C. gatti* are fluconazole (antifungal azole) and amphotericin B (antifungal polyene) (Reichert-Lima et al., 2016). Fluconazole is used in cases of pulmonary diseases with mild to moderate symptoms. In severe infections, amphotericin B is recommended (associated or not with 5-Flucytosine) followed by a prolonged therapy with fluconazole. Itraconazole, voriconazole and other azoles are recommended when the use of fluconazole is contraindicated or ineffective (Perfect et al., 2010). Resistance against antifungals (Zhai et al. 2013) enhances clinical failures and increases morbidity and mortality (Ghannoum and Rice, 1999). Furthermore, side effects due to the use of amphotericin B lead to dose-dependent nephrotoxicity frequently associated with increased mortality, requiring monitoring of the renal function of patients. Altogether, these findings increase the demand for new therapies against cryptococcosis.

Previous studies have shown that lectins from plants may have immunomodulatory effects: augmented recruitment and activation of mononuclear and polymorphonuclear leukocytes, development of Th1, Th2 and Th17 response and stimulation of phagocytosis (Da Silva and Correia, 2014). *Cratylia mollis* is a native forage plant endemic to the Semiarid Region of Brazil (Caatinga biome), and popularly known as camaratu bean. Four isolectins (Cramoll 1, 2, 3 and 4) can be purified from seeds of the plant. pCramoll (preparation containing isolectins 1 and 4) has binding sites for the specific recognition of glucose/mannose receptors and presents anthelmintic, antiprotozoal, antitumoral, healing and immunomodulatory effects (Maciel et al, 2004; Melo et al, 2011; Da Silva et al., 2014). Focusing on immunoregulatory properties, the aim of this study was to evaluate the immunomodulatory effect of pCramoll in a murine model of infection by *C. gattii*. Our results revealed that this lectin is able to increase survival, decrease fungal burden in organs and reduce the morbidity of cryptococcosis.

2. Material and methods

2.1 pCramoll

Cratylia mollis seed extract (10% w/v prepared in 0.15 M NaCl) was fractionated using ammonium sulphate (40–60% w/v) and the fraction obtained was submitted to affinity chromatography in a Sephadex G-75. The pCramoll preparation was bioselectively eluted with 0.3M of D-glucose in 0.15 M NaCl, dialyzed against 0.15 M NaCl for 24 h and subsequently lyophilized (Correia and Coelho, 1995) and diluted in PBS to analyze protein concentration, as determined by the BCA kit (Thermo Fisher Scientific Inc., Waltham, MA USA).

2.2 Phagocytosis assay, Intracellular Proliferation Rate (IPR), measurement of ROS and NO production by macrophages

Bone marrow cells were isolated as described previously (Weischenfeldt J, Porse B. 2008, Souza et al., 2016). Femurs and tibias from mice were removed, disinfected by immersion in 70% ethanol and the ends of each bone were cut. Both bones were flushed with 5mL of cold RPMI 1640 (HyClone, LGC Biotecnologia) using a 5-mL syringe and a 25-gauge needle. The cell suspension was centrifuged for 5 min at 1200rpm at 4°C and washed once with cold RPMI. Then, bone marrow cells were counted using a hemocytometer and the concentration was adjusted to 2×10^6 cells/mL for plating on tissue culture–treated petri dishes in BMM medium (RPMI supplemented with 30% L929 growth conditioning media, 20% bovine fetal serum [Gibco], 2 mM glutamine [Sigma], 25 mM HEPES pH 72, 100 units/mL of penicillin-streptomycin [Life Technologies]). Fresh media were added every 48 hours. Bone marrow–derived macrophages (BMDMs) were collected on day 7 and used for subsequent experiments. Under these conditions, macrophages/monocytes progenitors will proliferate and differentiate into a homogenous population of mature BMDMs, obtained >90% pure live macrophages (Zang et al., 2008, Bhattacharya et al., 2015, Heung and Hohl, 2016). For all analyses, the cells were infected with L27/01 yeasts cells (growth on Sabouraud Dextrose Agar (SDA) medium, for 48h at 35ºC) opsonized with 10% murine serum in RMPI suspension (0.4×10^6 cells/mL), at the proportion of 5 macrophages: 1 *C. gattii*, under cell treatment with 1μM or 5μM pCramoll and incubated for 3 or 24 hours at 37ºC under 5% CO_2.

The phagocytic index was performed using 24-well plates and a single coverslip 13mm in diameter. After 3 hours, these coverslips containing adherent cells were removed, stained with Giemsa and the index was calculated as the percentage of cells with internalized *C. gattii* 24 hours post-infection (Santos et al., 2014). Images from the phagocytosis assay were obtain using a Nikon COOLPIX 4500 camera coupled to a Nikon ECLIPSE E200 microscope with 100 x of magnification and analyzed using the ImageJ Protocol (http://rsbweb.nih.gov/ij/); the cell area (average of 100 cells per coverslip) was measuring as described by Baviskar (2011).

The intracellular proliferation rate (IPR) assay was performed as previously described (MA et al., 2009) with modifications. Non-internalized yeast cells in the supernatant were taken from the wells and the adherent phagocytes were washed with 200 µL PBS. These macrophages were lysed at 3h and 24 h with 200 µL of cold, sterile, distilled water and incubated for 30 minutes, then 100 µL was collected and plated on SDA medium, and the viable yeast cells were counted.

The intracellular proliferation rate IPR was calculated as the quotient of the intracellular yeast cell numbers at 24h (the point in time which featured the maximum intracellular yeast number) and 3h.The supernatant of phagocytosis assay was used for measurement of NO production using the Griess assay. The nitrite concentration was quantified by extrapolation from a sodium nitrite standard curve, determined at 540 nm with a microplate reader (Benchmark Plus, Bio-Rad, CA, US).

Macrophages/well in RPMI-1640 without phenol red (Sigma-Aldrich) were also infected with *C. gatti* (same conditions that phagocytosis assay), and the supernatant was used for ROS quantification using 2-7-dichlorofluorescein diacetate (DCFH-DA; Invitrogen, Life Technologies, Carlsbad, CA, USA) was used and the fluorescence was measured with a fluorometer (Synergy 2 SL Luminescence Microplate Reader; Biotek) the positive control was hydrogen peroxide at 10 µM (Ferreira et al. 2013).

2.3 Effect of pCramoll and fluconazole in the *Cryptococcus gattii* infection model

The protocol of the animal studies was approved by the Comissão de Ética no Uso de Animais (CEUA) at the Universidade Federal de Minas Gerais (Protocol 310/2014). All mice were housed in clean cages, with food and water *ad libitum*. The

controlled environment was set to a 12 h light/dark cycle at 23ºC. Mice C57BL/6, 5-6 weeks old (n= 6/group) were anesthetized by intraperitoneal (i.p.) injection of ketamine hydrochloride (60 $mg.kg^{-1}$) and xylazine (10 $mg.kg^{-1}$) in PBS, and then inoculated by an intratracheal infection with 30 µL of 10^4 CFU/animal of *C. gattii*, L27/01 strain. Intratracheal infection with the *C. gattii* L27/01strain (GVII molecular type) has been previously shown in other works (Santos et al., 2014; Ferreira et al., 2015).

Initially, infected mice were intraperitoneally (i.p.) treated every 10 days with 1µg, 250µg or 500µg of lectin alone (the first dose was administered one day before intratracheal infection). For the group treated with the combination fluconazole ($20mg.kg^{-1}$) and pCramoll at 1µg, 250µg or 500µg (the fluconazole was daily administered and every 10 days the association was used), or the mice were treated daily with fluconazole alone ($20mg.kg^{-1}$) for survival monitoring. The untreated group (NT), and not infected group (NI) were inoculated with PBS. All animals were monitored twice daily for survival and behavior parameter analysis (SHIRPA protocol). The SHIRPA protocol analyzes the behavioral and functional assessment of neurological diseases. The tasks are grouped into five functional categories: neuropsychiatric state, motor behavior, autonomic function, muscle tone and strength, and reflex and sensory function (Santos et al., 2014; Costa et al., 2016). All mice were examined daily and the score for each functional category was calculated as the total of the evaluated parameters according to Lackner et al. (2006) and Pedroso et al. (2010) using the EpiData 3.1 software. The supplementary Table S1 describes all the parameters analyzed in the SHIRPA protocol.

Furthermore, other groups of mice were i.t. infected and treated with pCramoll at 1µg per mouse (the concentration that provided the best results in the survival curve), combined or not with fluconazole to obtain lungs and brain at 15 or 35 days post-infection (d.p.i). The animals were euthanized by cervical dislocation under anesthesia. The lungs and brain were removed for determining the colony-forming units (CFU) as described previously (Santos et al., 2014). For this, the organs were homogenized with sterile PBS and plated in SDA medium. Specifically, 100 mg or 50mg of lung tissue was homogenized with 1 mL or 0.5 mL of extraction solution of protein, containing PBS (pH 7.0) and anti-proteases (0.1mM PMSF, 0.1mM benzethonium chloride, 10mM EDTA, 20 Kallikrein inhibitor units of aprotinin A, all purchased from Sigma-Aldrich and 0.05% tween 20). The samples were centrifuged for 10 min at 3000x g, at 4°C and

the supernatant was frozen at -20°C and utilized for cytokine analysis. The levels of IL-10 and IL-6 were determined using commercially available antibodies according to the manufacturer's instructions (R&B Systems, Minneapolis, MN, USA) and the levels of IFN-γ and IL-17A were measured by cytometric bead array (BD Biosciences, San Jose, CA, USA). Also, the bronchoalveolar lavage fluid (BAL) was obtained and centrifuged at 1200 rpm for 5 min at 4ºC. The cell pellet was suspended in 3% albumin solution (100 μl). A 10ul aliquot of albumin solution containing the cells washed was diluted in 10uL of Türck for cell count totals in a Neubauer chamber with an optical microscope at 40x magnification. For differential counts, smears were made in cellspin (CT-2000, CIENTEC), stained using the Giemsa method and cells were quantified by morphological criteria for the distinction of cellular types (mononuclear and polymorphonuclear) and the results were grouped according to the percent content of these two cells type (Maxeiner et al., 2007).

2.4 Myeloperoxidase (MPO) and N-acetylglucosaminidase (NAG) activities

The infiltration of neutrophil in the lungs was indirectly measured by the assay of myeloperoxidase activity (MPO) according to Costa et al. (2016), by measuring the change in optical density (OD) using 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma-Aldrich). The absorbance reading was taken at 450nm in a spectrophotometer. For N-acetylglucosaminidase activity (NAG), 2,24mMp-nitrophenyl-N-acetyl-β-D-glicosaminide (Sigma-Aldrich) was used and absorbance was determined at 400nm (Baltazar et al., 2014).

2.5 Histopathology

The lungs were removed during necropsy and immediately fixed in buffered 10% formalin (v/v). The tissue was embedded in paraffin, and the sections were stained with hematoxylin and eosin (Sigma) and examined under light microscopy.

2.6 Statistical analysis

Statistical analysis of all data were performed using GraphPad Prism version 5.0 with $p<0.05$ considered significant. The survival curve was plotted by Kaplan-Meier analysis and the results were analyzed using the log rank test, for behavior parameters the area under the curve was analyzed. Also, the results of the phagocytosis assay,

intracellular proliferation rate, measurement of ROS and NO production by macrophages, MPO and NAG activity, and quantification of cytokines were analyzed by analysis of variance (ANOVA) followed by Dunn's Multiple Comparison Test and the Student's t test.

3. Results

3.1 Preliminary tests of toxicity and antifungal activity of pCramoll

Previously, the pCramoll toxicity analysis in *Caenorhabditis elegans* model was performed complementarily. Non-toxicity was verified in these worms (data not shown) and confirms the non-cytotoxicity of the pCramoll concentration used in this study and published previously by Silva et al., 2014. Subsequently, we investigated the antifungal activity of pCramoll against *C. gattii* by screening and determining the minimum inhibitory concentration (MIC). Neither of the concentrations tested (0.93 to 120 µg/mL) were able to inhibit fungal growth (data not shown). Indeed, no interaction between pCramoll and fluconazole was obtained by the checkerboard test (data not shown).

3.2 pCramoll increases phagocytosis and fungicidal activity of bone marrow-derived macrophages (BMDM)

pCramoll at 1µM increased the phagocytic index after 3h of incubation (Figure 1A), compared with the untreated group. At the same time, the levels of ROS were increased in the presence of pCramoll at 1µM and 5µM, both for infected and uninfected macrophages (Figure 1B), especially with pCramoll at 1 µM with significant increase in the production of nitrosative species (1C). This increase in ROS levels was associated with a reduced intracellular proliferation rate (Figure 1D). Another interesting observation was the presence of macrophage aggregates, vacuoles and increased expansion of the cells (area) in the presence of increasing concentration of pCramoll (Figure 1E). Bone marrow-derived macrophages stimulated with pCramoll at 1µM and 5µM showed, 3h post-incubation, 0.080µm^2 and 0.197 µm^2 of area, respectively, while at 24h: 0.214µm^2 and 0.483µm^2 respectively. There was an increase of up to 5× in cell area compared with untreated macrophages infected with *C. gattii* after 3h and to 7× after 24h of incubation (Figure 1F).

3.3 pCramoll and Fluconazole increase the survival and improve the behavior of animals infected with *C. gattii*

An increase in survival of animals treated with pCramoll was observed. The untreated group (NT) showed a median survival (MS) of 20 days (Figure 2A), while the MS for groups treated with the lectin at 500µg, 250µg and 1µg, were 30.5 ($p < 0.05$) (data not shown), 30 ($p<0.01$) (data not shown) and 29.5 ($p < 0.01$) days (Figure 2A), respectively, an increase of 50% (approximately) for all doses tested, and an independent dose response. pCramoll at 500µg, 250µg and 1µg was also tested in uninfected animals and no change in the survival/behavior of animals compared to NI mice was found (data not shown). When combined with fluconazole, the influence of lectins on survival was dose dependent. The group treated with fluconazole alone showed an MS of 46 days, while the groups where pCramoll was combined with the antifungal demonstrated a MS of 51 days for 500µg of pCramoll (data not shown), 58 days for 250µg of pCramoll (data not shown) and 62 days for 1µg of pCramoll ($p < 0.05$) (Figure 2A). Based on these results, the dose of 1µg of pCramoll combined or not with fluconazole was used in the further experiments. Indeed, the cellular polysaccharide extracted from strain L27/01 (PSC) of *C. gattii* was associated with pCramoll and fluconazole, and were tested in our model. The administration of PSC only hurried the death of infected animals (MS of 17 days), compared to the NT group (20 days). The same was observed where PSC was associated with pCramoll and fluconazole (MS of 43 days), compared to the FCZ-treated group (MS of 46 days), where the animals had previously succumbed (data not shown).

The behavior analysis of basic functions such as sensory-reflex (Figure 2B), muscle tone and strength (Figure 2C) demonstrated improved behavior by day 20 post-infection when animals were treated with pCramoll, fluconazole and the combination compared to the untreated group (NT). Moreover, the combination is significantly more efficient than fluconazole alone ($p < 0.05$). A similar behavioral profile was observed for parameters involved in the neuropsychiatric state (Figure 2D) and body weight (Figure 2E), which improved by day 22 day post-infection ($p < 0.05$) for all the treated groups, but with better performance for groups treated with the combination ($p < 0.05$). There was no difference between the treated and untreated groups for motor behavior (Figure 2F). Finally, as shown in Figure 2G, autonomic function was only improved when the combination was used ($p < 0.05$).

3.4 The combination pCramoll + fluconazole reduces fungal burden and increases inflammatory response

All the treatments reduced the fungal burden in the lungs after 15 dpi. At 35 days post infection, fluconazole combined with pCramoll was better than fluconazole alone in reducing pulmonary fungal burden ($p<0.05$) (Figure 3A). In brain tissue, only the use of fluconazole reduced the fungal burden after 15dpi (Figure 3B), however, the combination (fluconazole + pCramoll) was able to reduce cerebral fungal burden significantly when compared with fluconazole alone after 35 dpi ($p <0.05$). Histological analysis of lung tissue from untreated infected mice at 15 dpi showed numerous yeasts in the pulmonary parenchyma, mild perivascular infiltrate and alveolar thickening. Moderate to intense perivascular inflammation was observed in lung tissue from pCramoll, and pCramoll + fluconazole groups. Lungs from treated groups showed less yeast in alveolar space than the untreated group (Figure 3C).

Regarding the inflammatory infiltrate in the bronchoalveolar lavage fluid (Figure 4A), we verified a predominance of mononuclear cells for all groups except the one treated with the combination, for which there was a balance between mononuclear and polymorphonuclear cells, at both 15 and 35 dpi (Figure 4B). Additionally, MPO (Figure 4C) and NAG (Figure 4E) activities were performed in order to confirm the presence of neutrophils and macrophages, respectively, where the combination demonstrated higher activities of both enzymes at 15 dpi.

The levels of IFN-γ (Figure 4F) were higher for the group treated with the combination at 35 dpi. The levels of IL-6 (Figure 4G) and IL-10 (Figure 4H) were augmented in the NT group. An increase in IL-17A (Figure 3K) was verified for the combination and for fluconazole alone at 15 dpi.

4. Discussion

We observed complementarily that pCramoll did not show toxicity in the *C. elegans* model, corroborating previously published *in vitro* cytotoxicity data in

mammalian cells (Melo et al., 2010; Melo et al., 2011, Da Silva et al., 2015). In addition, pCramoll did not present antimicrobial action against *C. gatti*, an activity rarely found in the legume lectin domain (Dias et al., 2015). However, a few studies have shown the immunomodulatory potential of plant lectins to combat bacterial and fungal infections (Alegre-Maller et al., 2014), in this context pCramoll influences the inflammatory responses against *C. gatti*.

pCramoll increased the phagocytosis of *C. gattii* and the production of ROS and NO, augmenting the fungicidal activity of macrophages. Furthermore, this effect was observed in the cellular morphology of infected macrophages. Previously, the immunoregulatory influence of pCramoll has been portrayed, showing increased phagocytosis of *S. aureus*, as well as enhancement of NO, ROS and pro-inflammatory cytokines (Da Silva et al., 2014). Phagocytosis is the main mechanism for fungal elimination (Leopold et al., 2016), and is associated with increased survival of infected animals. Artin M (another mannose-specific lectin, like pCramoll) demonstred that phagocytosis was also the mechanism responsible for *Candida albicans* elimination, reducing the fungal burden in target organs and increasing animal survival (Loyola et al., 2012).

The murine model used in this study mimics the natural route of infection by *C. gattii*, as confirmed by the results of fungal burden and alterations in histopathology and behavior. pCramoll alone, as well as when combined with fluconazole, enhanced the survival of animals and improved the behavioral alterations caused by neuro-cryptococcosis. pCramoll in association with fluconazole decreased distinct alterations in related parameters, such as sensory-reflex function, the neuropsychiatric state, muscle tone and strength, as well as body weight. In the course of the disease, these clinical manifestations are determinants for neurological sequelae and death (Chen et al., 2012).

The pCramoll and fluconazole treatments decreased CFU in the lungs, at 15 dpi. Not only does this association enable a significant reduction in CFU, it is also more effective than the use of fluconazole alone in treating pulmonary cryptococcosis. Although yeasts were recovered from the brain of animals treated with the combination, they were not found in animals treated only with fluconazole. This may be due to pCramoll not crossing the blood-brain barrier (Patricio et al. 2011) and not directly acting on the central nervous system (CNS). Meanwhile, fluconazole was present at higher concentration in the CNS after 14 days of use, reducing CFU in the brain

(Mendes et al, 2010). Furthermore, pCramoll also stimulates *C. gattii* phagocytosis, increasing the early combat of the pathogen in the lungs. However, some yeasts have the capacity to survive in phagolysosomes, enabling transmigration of the fungus (Charlier et al. 2009). This phenomenon reduces fungal permanence in lung tissue, inhibiting strategies of fungal adaptation, avoiding cellular modification which inhibit the phagocytosis, and more virulent growth, like giant, fluconazole-resistant cells (Kronstad et al. 2011).

Interestingly, 35 days post-infection, there was a significant decrease in CFU in the lungs and brain in animals treated with the combination compared to the use of fluconazole alone. Ongoing use of the combination resulted in better neurological and behavioral conditions. This therapeutic characteristic is very important because previous studies have demonstrated that most patients infected with *C. gattii* presented CNS cryptococcosis, especially with meningitis 64% - 76% (Chen et al., 2000; Chen et al., 2012). The reduced number of yeasts in the brain improves the general conditions of mice, as confirmed by the results shown in the survival curve.

Infections with *C. gattii* leads to suppression of the host's immune responses, including decreased leukocyte recruitment and pro-inflammatory cytokine production (Wright et al., 2002; Leopold et al., 2016). On the other hand, the therapy tested in this work has shown that, at 15dpi, the animals treated with pCramoll alone increase leukocyte migration with a predominance of monocytes to macrophages contained in the BAL, although with less expression of inflammatory cytokines IFN-γ, IL-4 and TNF-α in the lungs (data not shown). The recruitment of these cells and their presence in the inflammatory response are crucial for immunological responses and pathogen depletion, mainly by phagocytosis (Melo et al., 2010).

At 15dpi, when pCramoll was combined with fluconazole, despite the large inflammatory infiltrate, the leukocyte content was balanced (neutrophils and macrophages), with lower Th1 cytokine expression (IFN-γ) as well as IL-10 auto-regulator. This is very important because the highly detrimental effect of IL-10 in cryptococcal infection models, as shown in NT mice, plays a major role in downregulating cryptococcal clearance (Olszewski et a., 2010). The same way as at 15 dpi, the high IL-17A production was observed for the combination. This inflammatory profile is not limited to the Th1/Th2 balance polarization against the infection, this response has been credited to IL-17A cytokine, which is known to have a role in cellular

recruitment (Kolls and Linden, 2004; Steinman L, 2007) and contribute to anti-cryptococcal protection (Voelz et al., 2009).

Up to 35 dpi, an increase of IFN-γ was observed in the lungs of mice treated with the combination, followed by IL-17A production, however with lower inflammatory infiltrate compared to the fluconazole group. The early decrease of IL-10 as seen in the groups treated with pC and their combination enhances IFN-γ and IL-17A effector responses and promotes fungal clearance in mice with cryptococcal lung infection (Murdock, et al., 2014). Previous studies have shown that pCramoll lectin stimulates Th17- and modulate the Th1-type cytokines production (Oliveira et al., 2013). The influence of the Th17 pathway in resistance to fungal pathology is a prospective finding in experimental Arpergilosis and Candidiasis studies (Romani et al., 2006; Zelante et al., 2009), while the role of Th1-type cytokines has also been demonstrated (Voelz et al., 2009; Gibson and Johnston, 2015). Moreover, the moderate inflammation is fundamental to the mechanism of tissue repair, associated with antifungal pathways, as seen in associated response mechanisms of IL17/IL10 cytokines (Zizzo, 2013; Murdock et al., 2014). Lower/moderate inflammation contributes to better neurological conditions.

Therefore, pCramoll is an immunomodulatory lectin without antifungal activity but its combination with fluconazole increases the survival of animals with cryptococosis, improving aspects of morbidity present in disease progression.

Conclusion

Immunotherapy with lectins in the treatment of cryptococcosis has not been reported, making this work pioneering. The immunoregulatory effect of pCramoll increases the survival of animals and improves clinical signs. Combination with fluconazole was able to reduce fungal burden in the lungs and brain of infected animals, increase *C. gattii* phagocytosis and produce higher levels of ROS. As such, the combination thereby represents a promising alternative in the treatment of cryptococcosis.

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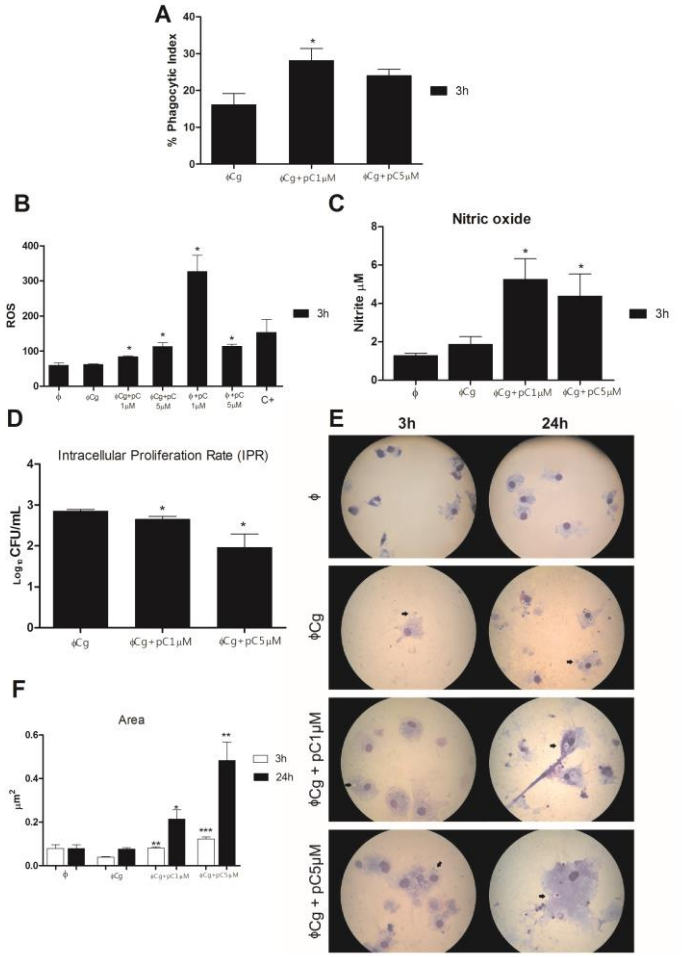

FIGURE 1: Immunomodulatory and fungicidal effect of pCramoll in murine bone marrow macrophages infected with *Cryptococcus gattii*. (A) After 3h hours of incubation, the phagocytic index increased under pCramoll treatment to 1 μM; (B) After 3h of incubation, treatment with pCramoll enhances ROS production in macrophages infected with *C. gattii*, in a concentration-dependent manner. (C) Oxide Nitric production after 3h after incubation; (D) Intracellular Proliferation Rate after 24h of incubation; (E) Macrophage morphology after 24h of incubation with *C. gattii*, showing *C. gattii* phagocytosis (arrows). Cell cultures were observed under 100× optical zoom and 10 fields per coverslip. (F) Area of macrophages stimulated with lectins and subsequently infected with *C. gatti*. Legend: * p <0.05; ** <0.01; *** p <0.005 (Difference between treatment groups and infected control); ϕ – Uninfected macrophages, ϕCg – *C. gattii*-infected macrophages; ϕpC - Uninfected macrophages treated with pCramoll; C+ 10μM Hydrogen peroxide. Data represent the means ± SE from two independent experiments consisting of ten replicate assays.

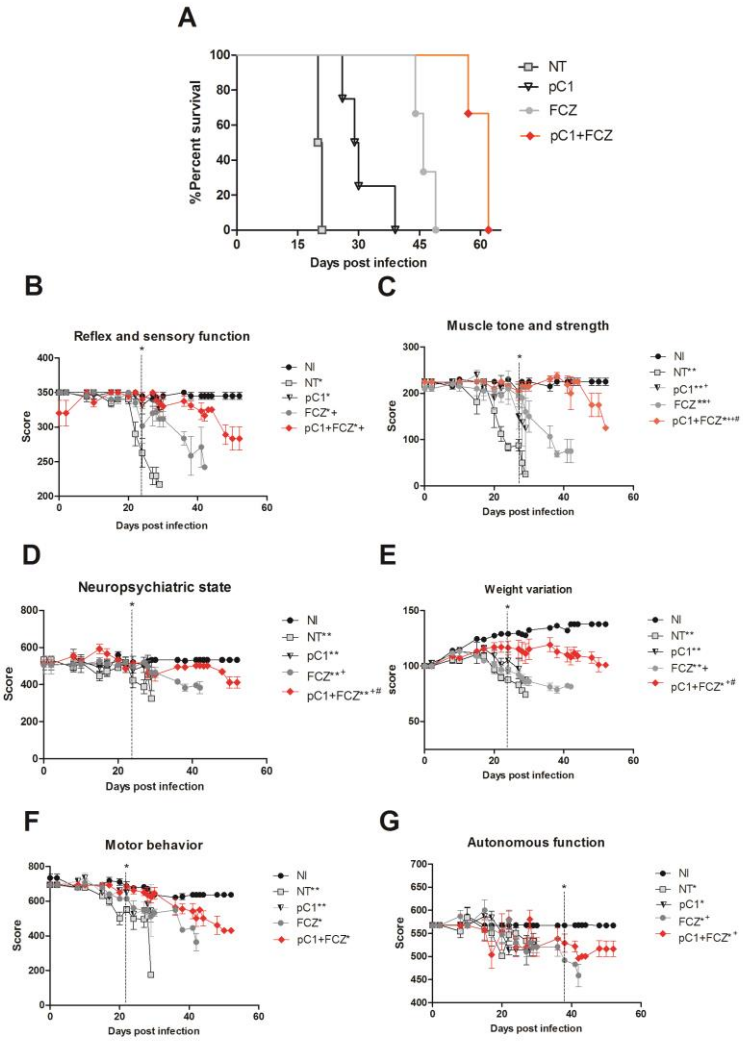

FIGURE 2: Survival curve and behavioral profile evaluation of mice infected with *Crytococcus gattii* and subjected to different treatments. (A) Six mice per group were inoculated with 10^4 cells of L27 / 01 strain by intratracheal line inoculation and treated with fluconazole at 20mg.kg^{-1} (FCZ), pCramoll at 1ug (pC1), or the combination (pC1 + FCZ). Animals treated by pC1 + FCZ had an average increase of over 34.8% in survival compared to those treated with FCZ alone * $p < 0.05$. (B-F) Five animals per group were submitted to the SHIRPA Protocol. There was no behavioral difference between uninfected mice (NI) and those treated with pC1 alone, which did also not influence the survival of mice. Legend: Untreated mice (NT); * $p < 0.05$; ** $p < 0.001$ (difference when compared to NI); $^{+}$ $p < 0.5$; $^{++}$ $p < 0.001$ (difference when compared to NT); $^{\#}$ $p < 0.05$ (difference between FCZ and combination). Data represent the means ± SE from three independent experiments.

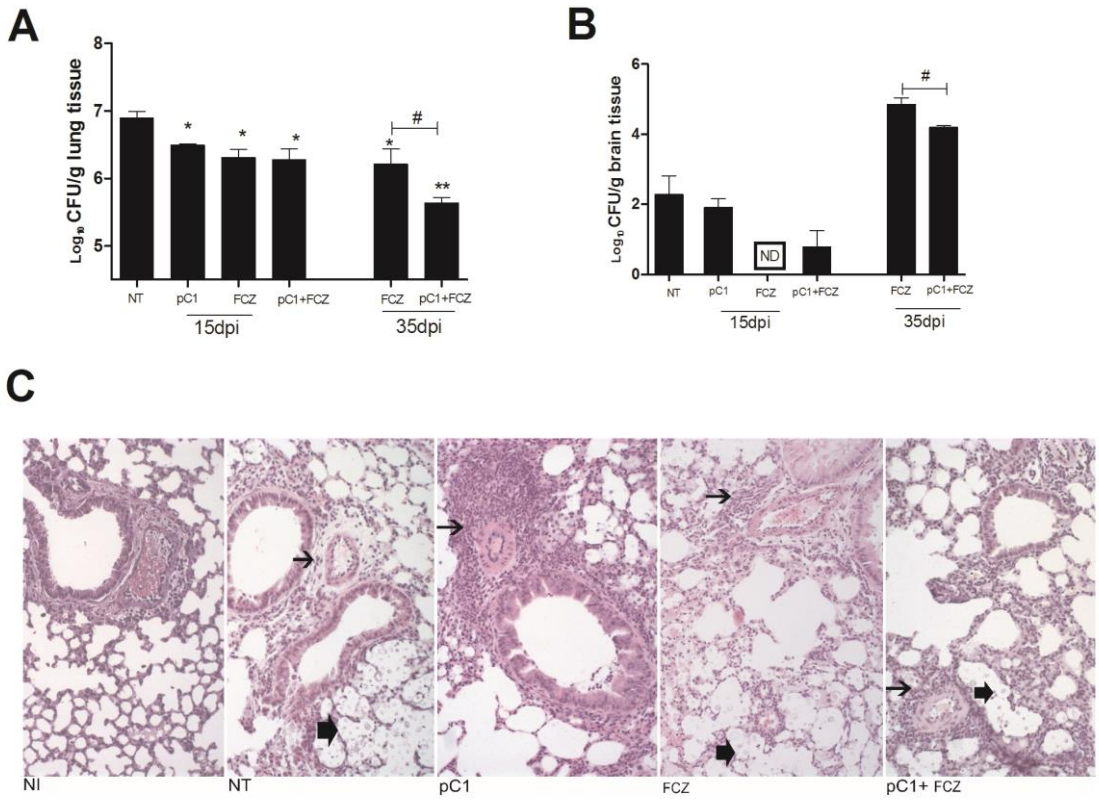

FIGURE 3: pCramoll in combination with fluconazole shows increased survival and reduced fungal burden in the lungs and brain of *Cryptococcus gattii*-infected mice. Six mice per group were inoculated with 10^4 cells of the L27/01 strain by intratracheal line inoculation and given the combination treatment, analyzed at 15 and 35 dpi: (A,B) Colony-forming Units (CFU) recovered from brain and lungs, respectively, at 15 and 35 days post infection; (C) Histological sections of lung tissue stained with H&E at 200×, 15 days post inoculation. Non-infected mice showed normal histology. Mice treated with pC1 showed more prominent perivascular inflammatory infiltrate (thin arrow) and less yeasts (thick arrow) in alveolar space than untreated, infected mice. Mice from FLZ or pC1+ FLZ showed moderate inflammation. Uninfected animals (NI), infected untreated control (NT), groups treated with pCramoll at 1μg (pC1), Fluconazole (FCZ), ND (not recovered CFU).* p <0.05; ** P <0.01; *** p <0.005 (difference between treated groups and NT); $^{\#}$p <0.05 (difference when comparing treated groups); $^{\#\#\#}$p <0.005 (difference between FCZ alone and combination, at 35 dpi). Data represent the means ± SE from three independent experiments consisting of triplicate assays.

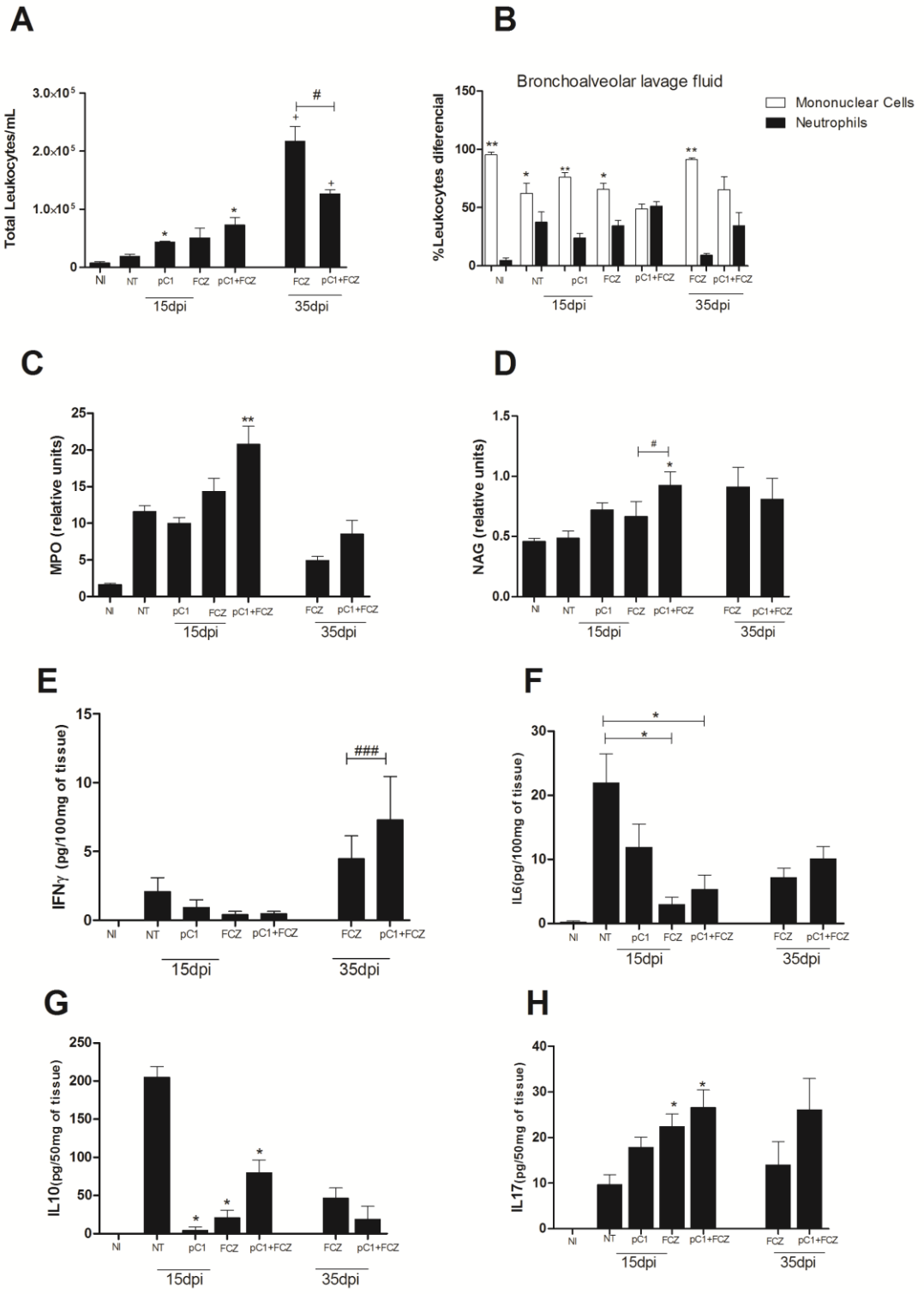
A
Total Leukocytes/mL
3.0x10^5
2.0x10^5
1.0x10^5
0
#
*
+
NI
NT
pC1
FCZ
pC1+FCZ
15dpi
35dpi
B
Bronchoalveolar lavage fluid
Mononuclear Cells
Neutrophils
%Leukocytes diferencial
150
100
50
0
**
*
C
MPO (relative units)
25
20
15
10
5
0
D
NAG (relative units)
1.5
1.0
0.5
0.0
E
IFNγ (pg/100mg of tissue)
###
F
IL6(pg/100mg of tissue)
G
IL10(pg/50mg of tissue)
250
200
150
100
50
H
IL17(pg/50mg of tissue)
40
30

FIGURE 4: Inflammatory response of pCramoll in combination with fluconazole in *Cryptococcus gattii*-infected mice, analyzed at 15 and 35 dpi: (A, B) Total and differential count of leukocytes (neutrophils and mononuclear cells) in the bronchoalveolar lavage, respectively; at 15 and 35 days after infection; (C, D) Myeloperoxidase and N-acetylglucosaminidase activities in lungs of infected animals; (E, F, G, H) Levels of cytokines IFN-γ, IL-6, IL-10 and IL-17A in lungs. Uninfected animals (NI), infected untreated control (NT), groups treated with pCramoll at 1μg (pC1), Fluconazole (FCZ), * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$ (difference between treated groups and NT); $^{\#}$ $p < 0.05$ (difference when comparing treated groups); $^{\#\#\#}$ $p < 0.005$ (difference between FCZ alone and combination, at 35 dpi). Data represent the means ± SE from three independent experiments consisting of triplicate assays.