Universidade Estadual Júlio de Mesquita Filho

UNESP

Faculdade de Medicina de Botucatu

Participação das NETs (*neutrophil extracellular traps*) na atividade fungicida de neutrófilos humanos contra o *Paracoccidioides brasiliensis*

Doutoranda: Tatiana Fernanda Bachiega Pinelli

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RESUMO

A paracoccidioidomicose é uma micose sistêmica causada pelo fungo dimófico *Paracoccidioides brasiliensis*, que é endêmico na América Latina. As células fagocitárias desempenham um papel importante durante a resposta imune inata contra o Pb, e se tornaram alvo de estudos neste sentido. Em especial, os neutrófilos são capazes de destruir microrganismos através de três mecanismos: fagocitose, seguida pela morte em fagolisossoma, secreção de agentes microbicidas e através do mais recente mecanismo descoberto, a netose, que consiste na liberação de redes extracelulares a partir dos neutrófilos (NETs), constituídas por proteínas granulares associadas a cromatina. Neste estudo demonstramos pela primeira vez que leveduras de *P. brasiliensis* induzem NETs in vitro principalmente através da ligação pelo receptor dectina-1 presente nos neutrófilos. Estas estruturas foram demonstrados através de microscopia eletrônica de varredura, e os componentes específicos das NETs: histona, elastase e DNA foram evidenciados a partir da microscopia eletrônica de imunofluorescência confocal. As leveduras foram capturadas pelas NETs, o que revelou um importante papel destas esrtruturas: impedir a disseminação do fungo. Além disso, o tratamento das co-culturas com DNAse, que degradou as NETs evidenciou aumento da sobrevivência do fungo, o que denota sua capacidade microbicida.

ABSTRACT

Paracoccidiodomycosis is a systemic mycosis caused by the dimorphic fungus *Paracoccidioides brasiliensis*, which is endemic in Latin America. Since phagocytic cells play an important role during innate immune response against this fungus, we have studied the relationship between human neutrophils (PMNs) and *P. brasiliensis*, focusing on the effector mechanisms of these cells. Neutrophils can destroy microrganisms using at least 3 distint mechanisms: phagocytosis followed by destruction in a phagolysossome, secretion of antimicrobial molecules, and the more recently identified netosis, involving the release of neutrophil extracellular traps (NETs) that are constituted by chromatin associated with different granule proteins with antimicrobial activities. Here, we showed for the first time, that yeast cells from *P. brasiliensis* strain 18 are able to induce the release of NETs *in vitro*, by binding to dectin-1 receptor on human neutrophils. These structures were evidenced by scanning electron microscopy, and specific NETs compounds such as histone and elastase were shown by confocal microscopy. Fungi were ensnared by NETs, denotting the role of these structures in confining infection, avoiding dissemination. In addition, disruption of NETs by treatment of cocultures with DNAse increased the fungi survival, evidencing their killing capacity.

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1. Revisão Bibliográfica

Formação e Função das NETs (neutrophil extracellular traps)

1.1 Neutrófilos

Descritos pela primeira vez por Paul Erlich como leucócitos polimorfonucleares, os neutrófilos desempenham função crucial na imunidade contra infecções microbianas, visto que a depleção destas células está relacionada a infecções graves e recorrentes provocadas por fungos e bactérias, que podem levar o hospedeiro à morte (Kaufmann and Steward, 2005; Nathan, 2006).

Os neutrófilos constituem cerca de 50 a 70% do total de leucócitos circulantes na corrente sanguínea e são recrutadas para o sítio de infecção por sinais quimiotáticos. Inicialmente, através de interações transitórias entre moléculas de adesão da família das selectinas (presentes em células endoteliais e neutrófilos) ocorre o rolamento pela parede do vaso sanguíneo adjacente ao sítio de inflamação. Após firme adesão, os neutrófilos realizam a diapedese, processo pelo qual migram entre as células endoteliais para alcançar o sítio de infecção extravascular em que ocorrerá a resposta (Dallegri *et al.,* 1997; Kobayashi *et al.,* 2003; Liu *et al.,* 2004).

Após o contato com o patógeno, os neutrófilos podem fagocitar os microrganismos, que são aprisionados dentro destas células em vesículas chamadas fagossomos. Posteriormente, os grânulos intracelulares se fundem ao fagossomo, dando origem aos fagolisossomos, estruturas nas quais os

patógenos são mortos pela combinação de mecanismos dependentes e independentes de oxigênio (Nathan, 2006).

No mecanismo dependente de oxigênio, a enzima NADPH-oxidase localizada na membrana e citoplasma celular se liga à membrana fagossomal e transfere elétrons ao oxigênio molecular, o que resulta na formação do ânion superóxido (O_2^-). O superóxido é decomposto através da enzima SOD dismutase, gerando peróxido de hidrogênio (H_2O_2), que por sua vez pode atuar como substrato para ação da mieloperoxidase (MPO) que culmina na produção de ácido hipocloroso (HOCl), o mais importante composto oxidante com função bactericida dos neutrófilos (Hampton *et al.,* 1998).

O mecanismo independente de oxigênio consiste na descarga direta do conteúdo dos grânulos presentes nestas células. Os grânulos primários, secundários e terciários possuem proteínas com funções microbicidas tais como: lactoferrina, lipocalina, lisozima, metaloproteinases, α-defensina, catepsina G, mieloperoxidase entre outras.

No ano de 2004, foi descrito por Brinkmann e colaboradores uma terceira função efetora de neutrófilos contra microrganismos. Esta nova estratégia se refere a capacidade dos neutrófilos lançarem redes extracelulares compostas por proteínas derivadas do citoplasma celular, e surpreendentemente, por DNA. O mecanismo de extrusão destas redes foi denominado NETose, e as redes em si, NETs (neutrophil extracelular traps). As NETs são estruturas complexas, compostas por milhares de filamentos de aproximadamente 15nm de diâmetro que se entrelaçam formando redes extracelulares (Brinkmann *et al.,* 2004). Vários estímulos são capazes de induzir a liberação das NETs, entre eles citocinas como IL-8, IFN-γ, GM-CSF;

PMA, LPS, *Mycobacteruim tuberculosis*, *Escherichia coli*, *Leishmania amazonesis*, *Candida albicans*, *Aspergillus fumigatus* entre outros de uma lista crescente de indutores de NETs (Brinkmann *et al.,* 2004; Martinelli *et al.,* 2004; Urban *et al.,* 2006; Ramos-Kichik *et al.,* 2008; Grinberg *et al.,* 2008; Guimarães Costa *et al.,* 2009; Bianchi *et al.,* 2009). Sabe-se que apenas um terço dos neutrófilos é capaz de lançar mão desta estratégia e sugere-se o envolvimento de receptores como os do tipo Toll, Fc e citocinas nesta via de ativação (Brinkmann *et al.,* 2004; Clark *et al.,* 2007).

A formação das NETs aparenta ser uma nova forma de morte celular ativa que difere em muitos aspectos da necrose bem como da apoptose conforme demonstrado na tabela abaixo:

Tabela 1. Diferenças entre Necrose, Apoptose e Netose (*Adaptado a partir de Goldmann and Medina, 2013*).

Necrose	**Apoptose**	**Netose**
Desintegração de membranas e organelas	Bolhas na membrana celular	Vacuolização
Exposição de fosfatidilserina	Exposição de fosfatidilserina	Não exposição de fosfatidilserina
Inchaço celular seguido por ruptura	Condensação da cromatina sem desintegração da membrana nuclear	Descondensação da cromatina com desintegração da membrana nuclear

1.2 Mecanismos de formação das NETs

Por ser um mecanismo recém-descoberto, a netose representa um campo apenas parcialmente conhecido.

Há pouco tempo, havia o relato de apenas uma sucessão de eventos que culminava na extrusão das redes extracelulares: acreditava-se que durante a netose o núcleo celular perdia sua forma, a cromatina era descondensada e o material nuclear extravasava para o citoplasma, concomitantemente ao rompimento das membranas dos grânulos e total mistura entre os materiais citoplasmáticos e nucleares. Por fim, a membrana celular passava a apresentar um poro, por onde as NETs eram liberadas e ocorria a morte da célula fonte de liberação das redes (neutrófilos) (Fuchs *et al.,* 2007). Recentemente, foram relatados indícios de que os neutrófilos ainda poderiam permanecer vivos e operantes após a netose, e foi proposto o uso de dois termos a fim de diferenciar os processos: “netose suicida” e “netose vital” (Fig. 1). A netose vital, descrita pelo grupo de Kubes (2013) caracteriza-se pela extrusão das NETs sem morte celular. Evidências que fortaleceram esta afirmação se referem a experimentos em que os neutrófilos após lançarem as NETs não podiam ser corados com SYTOX green, um indicativo de que as células permaneciam intactas e inacessíveis ao corante (Clark *et al.,* 2007). Yousefi e colaboradores (2009) descreveram também que neutrófilos ativados por GM-CSF e LPS e C5a foram capazes de liberar NETs, sendo que a origem do DNA das redes era mitocondrial e não nuclear, fato que não culminou com a morte celular.

Algumas diferenças entre as duas formas de netose foram descritas (Fig.1). A primeira se refere ao estímulo iniciador do processo. A netose suicida parece estar mais relacionada ao estímulo químico com PMA, e ser um processo mais lento. Por outro lado, estímulos que são reconhecidos por receptores celulares, tais como a ativação de neutrófilos por hemácias, LPS e diversas classes de patógenos parecem constituir um processo mais rápido e vital para os neutrófilos, não relacionado exclusivamente com a liberação extracelular de DNA mitocondrial (Clarck *et al.,* 2007; Yipp and Kubes, 2013). Em especial, foi demonstrado que a netose em resposta a *S. aureus* foi mediada pelo surgimento de bolhas no envelope nuclear e consequente exportação vesicular,com preservação da membrana plasmática (Pilskzek *et al., 2010).*

1.3 Pré-requisitos para formação de NETs – um campo controverso

O mais importante indutor de NETs, muitas vezes utilizado como controle positivo para liberação das redes extracelulares é o PMA, um ativador sintético da família de enzimas PKC, responsáveis diretas pela ativação de NADPH oxidase e produção de espécies reativas de oxigênio (ROS). Estes produtos aparentam ser importantes na geração das NETs, uma vez que ROS¨scavengers¨ foram capazes de inibir a produção das NETs (Fuchs *et al.,* 2007; Raad *et al.,* 2007), e além disso, pacientes portadores de doença granulomatosa crônica, os quais não possuem NADPH oxidase funcional,

também não foram capazes de gerar NETs, sendo esta capacidade restaurada após terapia gênica (Bianchi *et al.,* 2009).

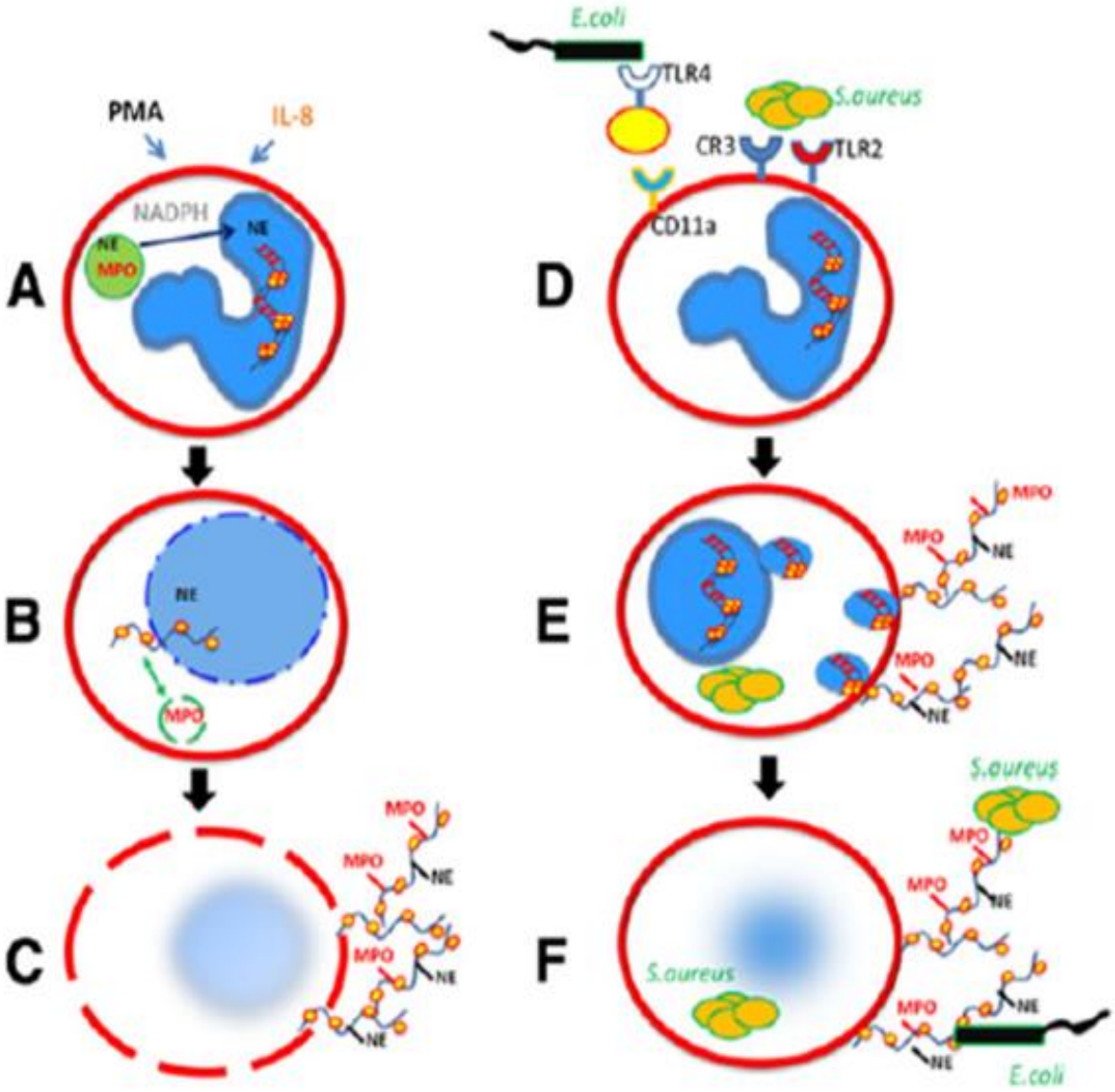

Figura 1. A-C Netose suicida a partir da ativação com LPS. Formação de poros ao longo de toda a membrana do neutrófilo seguido por morte celular. D-F Netose Vital, transporte do DNA em vesículas, preservando o citoplasma celular. Extrusão de NETs através da membrana celular sem abertura de poros. Integridade celular. (*Adaptado a partir de Yinn and Kubes, 2013*).

A ação dos ROS sobre a formação das NETs ainda não foi elucidada, sendo que podem ser os responsáveis pelas alterações morfológicas que levam a liberação das NETs e também responsáveis pela inativação de caspases, o que impede a apoptose e promove a autofagia, outro processo que leva a dissolução de membranas celulares e facilita a netose (Papayannopoulos et al., 2010; Remijsen et al., 2011).

Por outro lado, outras pesquisas indicam que os ROS em si não são suficientes para indução de netose: a incubação de neutrófilos com peróxido de hidrogênio não foi capaz de gerar NETs, apenas induzir apoptose (Remijsen *et al.,* 2010), neutrófilos de recém-nascidos não foram capazes de realizar netose, apesar de gerarem níveis semelhantes de ânion superóxido (Yost et al, 2009), o estímulo de neutrófilos com fMLP, um importante indutor de NADPH não induziu netose (Urban *et al.,* 2009; Remijsen *et al.,* 2010) e também foi demonstrada produção de NETs após estímulo com IL-8, que não participa da ativação de NADPH oxidase (Brinkmann *et al.,* 2004; Gupta *et al.,* 2005; Sheppard *et al.,* 2005). Portanto, a função bem como a dependência dos ROS na geração das NETs permanecem controversas, e indica que a netose é dependente dos diferentes estímulos do microambiente que envolve estas células.

Outra enzima que parece estar relacionada com a formação das NETs é a mieloperoxidase. A função chave desta enzima parece estar na ação em conjunto com a elastase na descondensação da cromatina (Papayannopoulos *et al.,* 2010). Há evidências de que sua inibição *in vitro* acarreta diminuição da formação das NETs por neutrófilos humanos (Akong-Moore *et al.,* 2012), assim

como em pacientes com deficiência na função desta enzima (Metzler *et al.,* 2011).

Um ponto de concordância entre os trabalhos é a importância da descondensação da cromatina devido a ação da enzima peptidil arginina deiminase 4 (PAD4). A conversão de resíduos de arginina em citrulina no núcleo celular elimina a sua carga positiva, contribuindo para a destruição da ligação iônica entre o DNA e as histonas e permite que estas estruturas se dispersem para a formação das NETs. A importância desta enzima foi evidenciada em camundongos deficientes em PAD4, que apresentaram diminuição significativa da netose *in vitro*, o que resultou em aumento da gravidade das infecções bacterianas (Li *et al.,* 2010; Rohrback *et al.,* 2012).

1.4 Funções efetoras das NETs: aprisionamento e morte

As NETs possuem duas funções efetoras importantes na imunidade contra os mais diferentes patógenos. As redes extracelulares podem apenas aprisionar o patógeno, impedindo sua disseminação, ou podem ainda ter uma ação microbicida direta sobre os microrganismos aprisionados. Em relação a primeira função, esta estratégia foi demonstrada através dos mais diversos ensaios de microscopia com vários microrganismos: *S. aureus, S. flexneri, E. pneumoniae, L. amazonensis, A. fumigatus e C. albicans* entre outros (Brinkmann *et al.,* 2004; Urban *et al.,* 2006; Wartha *et al.,* 2007; Guimarães-Costa *et al.,* 2009; Bruns *et al.,* 2010). Os mecanismos pelos quais as NETs capturam os microrganismos vêm sendo pesquisados e até agora há evidências que a carga do ácido nucléico é importante, porém há muitas outras

ligações específicas que podem atuar neste processo (Douda *et al.,* 2011). *In vivo*, especula-se que a fibronectina (expressa no interstício celular) represente o elo entre *Candida albicans* e o DNA das NETs (Byrd *et al.,* 2013).

Em relação a segunda função efetora, a atividade microbicida direta das NETs tem sido demonstrada para alguns microrganismos (Urban *et al.,* 2006; Bianchi *et al.,* 2009; Pilsczek *et al.,* 2010) e os componentes individuais das redes tem sido alvo de pesquisas neste sentido. A elastase, presente em grande quantidade das NETs, representa o principal fator contra *Shigella flexneri* (Weinrauch *et al.,* 2002; Brinkmann *et al.,* 2004) enquanto a quelação de cátions exercida pelas NETs contribui para morte de *Pseudomonas aeruginosa* (Mulcahy *et al.,* 2008). Em relação aos fungos patogênicos, NETs induzidas por PMA foram capazes de inibir o crescimento de *Aspergillus*, possivelmente pela quelação de zinco mediada pela calprotectina (McCormick *et al.,* 2010). Contra *Candida albicans* especificamente, as NETs desempenharam atividade fungicida a partir de mecanismos dependentes de DNA e calprotectina, uma proteína constitutiva de neutrófilos relacionada a atividade microbicida destas células (Urban *et al.,* 2006; Urban *et al.,* 2009).

1.5 Mecanismos de escape das NETs por patógenos

A medida que estudos demonstram a atividade microbicida das NETs, outros destacam mecanismos de escape presentes em alguns patógenos que impedem a eficiência da netose.

O mais relatado é a produção de nucleases que degradam o DNA e consequentemente, as redes extracelulares. Streptococcus do grupo A (GAS)

representados por *S. pyogenes* produzem DNAses capazes de desintegrar as NETs e conferir maior virulência às bactérias (Sumby *et al.,* 2005; Buchanan *et al.,* 2006). Da mesma forma, *S. pneumoniae* também degrada as NETs via expressão de DNAse, além de possuírem uma cápsula polissacarídica, que impede a adesão às NETs (Beiter *et al.,* 2006; Wartha *et al.,* 2007).

Uma proteína de superfície de *S. pyogenes*, M1, é capaz de se ligar a catelecidinas LL-37 e prevenir a atividade microbicida das NETs (Lauth *et al.,* 2009) enquanto uma peptidase, denominada spyCEP tem a capacidade de clivar a IL-8, uma citocina produzida por células endoteliais e indutora de NETs (Fuchs *et al.,* 2007; Wartha *et al.,* 2007; Zinkernagel *et al.,* 2008).

S. agalactiae expressa um exopolissacarídeo com resíduos de ácido siálico, mimetizando um glicoepítopo normal em células humanas, é reconhecido por receptores Siglec em neutrófilos como próprio, não deflagrando sinal de fagocitose, nem induzindo o “burst” respiratório e também inibindo a formação das NETs. Além disso, expressa nuclease a fim de degradar as NETs (Carlin *et al.,* 2009; Derré-Bobillot *et al.,* 2013).

Estrategicamente, lipooligossacarídeos presentes em *H. influenzae* promovem a formação de um biofilme por estas bactérias e esta comunidade de microrganismos funcionais favorece as relações simbióticas além de aumentar a sobrevivência, permanência e virulência desta espécie (Hong *et al.,* 2009).

1.6 NETs como promotoras de doenças

Muitas moléculas presentes nas NETs, tais como mieloperoxidase, histonas e DNA representam auto-antígenos em doenças autoimunes sistêmicas. Em vasculites, muitos pacientes desenvolvem anticorpos contra citoplasma de neutrófilos (ANCA), os quais apresentam reatividade específica contra os marcadores moleculares desta doença: proteinase 3 (PR3) e MPO, constituintes das NETs (Kessembrock *et al.,* 2009). Sabe-se que durante a netose, há liberação de IL-17 por neutrófilos e mastócitos, e esta citocina é um fator chave para o desenvolvimento de psoríase (Lim *et al.,* 2011), fato que correlaciona o desenvolvimento da doença e produção de NETs. Evidências indicam o desbalanço entre produção/degradação de NETs em pacientes com lupus eritematoso sistêmico, sendo que a produção aberrante de NETs é responsável pelos danos teciduais e produção de auto antígenos (Garcia Romo *et al.,* 2011; Lande *et al.,* 2011; Villaneuva *et al.,* 2011). A síndrome de Felty é uma doença autoimune caracterizada pela presença de artrite reumatóide, esplenomegalia e neutropenia. Nesta doença, auto anticorpos contra histonas deiminadas por PAD-4 são marcadores moleculares, o que também relaciona a presença de NETs com a síndrome (Dwivedi *et al.,* 2011).

As NETs, quando presentes em vasos sanguíneos podem representar estímulo para a formação de trombos por promover adesão de hemácias à estas estruturas e concentrar proteínas e fatores envolvidos na coagulação. Esta relação se dá através da ligação de LPS circulante ao TLR-4 das hemácias, que por sua vez unem-se aos neutrófilos, deflagrando um sinal positivo para a formação das NETs, através da ação de um composto das hemácias, as β-defensinas (Clarck *et al.,* 2007; Fuchs *et al.,* 2010; Brill *et al.,* 2011; von Brühl *et al.,* 2012).

Um número exacerbado de NETs foi encontrado no espaço interviloso de pacientes com pré-eclampsia devido à ativação de neutrófilos por IL-8 e micropartículas inflamatórias secretadas a partir de sinciotrofoblasto, correlacionando a gravidade da doença com a presença das NETs (Gupta *et al.,* 2005).

Células tumorais circulantes são retidas nas NETs, fato relacionado a metástase em muitos casos, um indicador de que as NETs podem representar um alvo terapêutico importante nestas condições (Cools-Lartigue *et al.,* 2013).

1.7 NETs e fungos

Candida albicans representou o primeiro patógeno eucariota em que as NETs foram descritas. As redes extracelulares foram capazes de matar ambas as formas do fungo, hifa e levedura de modo independente da opsonização. Concluiu-se que componentes dos grânulos dos neutrófilos foram responsáveis pela atividade fungicida, sem a participação de histonas (Urban *et al.,* 2006). Posteriormente, o mesmo grupo de pesquisa identificou 24 proteínas nucleares, granulares e citoplasmáticas que compunham as NETs, dentre as quais a calprotectina desempenhou maior atividade fungicida contra *C. albicans* (Urban *et al.,* 2009). Mais recentemente foi demonstrado que o receptor CR3 de neutrófilos é o responsável por reconhecer hifas deste fungo e iniciar Netose independente de geração de ROS, porém dependente de fibronectina, componente da matriz extracelular (Byrd *et al.,* 2013).

A aspergilose invasiva é uma causa comum de morte em pacientes com doença granulomatosa crônica (DGC), os quais possuem defeito em NADPH, o

que resulta na deficiência da produção de ROS e NETs. Após terapia gênica em pacientes de DGC, portanto com NADPH oxidase operante, foi possível restaurar a resposta contra conídeos e hifas de *A. nidulans* mediada por NETs, dependente de calprotectina (Bianchi *et al.,* 2009; Bianchi *et al.,* 2011). *A. fumigatus* também foi capaz de induzir a formação de NETs *in vitro*, de maneira dependente da hidrofobina RodA, uma proteína de superfície que mantém o conídio inerte (Bruns *et al.,* 2010). McCormick e colaboradores (2010) demonstraram que as NETs, apesar de induzidas pelo *A. fumigatus* não foram capazes de matar o fungo, e propuseram que as redes extracelulares teriam o papel de capturar e impedir a disseminação do mesmo, bem como reduzir o seu crescimento.

Apesar das evidências acima apresentadas, não há relatos sobre a possível participação das NETs na infecção pelo *P. brasiliensis.* Desse modo, o objetivo do presente estudo foi testar a hipótese de que a formação das NETs induzida pelo fungo pode estar relacionada a atividade fungicida de neutrófilos. Assim, avaliamos a formação *in vitro* das NETs contra o *P. brasiliensis* e se parte da atividade fungicida atribuída aos neutrófilos poderia ocorrer através da ação das redes extracelulares, bem como a participação de receptores de reconhecimento de padrões na sua formação.

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Manuscrito

Role of Dectin-1 and NETs release by human neutrophils in the extracelular killing of *P. brasiliensis*

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Abstract

Paracoccidiodomycosis is a systemic mycosis caused by the dimorphic fungus *Paracoccidioides brasiliensis*, which is endemic in Latin America. Since phagocytic cells play an important role during innate immune response against this fungus, we have studied the relationship between human neutrophils (PMNs) and *P. brasiliensis*, focusing on the effector mechanisms of these cells. Neutrophils can destroy microrganisms using at least 3 distint mechanisms: phagocytosis followed by destruction in a phagolysossome, secretion of antimicrobial molecules, and the more recently identified netosis, involving the release of neutrophil extracellular traps (NETs) that are constituted by chromatin associated with different granule proteins with antimicrobial activities. Here, we showed for the first time, that yeast cells from *P. brasiliensis* strain 18 are able to induce the release of NETs *in vitro*, by binding to dectin-1 receptor on human neutrophils. These structures were evidenced by scanning electron microscopy, and specific NETs compounds such as histone and elastase were shown by confocal microscopy. Fungi were ensnared by NETs, denotting the role of these structures in confining infection, avoiding dissemination. In addition, disruption of NETs by treatment of cocultures with DNAse increased the fungi survival, evidencing their killing capacity.

1. Introduction

Paracoccidioidomycosis (PCM), caused by the dimorphic fungus *Paracoccidioides brasiliensis* (Pb) is a systemic mycosis; endemic to most Latin America countries, especially in Brazil (Brummer *et al*, 1994). Infectious propagules (i.e.,hyphal fragments or conidia) are inhaled, deposited in the lungs and due to an increase in the temperature to 36–37°C, they differentiate in the pathogenic yeast form, thus establishing the disease (Silva-Vergara *et al*, 1998, Theodoro *et al*, 2005, Terçarioli *et al*, 2007, McEwen *et al*, 1987). Thereafter, yeasts can disseminate by lympho-haematogenous route, inducing a disease characterized by a wide spectrum of clinical symptoms (Shikanai-Yasuda *et al*, 2006). Many people are exposed to the fungus, but only small number develop clinical symptoms, suggesting that both innate and adaptive mechanisms are important for fungus clearance (Calich *et al*, 2008, Benard *et al*, 2008).

During innate immune response, phagocytic cells play a critical role against this fungus performing both fungicidal activity and modulation of the inflammatory responses. Among these cells, neutrophils have attracted the attention of researchers in recent years, because a massive infiltration of PMNs is found in the inflammatory sites of human disease (Franco *et al*, 1982) and different experimental models (Iabuk *et al*, 1979, Kerr *et al* 1988 Calich, 1994) probably as a result of chemokines action (Souto *et al*, 2003). It is suggested, that chemokines can be released by macrophages that have established an initial contact with the fungus (Calich *et al*., 1985). Once infiltrated, these cells are extremely important for the defense mechanisms of the host (Meloni -

bruneri *et al*. 1996) particularly in animals with increased susceptibility to infection (Pina *et al*., 2006).

The effector and modulatory functions of phagocytic cells, including neutrophils, are initiated by their by microorganism they recognize. This process involves a number of receptors denominated “pattern-recognition receptors” (PRRs), that recognize molecular structures shared by a large number of microorganisms, termed “pathogen-associated molecular patterns” (PAMPs). Among the PRRs involved in fungi recognition are the Toll-like (TLRs) and C-type lectin receptors (Romani, 2004; Robinson *et al*., 2006).

Traditionally, neutrophils engulf the microbes into a phagocytic vacuole and subsequently intracellular granules fuse with phagosome and release their contents to form a phagolysossome, where microbes are killed by both non-oxidative and oxidative mechanisms (Nathan, 2006). Early studies showed that nonactivated human PMNs have fungistatic (Kurita *et al*., 1999) and fungicidal activity against the *P. brasiliensis* (Kurita *et al*. 2005). However, these antifungal effects are significantly increased after activation with cytokines (Kurita *et al*. 1999; Kurita *et al*. 2000). Similar study conducted in our laboratory showed that non-activated PMNs lack fungicidal activity against Pb18. However, a significant activity is obtained after cell priming with IFN-γ, TNF-α, GM-CSF or IL -15 (Rodrigues *et al*. 2007; Tavian et al. 2007). The studies also showed that the effector mechanisms of activated PMNs against fungus involve the participation of O_2^- and hydrogen peroxide (H_2O_2). Despite these results, other effector mechanisms involved in *P. brasiliensis* destruction should be explored, especially on those that does not depend on the direct action of the oxidative metabolism products.

In this sense, Brinkmann *et al* (2004) described a mechanism of extracellular killing by neutrophils called netosis. This phenomenos can bem triggered by IL-8, lipopolysaccharide (LPS), PMA, microrganisms and activated platelets inducing death of neutrophils that produce and release neutrophil extracellular traps (NETs) to the extracellular medium. NETs are composed by chromatin associated with different antimicrobial proteins from cytoplasmatic granules, including enzymes (lysozyme, proteases), antimicrobial peptides (BPI, defensins), ion chelator (calgranulin) and histones (Brinkmann *et al*., 2004). The high concentrations of these components in NETs, as well as their synergistic actions are responsible for the effective antimicrobial activity of these structures (Brinkmann and Zychlinsky, 2012). In the light of these studies, we asked whether *Paracoccidioides brasiliensis* is able to induce NETs release by human neutrophils, as well as whether these structures contribute for the killing of this pathogen.

Our results show the first time that Pb induces NETs release by human neutrophils and we identified dectin-1 as the mais PRRs involved in the NET formation.

2. Materials and methods

2.1 Subjects

Healthy blood donors from University Hospital of the Botucatu Medical School, São Paulo State University, UNESP, were evaluated in this study. The

study was approved by Ethics Committee of this School, and informed consent was obtained from all the blood donors.

2.2 Isolation, purification and culture of human blood PMNs

Blood was collected and neutrophils were isolated by density gradient centrifugation (Histopaque 1119 and 1083g/mL - Sigma–Aldrich) followed by hypotonic lysis of erythrocytes. Purified neutrophils (≥95% of the cells. Cellular viability was assessed by trypan blue dye exclusion test) cells were ressuspended in complete medium (RPMI medium 1640 suplemmented with 2 mM L-glutamine, 40 µg/mL gentamicine and 10% inativated fetal calf serum) and kept on ice until use. Cell suspensions were adjusted for $2x10^6$ cells/mL or $1x10^6$ cells/mL depending on the assay to be performed.

2.3 Fungi

Yeast cells of *P. brasiliensis*: Pb 18 (high virulence) was used in this study. To ensure virulence, the isolate was used after three serial animal passages. The yeast cells were then maintained by weekly sub-cultivation in the yeast-form cells at 35ºC on 2% glucose, 1% peptone, 0.5% yeast extract and 2% agar medium (GPY medium) and used on the sixth day of culture. Yeast cells were washed and suspended in 0.15 M phosphate-buffered saline (PBS pH 7.2). To obtain individual cells, the fungal suspension was homogenized with glass beads in a Vortex homogenizer (three cycles of 10s). Cell suspensions were then maintained at 37°C for 5 minutes for sedimentation of undissolved

during stirring. After this period, the supernatant was collected and yeast cells were counted using microscope with phase contrast, followed by adjustment of concentration to $4x10^4$ or $2x10^4$ viable yeast cells/mL.

2.4 Scanning Electron Microscopy

Neutrophils ($1x10^6$/mL) were adhered on coverslips treated with 0.01% polylysine (Sigma). After adherence, cells were treated with PMA (100ng/mL), challenged with 100µL of a Pb18 suspension, containing $2x10^4$ yeasts/mL of complete medium (50:1neutrophils/fungus ratio), or previously treated with DNAse(100U/mL – Fermentas Life Science) for 30 minutes and after challenged with Pb 18. After 2 h in 5% CO_2 at 37^{o}C, cultures were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, postfixed with 1% osmium tetroxide and dehydrated with an ascending ethanol series. After dehydration and critical-point drying, the specimens were coated with gold and analyzed in a FEI QUANTA 200 scanning electron microscope.

2.5 Confocal laser scanning microscopy

Cells ($1x10^6$/mL) were adhered on coverslips treated with 0.01% polylysine (Sigma) and challenged with Pb18 (according item 2.4). After 2h coverslips were stained with anti-elastase (Calbiochem) and antihistone H2A antibodies (Millipore), followed by antibodies anti-rabbit-FITC (Millipore) and anti-mouse-Texas red (Calbiochem), respectively. Slides were mounted using

mounting medium for fluorescence with DAPI (Vectashield-Vector Labs). Confocal images were taken in a Leica TCS SP5 microscope.

2.6 Evaluation of fungicidal activity

Neutrophil suspensions ($2x10^6$ cells/mL) were dispensed into 100 µL/well in 96-well flat-bottom plates (Nunc Life Tech., Inc., MD, USA) and activated with GM-CSF, IFN-γ or TNF-α (R&D Systems, Minneapolis, MN, USA), at different concentrations, for 18 h in 5% CO_2 at 37°C in the presence of Cytochalasin D (10 µg/mL; Sigma) or DNAse (100U/mL) thirty minutes before the challenge with Pb18. The cultures were challenged for 4 h in 5% CO_2 at 37°C with 100µL of a *P. brasiliensis* suspension, containing $4x10^4$ yeast/mL (50:1 neutrophil/fungus ratio) prepared in complete medium plus 10% fresh human AB serum, as the source of complement for yeast opsonization. Neutrophils-fungus cocultures were harvested by aspiration with sterile distilled water to lyse neutrophils. Each coculture well washing resulted in a final volume of 2.0 mL and 0.1 mL was plated in triplicate on supplemented brain-heart infusion (BHI) agar medium (Difco Laboratories, Detroit, MI, USA) containing 0.5% of gentamicin, 4% horse normal serum and 5% of a water-extract from Pb 192 yeast cells cultured in liquid medium for 7 days and considered as a source of growth-promoting factor (Kurita *et al*, 1999). The fungus inoculum used for neutrophil challenge was also plated according to the same conditions. Then, the plates containing the product of neutrophil-fungus cocultures were considered as experimental plates and those plated with the inoculum alone were used as controls. Control and experimental plates were incubated at 35°C

in sealed plastic bags to prevent drying and after 10 days, the number of colony forming units (CFU) per plate was counted. The fungicidal activity percentage was determined by the following formula:

% Fungicidal activity = [1-(mean CFU recovered on experimental plates/
Mean CFU recovered on control plates)] x 100

2.7. Quantification of NETs

Neutrophil cultures ($2x10^6$/mL) were *in vitro* stimulated with PMA, challenged with Pb 18 (according item 2.6). Supernatants were then collected and submitted to treatment for two hours with restriction enzymes (EcoR1 and HindIII, 20 units/mL each; BioLabs), according to the manufacturer's instructions. NETs were quantified by using the Picogreen dsDNA kit (Invitrogen). The λ-DNA standard provided with the kit (100µg/mL) was diluted with Tris-EDTA (TE buffer) to the concentration of either 25pg/mL or 1ng/mL for the low and high range curves respectively, and received the same treatment with restiction enzimes. Plates were incubated at room temperature in the dark for 5 min prior reading on a SpectraMax M2® (Molecular Devices) using an excitation wavelength of 480 nm and emission wavelength of 520 nm. In some assays, before Pb challenge, neutrophils were incubated with monoclonal antibodies: anti-TLR2 (3,5ug/10^6 cells), anti-TLR4 (4ug/10^6 cells) and anti-dectin-1 (3ug/10^6 of cells) for 2h. These concentrations were chosen because they induced the highest percentages of blockage in previous experiments (data

not shown). We adopted a protocol which allows that in each evaluation, two receptors become blocked and only one remains available.

2.8. Statistical analysis

Data were statistically analyzed using the INSTAT software (Graph Pad, San Diego, CA, USA). The results were compared by analyses of variance (ANOVA) followed by Tukey's test, with the level of significance set at $p < 0.05$.

3. Results

3.1. Scanning electron Microscopy

To test whether Pb18 can induce the release of NETs by human neutrophils, yeast cells were incubated with human neutrophils at 1:50 ratio, for different time periods, and cocultures analysed by scanning electron microscopy. This fungus/neutrophils ratio was chosen, as it was previously standardized in our studies of antifungal activities of human phagocytic cells, such as killing. PMA was used as a positive control, since it is a potent inducer of NETs. Neutrophils alone, without PMA or fungi did not release NETs. However, these structures were visible after 45 min of cell incubation with PMA and increased during the following periods (Fig. 1A: white arrowhead). It is noteworthy that in the neutrophil/fungus cocultures, similar structures were detected, proving that neutrophils release true NETs in response to the fungus (Figs 1B, 1C, 1D). In addittion, the periods were coincident and the structures

were similar to those showed by Brinkmann *et al*, 2004, who, identified NETs for the first time. Small or large number of fungi can be seen trapped by the structures (Figs 1B and 1C, respectively). Neutrophils had lost their round shape leading to the release of their constituents, including NETs. Likewise, some fungi trapped by NETs also exhibit morphological changes such as a certain flattening, which may be attributed to the effect of NETs (Fig. 1D). Since NETs are composed by nuclear material, the addition of DNAse on cell culture was expected to result in their degradation. Indeed, in the assays in which this enzyme was used, before Pb challenge, there was no evidence of such corroborating our conclusion on NETs release (Fig 1E). In addittion, in DNAse treated cultures, images showing fungus phagocytosis by neutrophils can be identified (square) in which both seems to be morphologically intact (Fig. 1F).

3.2 Confocal laser scanning microscopy

Individual components of NETs are among histones, elastase and descondensed cromatine. Then, we decided to check if they could be identified into the traps by confocal microscopy.

Immunostaining with antibodies anti-histone followed by secundary antibodies Texas red (Fig 2A) showed an immunofluorescence image confirming the presence of histone in NETs. A similar image was detected when antibodies anti-elastase followed by secundary antibodies stained with FITC were used (Fig 2B). Fig 2C shows the presence of DNA by staining with

DAPI. Interestingly, overlay of images evidences the co-existence of the 3 components (Fig. 2 D).

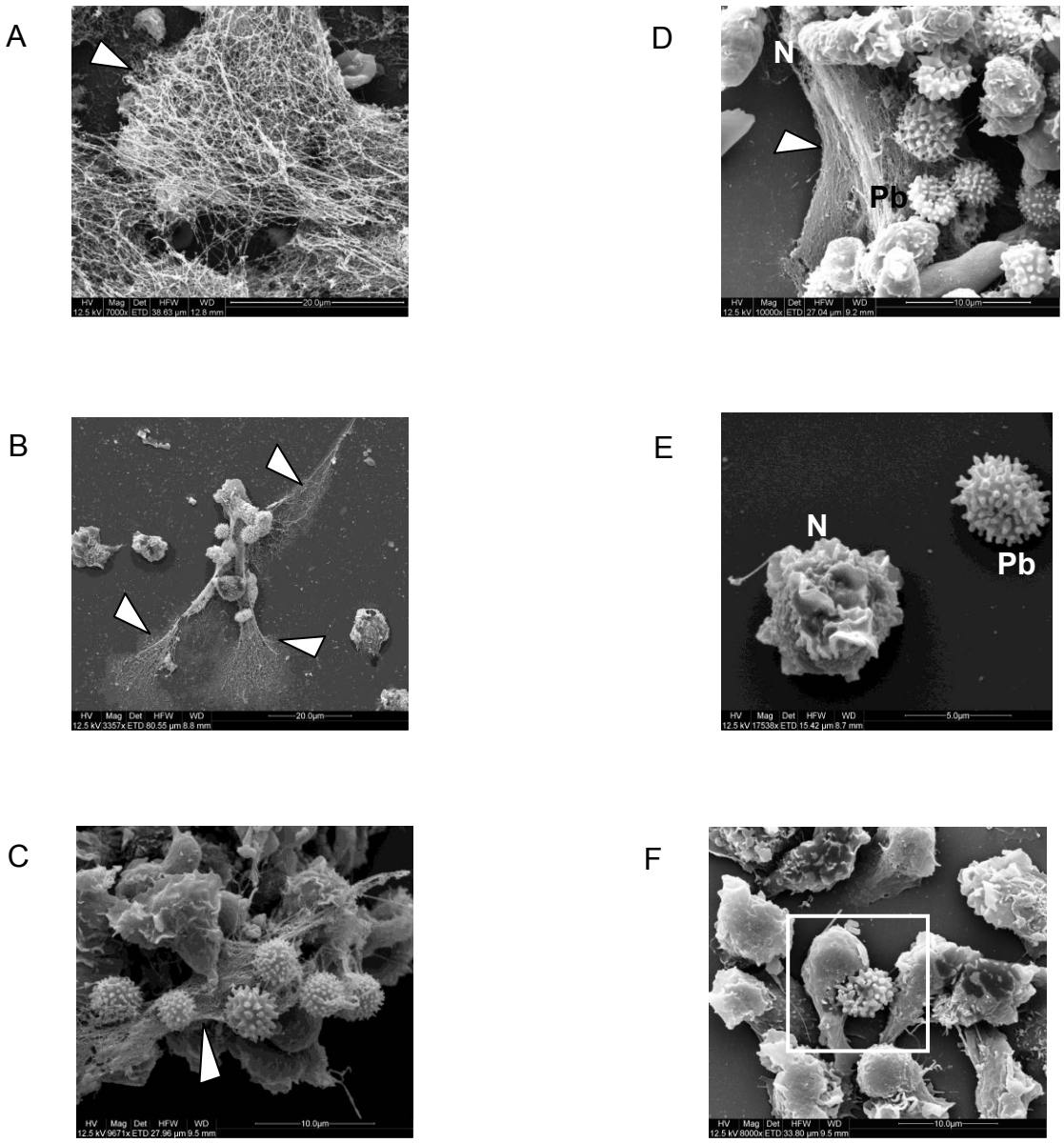

Fig.1. Scanning electron microscopy micrographs. Neutrophils treated with PMA (A) or Pb 18 for 2 h (B, C and D) showing NETs formation (arrowheads). Small (B) or large (C) numbers of fungi traped by NETs. Neutrophils (N) and fungi (Pb) showing altered morphology (D). Neutrophil cultures treated with DNAse before fungus challenge showing absence of NETs (E) and fungus phagocytosis (square) (F).

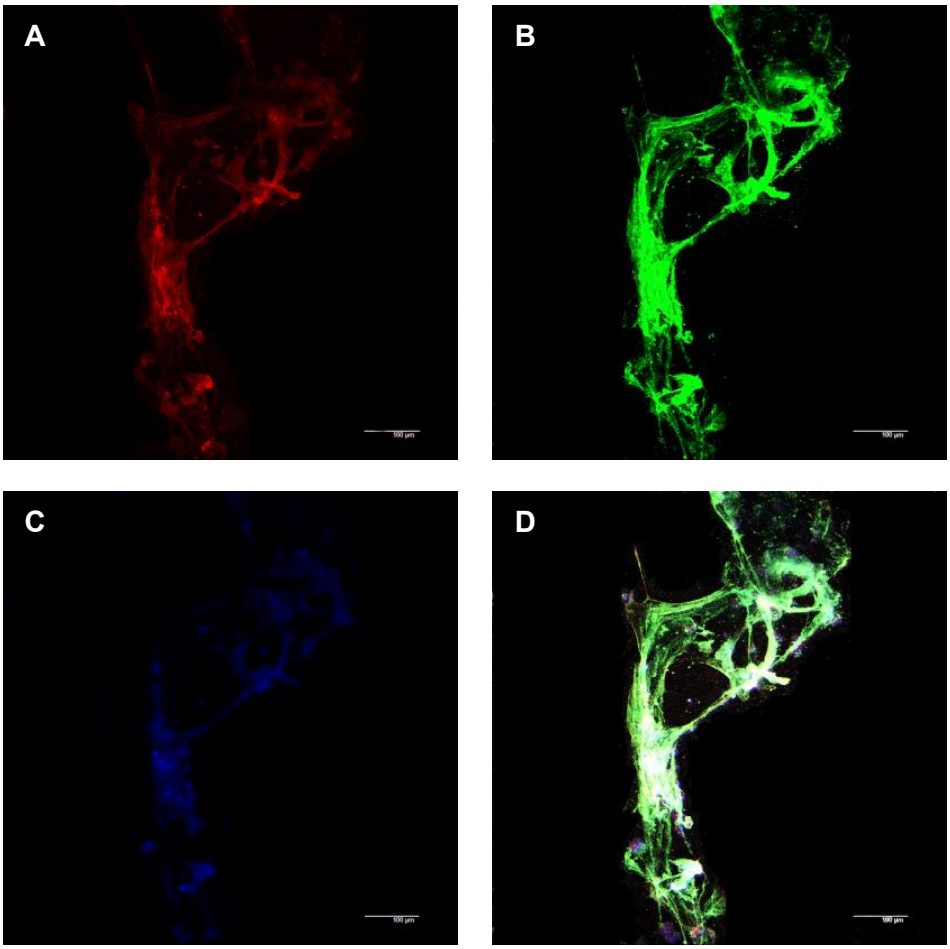

Fig. 2. Immunostaining of NETs identified after neutrophils /Pb18 cocultures for 2h. (A) Presence of histone, identified by specific primary antibody followed by secundary antibody conjugated with texas red. (B) Presence of elastase identified with specific primary antibody followed by secundary antibody co njugated with FITC. (C) Identification of DNA staining with DAPI. (D) Overlay of the 3 imunofluorescence images showing the concomitant presence of the 3 components. Bar 100µm.

3.3. Involvement of PRRs in NET formation.

Having detected that *P. brasiliensis* induces NETs release by human PMNs we aimed to evaluate the role of TLR2, TLR4 and Dectin-1 in this phenomenon. Our experimental approach was to block these receptors with monoclonal antibodies, before fungus challenge and analyse DNA amounts. We

adopted a protocol which allows that in each evaluation, two receptors become blocked and only one remains available for recognition (Fig. 3). Control cells released low levels of DNA (200ng/mL), which however, increased significantly after treatment with PMA (435ng/mL), or challenge with the fungus (410ng/mL). After blockade of the three receptors, NETS production decreased significantly (336ng/mL), indicating the involvement of at least one of the receptors tested. The individual evaluation of each receptor showed that when only TLR-2 or TLR-4 was available, DNA production decreased to 353 and 336ng/mL, respectively. On the other hand, in the experiments in which only dectin was available, the amounts of DNA were similar to those produced by no blocked neutrophils. These results strongly suggest that among the PRRs studied, dectin -1 seems to be the one involved in NETs release by human neutrophils in response to Pb.

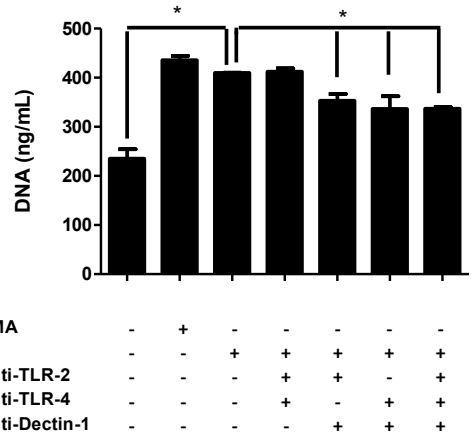

Fig.3. Involvement of TLR2, TLR4 and dectin-1 on NETs release by human neutrophils. Cells were stimulated with PMA, challenged with Pb or previously treated with anti-TLR2, anti-TLR4 and /or anti-Dectin-1 monoclonal antibodies. After challenge with Pb, supernatants were recovered and DNA quantified. Results are expressed as mean±SD of evaluations performed with cells of 6 individuals. *p<0.05.

3.4. Fungicidal Activity

As the microscopical observations showed that NETs can cover and trap yeasts of *P. brasiliensis*, our next question was whether these structures can also be involved in fungus killing. Previous studies showed that an effective fungicidal activity by neutrophils is observed after priming with GM-CSF, IFN-γ or TNF-α. Then, to test the participation of NETs in fungus killing, neutrophils were preincubated with each of these cytokines in an ideal concentration, and later incubation with cytochalasin D, an actin inhibitor used to block phagocytosis.

Extracelular killing was measured with or without DNAse (Fig.4) followed by challenge with Pb18. Cells primed with each of the cytokines presented high percentages of fungicidal activity (GM-CSF= 72 %, IFN-γ=59% and TNF-α= 67%) that reflects both intracellular and extracellular killing. These percentages were significantly reduced when neutrophils were treated with cytochalasin D (GM-CSF: 72% to 21%; IFN-γ: 59% to 23% and TNF-α: 67% to 32%), showing that a large percentage of killing occurs intracellularly. Moreover, the addition of DNase, though a lesser extent, also reduced the percentage of killing (GM-CSF: 72% to 42%; IFN-γ: 59% to 33%; TNF-a 67% to 56%) showing that fungicidal activity can also occur extracellularly and was probably mediated by NETs.

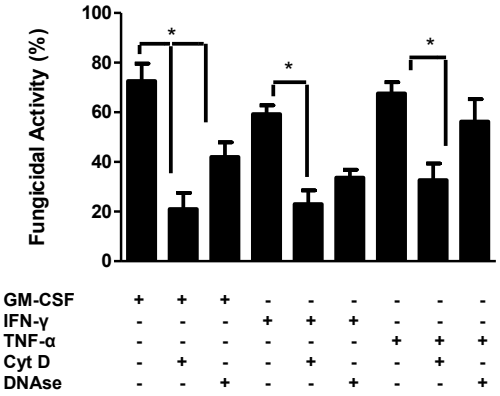

Fig. 4. Fungicidal activity. Neutrophils were preincubated for 18h with GM-CSF (250U/mL), IFN-γ (250U/mL) or TNF-α (500U/mL) and after with cytochalasin D (10µg/mL) or with DNAse (100U/mL) for 30 min, challenged with Pb18 for 4 h (PMN/fungus ratio :50:1) and fungicidal activity evaluated. *p<0,05 versus respective controls treated only with cytokines.

4. Discussion

In recent years, the release of NETs has been identified as a novel mechanism used by neutrophils to fight microorganisms. These structures have been shown to bind and in some cases to kill several bacteria, protozoa, virus and fungi species (Brinkmann *et al*., 2004; Urban *et al*; 2006; Guimarães Costa *et al*., 2009; Bruns *et al*., 2010; Mc Cormick *et al*., 2010; Saitoh *et al*., 2012). Here, we demonstrated, for the first time, that Pb18 yeast cells are able to induce the release of NETs, by human neutrophils, *in vitro*. The evidence that the structures visualized by scanning microscopy were NETs, was obtained by confocal microscopy in which the images of fluorescence showed the presence of individual components of NETs as histones and elastase. The finding that the

structures observed are highly sensible to DNAse degradation represents an additional confirmation. Of note, our images are similar to those detected in studies with other fungi. Such as *Aspergillus fumigatus*, *Aspergilus nidulans, Cryptococcus gattii* and *Candida albicans* (Urban *et al*., 2006; 2009, Bianchi *et al*., 2009; Bruns *et al*., 2010; MacCormick *et al*, 2010, Springer *et al*., 2010).

The elucidation of signalling mechanisms of NET-induction would help us to understand the process of NET-formation which is still unknown. In this aspect, pattern recognition receptors, such as Toll-like receptors (Netea *et al*., 2002) and/or and C-type lectin receptors (Brown and Gordon, 2001) may be involved. Studies showing the involvement of PRRs in NETs formation in response to microrganismos are scarce. Here, we demonstrated that dectin-1 is involved on fungus recognition and subsequent NETs release. It is the first study showing the role of PRRS in NETs release in response to fungi. On the other hand, TLR2 and TLR4 did not seem to have an important role, despite the fact that some studies showed that in human monocytes and PMNs both receptors are involved in the recognition, internalization and consequent activation of the immune response against the fungus (Bonfim *et al*., 2009). In addition, TLR4 has been implicated in NET formation that entangles circulating bacteria and provides intravascular immunity, avoiding bacterial dissemination during septic infections (Clark *et al*., 2007).

Of course the role of other candidate PRRs should be further evaluated. We would like to highligh that Pb not only induces the release of NETs, but also interacts with them. Treatment with DNAse comprovate this attachment, since as in treated cocultures, both, neither NETs and nor fungi were visualized. Since fungi are unable to adhere to the coverslips alone, their absence in

cultures after NETs disintegration means that they are strongly attached to these structures. The nature of the binding sites in the NETs for the various microorganisms is unknown. However, it is suggested that it can be mediated by ionic forces. This mechanism has been speculated for binding of yeasts of *Candida albicans* to NEts. Some sugars, such as mannans- very frequent in this fungus wall- have a negatively charged chemical composition that easily bind to NETs components such as highly cationic granule proteins and histones (Urban *et al*, 2006). In this context, the same process may be considered for other fungi, including Pb, that share wall sugar composition with *Candida*. Further studies are needed to confirm this possibilities.

Previous reports showed that human neutrophils are able to kill *P.brasiliensis* after activation with cytokines. As fungi trapped by NET-fibers presented an altered morphology, featuring cell damage, we tested whether these estructures were involved in Pb killing .Our hipothesis, is that some *P. brasiliensis* yeasts are too large to be up taken by phagocytosis requiring extracellular mechanism for destruction, probably mediated by NETs. Indeed, we found that most killing is mediated by phagocytosis and subsequent phagolysoma formation, but a significative extracellular killing still occurs and is mediated by NETs, in fact killing is significantly reduced when NETs are disrupted by DNAse treatment. An important question is which components of NETs were involved in Pb destruction. Although these components were not assessed in the present study, we can suggest the participation of some potential candidates. *Bacteria* and *Leishmania* are destroyed by histones (Brinkmann *et al*., 2004; Guimarães Costa *et al*., 2009). However, despite cationic properties of histones facilitate the binding of NETs to

fungi, these molecules do not seen to take part in the destruction process (Urban *et al*, 2006). On the other hand, the antimicrobial heterodimer calprotectin that is microbiostatic *in vitro*, due to its ability to chelate iron, (McNAmara *et al*, 1988, Steinbak *et al*, 1990, Murthy *et al*, 1993, Sohnle *et al*, 1996) has been identified as a major NETs component present, involved in fungi killing (Urban *et al*, 2009).

In summary, our data indicate that P. brasiliensis bind to human neutrophils via dectin-1 to induce the development of NETs, in order to trap Pb yeast cells, and promote their immobilization and prevent their spread and eventual killing.

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Conflict of interest

The authors declare no conflicts of interest of any kind.

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