Microbiology (2015), **161**, 1008–1017 DOI 10.1099/mic.0.000059

Human neutrophils produce extracellular traps against *Paracoccidioides brasiliensis*

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Neutrophils play an important role as effector cells and contribute to the resistance of the host against microbial pathogens. Neutrophils are able to produce extracellular traps (NETs) in response to medically important fungi, including *Aspergillus* spp., *Candida albicans* and *Cryptococcus gattii*. However, NET production in response to *Paracoccidioides brasiliensis* has yet to be studied. We have demonstrated that human neutrophils produce NETs against both conidia and yeasts of *P. brasiliensis*. Although the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI) did not alter NET production against conidia, it partially suppressed NET formation against *P. brasiliensis* yeasts. Cytochalasin D or IFN-γ did not affect the production of NETs against the fungus. Additionally, a mutant strain of *P. brasiliensis* with reduced expression of an alternative oxidase induced significantly higher levels of NETs in comparison with the WT strain. Finally, c.f.u. quantification of *P. brasiliensis* showed no significant differences when neutrophils were treated with DPI, DNase I or cytochalasin D as compared with untreated cells. These data establish that NET formation by human neutrophils appears to be either dependent or independent of reactive oxygen species production, correlating with the fungal morphotype used for stimulation. However, this mechanism was ineffective in killing the fungus.

Received 11 December 2014
Accepted 13 February 2015

INTRODUCTION

The fungus *Paracoccidioides brasiliensis* is a dimorphic and pathogenic species that causes paracoccidioidomycosis (PCM), the most frequent systemic mycosis in Latin America. This mycosis is predominantly found in South America, especially in Brazil, Colombia, Venezuela and Argentina (Brummer *et al.*, 1993; Negroni, 1993; Colombo *et al.*, 2011). *P. brasiliensis* is characterized by its ability to switch from the mycelial to the yeast morphotype; the former occurs at temperatures of 18–24 °C and is found in the external environment while the latter grows at 36 °C in culture or the host (Kanetsuna & Carbonell, 1970; Kanetsuna *et al.*, 1972; Brummer *et al.*, 1993; Tomazett *et al.*, 2010). Infection is caused after inhalation of *P. brasiliensis* conidia, small propagules produced by the mycelia being able to reach the lungs. In 90 % of clinically active cases, PCM produces a chronic systemic and progressive disease, which progresses slowly and can take months or even years to develop, while the remaining 10 % of patients develop an acute form (Londero & Melo, 1983; Brummer *et al.*, 1993; Restrepo *et al.*, 2008; Marques, 2013; Restrepo & Tobon, 2010).

In experimental animal models, it has been shown that an acute inflammatory process takes place during the early stages of the infection, with involvement of phagocytic cells, mainly neutrophils and macrophages (González *et al.*, 2003, 2008; Lopera *et al.*, 2011). This initial inflammatory response is associated with a decrease in the fungal burden in the lungs of infected mice (Pina *et al.*, 2006; González *et al.*, 2008).

It has been demonstrated *in vitro* that human neutrophils are involved in resistance to *P. brasiliensis* infection. These cells can ingest *P. brasiliensis* yeasts through phagocytosis, but when the yeast cells are too large, the neutrophils form an extracellular vacuole before killing the fungus (Dias

Abbreviations: AOX, alternative oxidase; CR3, complement receptor 3; DPI, diphenyleneiodonium chloride; NET, neutrophil extracellular trap; PMA, phorbol myristate acetate; ROS, reactive oxygen species.

Two supplementary figures are available with the online Supplementary Material.

et al., 2004). High activities of NADPH oxidase, endogenous peroxidase and acid phosphatase have also been demonstrated during the fungal phagocytosis process (Dias *et al.*, 2004). Nonetheless, although *P. brasiliensis* is phagocytosed and the respiratory burst is activated, these mechanisms do not seem to be sufficient to kill the fungus (Kurita *et al.*, 1999a, b; Dias *et al.*, 2008).

Other studies have shown that cytokines such as IL-15, IL-8, IFN-γ, granulocyte macrophage colony-stimulating factor (GM-CSF) and TNF-α increase the fungicidal/fungistatic effect of these cells (Kurita *et al.*, 1999a, 2000; Rodrigues *et al.*, 2007; Tavian *et al.*, 2008). Interestingly, the antifungal activity of IFN-γ, TNF-α and GM-CSF is exerted in a dose-dependent manner and this response is correlated with production of reactive oxygen species (ROS) (Rodrigues *et al.*, 2007).

In the last decade, a new neutrophil defence strategy has been described, namely neutrophil extracellular traps (NETs) (Brinkmann *et al.*, 2004). These structures are produced after cell activation and are composed of decondensed chromatin complexed with different cytoplasmic proteins, such as histones, as well as over 30 different neutrophil proteins (Brinkmann *et al.*, 2004; Fuchs *et al.*, 2007; Medina, 2009; Papayannopoulos & Zychlinsky, 2009). NETs can be produced by NADPH oxidase activation-dependent or -independent mechanisms and subsequent ROS production (Brinkmann *et al.*, 2004; Pilsczek *et al.*, 2010). These network structures can capture microbes, degrade their virulence factors and finally eliminate the pathogens (Brinkmann *et al.*, 2004; Fuchs *et al.*, 2007).

An increasing number of bacteria, fungi, viruses and protozoan parasites have been shown to induce NETs (Brinkmann *et al.*, 2004; Guimarães-Costa *et al.*, 2009; Urban *et al.*, 2009; Saitoh *et al.*, 2012; Jenne *et al.*, 2013) but to date only three fungal species (*Candida albicans*, *Aspergillus* spp. and *Cryptococcus gattii*) have been reported to activate their production (Urban *et al.*, 2006; Bruns *et al.*, 2010; Springer *et al.*, 2010; Bianchi *et al.*, 2011). NET production is induced after recognition of β-glucan present on the cell wall of *Ca. albicans,* through interaction with complement receptor 3 (CR3) located on the surface of the phagocytic cells (Byrd *et al.*, 2013). NETs play an important role against *Ca. albicans* and *Aspergillus fumigatus* hyphae. These structures are not phagocytosed owing to their large size, and NET formation may be a good strategy to arrest fungal spread (Urban *et al.*, 2009; Bianchi *et al.*, 2011). Although *Cr. gattii* induces NET production, these structures do not kill the fungus. It is noteworthy that these capsulated yeasts produce extracellular fibrils, which are associated with its virulence and the inhibition of neutrophil-mediated killing (Springer *et al.*, 2010).

Interestingly, the induction of NETs by *P. brasiliensis* has yet to be described. Here we report that human neutrophils can produce NETs after interaction with both morphotypes of *P. brasiliensis* (conidia and yeast cells). The production of these structures appears to be either dependent or independent of ROS production, correlating with the fungal morphotype used for stimulation. However, this mechanism was ineffective in killing the fungus.

METHODS

Strains and media. A strain of *P. brasiliensis* (ATCC 60855) originally isolated from a Colombian patient and characterized by its abundant conidia production was used in this study. The fungus was maintained at 18 °C by passage every 2 weeks on solid synthetic McVeigh–Morton modified (SMVM) medium (Restrepo & Jiménez, 1980). Conidia production and purification were assessed as described previously (Restrepo *et al.*, 1986; Del *et al.*, 2004). The numbers and viability of conidia were evaluated by staining with Janus Green (Invitrogen). Conidia suspensions with viabilities higher than 90 % were used throughout the experiments.

Yeast morphotypes were maintained by weekly subcultures in solid Sabouraud medium (Becton Dickinson) supplemented with 0.01 % thiamine (Becton Dickinson), 0.014 % asparagine (Sigma-Aldrich), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Sigma) at 36 °C with 5 % CO_2. Unless otherwise indicated, yeast cells were grown in broth Sabouraud medium supplemented with 0.01 % thiamine and 0.014 % asparagine plus 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 36 °C with aeration on a mechanical shaker, and were routinely collected during the exponential growth phase (at 72 h). Fungal growth was collected in PBS, and passed 12 times through a 5 ml 21G syringe with a 1.5 inch needle to eliminate clumped fungal cells. The fungal suspension was centrifuged at 1500 ***g*** and 4 °C for 5 min. Viability and numbers of yeasts were determined by staining with Janus Green. The fungal unit was considered to be equivalent to a single cell or mother cell with two to seven daughter cells. Fungal cells were counted in a haemocytometer and resuspended in RPMI 1640 culture medium (Gibco) to obtain the desired number.

Moreover, a mutant strain of *P. brasiliensis* (PbAOX) (Ruiz *et al.*, 2011) with modified expression of an alternative oxidase (AOX) gene, involved in the detoxification or reduction of ROS (Ruiz *et al.*, 2011), was also employed in the present investigation using antisense RNA technology.

A *Ca. albicans* strain (ATCC 90028) was used as a positive control for NET production by neutrophils (Urban *et al.*, 2006; Byrd *et al.*, 2013). This strain was maintained by 3 days of subculturing in solid Sabouraud medium at 35 °C with 5 % CO_2, and harvested during the exponential growth phase (48 h). Fungal mass was isolated by scraping the surface of the medium and resuspending this material in RPMI 1640 medium. Viability and numbers of yeast cells were determined by staining with Janus Green and counting in a haemocytometer.

Reagents. Recombinant human IFN-γ, phorbol myristate acetate (PMA), diphenyleneiodonium chloride (DPI), cytochalasin D, LPSs and luminol were purchased from Sigma-Aldrich. DNase I was provided by Mo-BIO. SYTOX Green was obtained from Molecular Probes (Invitrogen).

Isolation of human neutrophils. Blood samples were obtained from healthy human volunteers by following the guidelines of resolution 8430 of 1993 of the Colombian Ministry of Health. Blood was collected in EDTA-containing Vacutainer tubes (BD Biosciences). Polymorphprep (density 1.113 g ml^{-1}, 460 mOsM; Axis Shield) was used for blood gradient centrifugation at 550 ***g***. Neutrophils were collected and washed with HBSS buffer (Gibco) and diluted in RPMI 1640 medium. The number and viability of cells were evaluated by Trypan Blue exclusion (Invitrogen). Neutrophil cells with viability higher than 95 % were used.

NET visualization. For staining techniques, we used 12 mm glass coverslips (Fisherbrand) in 24-well non-treated culture plates (Brand). As a first step, neutrophils were adhered onto the glass coverslip, 2×10^5 neutrophils (PMNs) were seeded into each well and the preparations incubated for 30 min at 37 °C. They were then inoculated with 4×10^4 *P. brasiliensis* yeast cells in 250 µl RPMI 1640 medium and incubated for 3 h at 37 °C. In some wells, PMA (25 nM) was added to stimulate neutrophils. Furthermore, in some experiments 100 U ml^{-1} DNase I was added at the same time as the stimulus in order to degrade any DNA that had been released. Then, monolayers were fixed with 4 % paraformaldehyde for 10 min at room temperature. Additionally, cells were permeabilized using 0.5 % Triton X-100 for 1 min. NETs were stained with rabbit anti-human elastase AB21595 (Abcam), 0.6 µg in a 10 % PBS/BSA solution (Gibco); finally, an Alexa 488-conjugated goat anti-rabbit IgG (Invitrogen), 0.3 µg in PBS, was used as the secondary antibody. Additionally, DNA was stained with 1 µg ml^{-1} propidium iodide (Invitrogen) and the fungus with 100 µg ml^{-1} Calcofluor White (Fluka-Sigma-Aldrich) following the manufacturer's instructions. NETs were visualized using an Axio Vert.A1 inverted fluorescence microscope (Carl Zeiss) and the images captured using an Axiocam IC camera (Carl Zeiss).

Determination of NET formation (area of NETs). The area of NETs (%) was determined with ImageJ version 1.48 software (National Institutes of Health). Forty images were captured with a ×20 objective and analysed randomly from different regions of each coverslip; individual cell and NET areas were framed with the free-hand selection (yellow line) and the inner region was measured; the sum of these areas was taken as the total area. With these data, the area of NETs was calculated with Microsoft Excel 2010.

Quantification of extracellular DNA. Co-cultures of *P. brasiliensis* at the concentrations described above with 2×10^5 freshly isolated human neutrophils were grown in 200 µl RPMI 1640 medium using black 96-well culture plates (Greiner) for 3 h at 37 °C. In some wells, PMA (25 nM), LPS (3 µg ml^{-1}) or *Ca. albicans* (4×10^4 yeasts) was added to stimulate neutrophils.

In order to inhibit NET formation, neutrophil cultures were pre-incubated with 10 µg ml^{-1} cytochalasin D (an inhibitor of phagocytosis) and 16 µM DPI (an inhibitor of NADPH oxidase) for 30 min at 37 °C, after which *P. brasiliensis* yeasts or conidia were added. In addition, in some experiments 100 U ml^{-1} DNase I was added at the same time as the stimulus in order to degrade any DNA that had been released. Some neutrophils were also pre-treated with IFN-γ (100 U) 1 h before the second stimulus was added. Finally, 5 µl SYTOX Green (a fluorescent high-affinity nucleic acid stain) at a concentration of 5 µM was added to the wells and the fluorescence measured (excitation 480 nm, emission 530 nm) in a spectrofluorometer (Spectra Max Gemini; Molecular Devices). To quantify the amount of extracellular DNA, total DNA from 2×10^5 neutrophils was obtained using a commercial extraction kit (QIAamp DNA Mini kit; Qiagen); this DNA was taken as 100 % of total and used to calculate the extracellular DNA released from cells.

ROS production by human neutrophils. Activation of neutrophils upon stimulation with *P. brasiliensis* yeasts [WT (Pb60855), yeasts carrying the empty vector (PbEv60855), or a mutant strain with diminished expression of AOX (PbAOX)] was determined by measuring ROS production. Quantification of ROS was performed using a luminol-enhanced chemiluminescence method. Briefly, freshly isolated human neutrophils were seeded into white 96-well culture plates (Greiner) at 2×10^5 cells per well in PBS with glucose and luminol (50 µM; Sigma-Aldrich). Cells were incubated for 10 min at 37 °C and infected with 4×10^4 *P. brasiliensis* yeasts. As positive control, neutrophils were stimulated with 25 nM PMA (Sigma-Aldrich). NADPH oxidase activity inhibited prior incubation (20 min) with 10 µM DPI (Sigma-Aldrich). Unstimulated neutrophils were included in each experiment as negative control. An additional negative control consisting of *P. brasiliensis* yeasts alone was also included, and the luminescence of this control was subtracted from co-cultures. Finally, the chemiluminescence was measured every 2 min for 2 h in a Varioskan Flash Multimode Reader (Thermo Scientific). The area under the curve was calculated using Excel 2010.

Killing assay. In order to determine the efficiency of NETs in killing *P. brasiliensis*, a c.f.u. assay was performed. Co-incubation of fresh human neutrophils with *P. brasiliensis* yeasts (Pb60855 and PbAOX strains) in 96-well culture plates (Brand) was carried out as described previously. In additional experiments, some wells were treated with 16 µM DPI, 100 U ml^{-1} DNase I and 10 µg ml^{-1} cytochalasin D. After 3 h of incubation at 37 °C, wells were aspirated and washed with PBS. Dilutions of 1 : 10 and 1 : 100 were made and 100 µl of each dilution was plated onto BHI agar supplemented with 4 % (v/v) horse serum, 500 µM EDTA and 1 % glucose (Sigma-Aldrich). Cultures were incubated at 36 °C with 5 % CO_2. The c.f.u. values were counted after 3 days of culturing and thereafter until no further increase in colony numbers could be observed.

Statistical analysis. All statistical analysis was performed using GraphPad Prism software, version 5.0. Normal distribution was determined using ANOVA and verified by the Kolmogorov–Smirnov normality test. Differences between groups were analysed using Student's *t*-test or the Mann–Whitney test according to the Gaussian distribution of data. A P-value $\leqslant 0.05$ was considered to be statistically significant.

RESULTS

P. brasiliensis induces NET formation

Fluorescence microscopy was used to evaluate induction of NETs by human neutrophils after interaction with the *P. brasiliensis* yeast morphotype. PMA was used as positive control as previously reported (Brinkmann *et al.*, 2004; Urban *et al.*, 2006). NET formation was corroborated in confocal images using both anti-elastase and propidium iodide. Non-stimulated neutrophils did not produce NETs (Fig. 1a, b); by contrast, activation of these phagocytic cells with PMA showed NET production. Furthermore, most of the cells treated with PMA reached the final phase of NETosis, with most of the cells displaying these characteristic structures (Fig. 1a). As shown in Fig. 1, NET formation occurred in human neutrophils after 3 h of infection with *P. brasiliensis* yeasts. As indicated in Fig. 1a, *P. brasiliensis* yeast cells stained with Calcofluor White were in close contact with NET structures.

Quantification of NETs induced by *P. brasiliensis*

In order to confirm that the structures observed were extracellular DNA, co-cultures were treated with DNase I. As shown in Fig. 1b, treatment with DNase I reduces the percentage area of NETs to values equivalent to those observed in non-stimulated neutrophils (negative control). Using an additional quantification method (spectrofluorometry), we observed that different ratios of neutrophil and

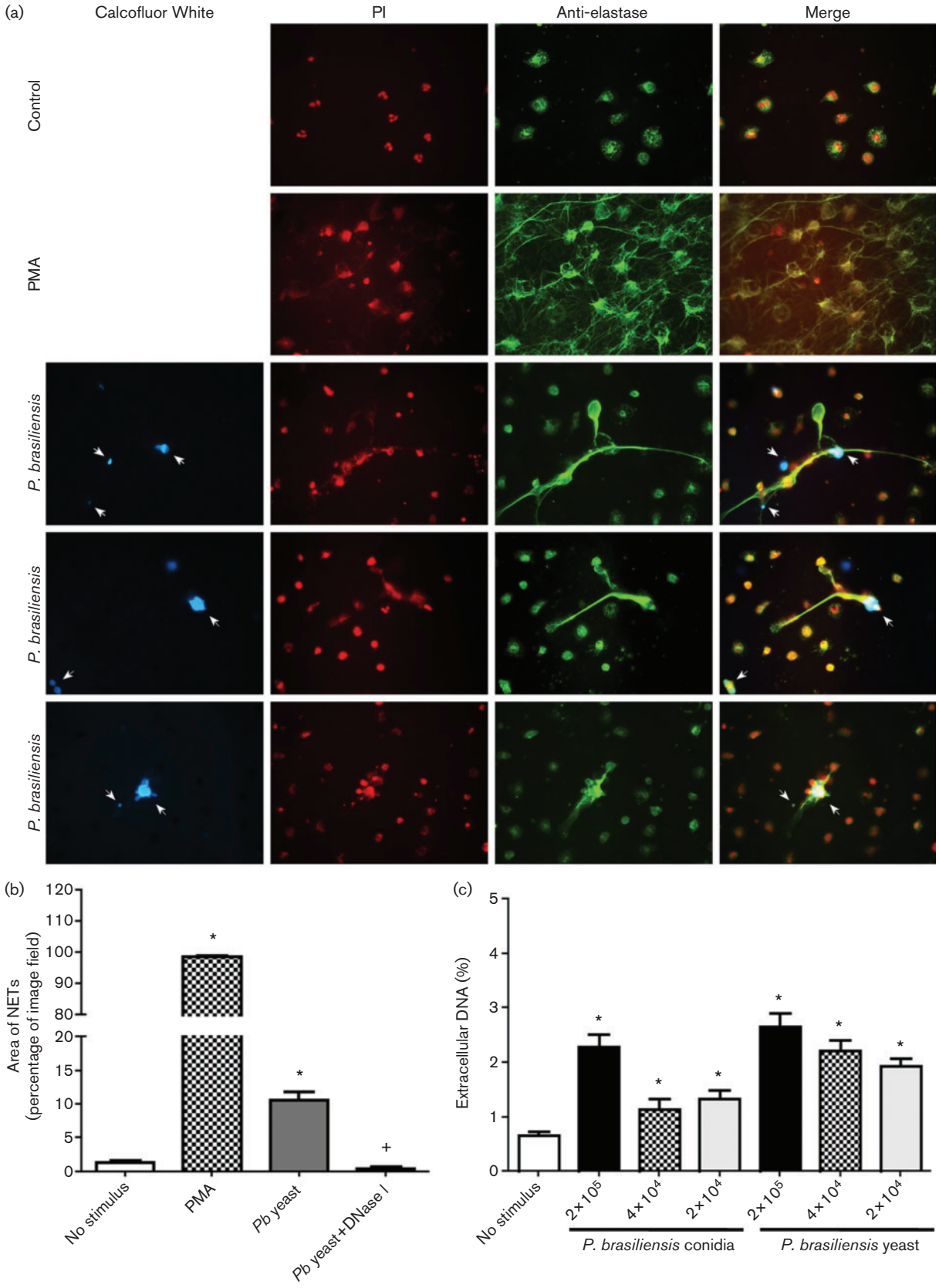

Fig. 1. *Paraoccidioides brasiliensis* promotes NET formation by neutrophils. (a, b) Human neutrophils were isolated and infected with *P. brasiliensis* yeasts at a ratio of 1 : 5 (*P. brasiliensis* : neutrophil); these co-cultures were incubated for 3 h as

described in Methods. Alternatively, neutrophils were treated with PMA (positive control) to induce NET formation (a, b) and with DNase I (b). Extracellular DNA was confirmed by immunofluorescence microscopy to be NETs by staining for elastase (anti-elastase; green), and DNA (PI, propidium iodide; red) (a); in addition, fungal cells were stained with Calcofluor White (blue) (a). Arrowheads indicate *P. brasiliensis* yeast cells in close contact with NET structures (images were captured using an Axiocam ICm 1 camera with ×63 or ×20 objective). (c) Induction of NET formation depends on the dose of fungal propagules. Neutrophils were incubated for 3 h at 37 °C with different ratios of *P. brasiliensis* morphotypes (conidia or yeasts) (m.o.i. 1, 5 and 10). Extracellular DNA was quantified using SYTOX Green by measuring its fluorescence intensity as described in Methods. Data are presented as mean ± SE of three independent experiments. *Statistically significant difference in percentage area compared with control ($P<0.05$). $^{+}$Statistically significant difference in comparison with *P. brasiliensis* yeast ($P<0.05$) by Mann–Whitney test. *Pb*, *P. brasiliensis*.

P. brasiliensis morphotypes showed dose-dependent production of NETs after 3 h incubation (Fig. 1c); both conidia and yeasts of *P. brasiliensis* induced significantly higher levels of fluorescence compared with the control ($P<0.05$). The quantity of NETs induced by *P. brasiliensis* morphotypes was comparable to that induced by LPS and *Ca. albicans*; it is noteworthy that PMA induced greater amounts of NETs, as evidenced by higher fluorescence observed in spectrofluorometry assays (Fig. 2a). To confirm that the observed fluorescence was due to extracellular DNA released, co-cultures were treated with DNase I. We observed that, after 3 h of incubation with DNase I, DNA decreased to levels equivalent to the values observed for non-stimulated neutrophils (negative control) (Fig. 2a). This phenomenon was observed with all other stimuli, demonstrating that NETs were composed of DNA (Fig. 2a). Furthermore, to evaluate whether phagocytosis is an important step in the production of NETs against *P. brasiliensis*, cytochalasin D (an inhibitor of actin polymerization) was added to the neutrophil/fungus co-cultures. After 3 h of incubation, treatment with cytochalasin D did not affect NET formation (Fig. S1, available in the online Supplementary Material). Additionally, we found that neutrophils pre-stimulated with recombinant IFN-γ did not have any effect on NET production against *P. brasiliensis* (data not shown).

Formation of NETs by *P. brasiliensis* conidia is independent of ROS production, while yeast morphotype partially depends on NADPH activation

An inhibitor of NADPH oxidase, DPI, was used to investigate whether NET formation depends on ROS production (Fig. 2b). Previous reports showed that inhibition of NADPH oxidase with DPI effectively blocks NET production (Fuchs *et al.*, 2007). DPI was added to the cultures of neutrophils along with different stimuli, including PMA, LPS, *Ca. albicans* and *P. brasiliensis* conidia or yeasts, and followed by 3 h of incubation. PMA treatment was used as a positive control in this experiment, as this stimulus activates the NADPH oxidase able to induce formation of NETs. We observed that NET production decreased significantly when DPI was added to neutrophils treated with PMA (Fig. 2b); this result confirms that NET induction by this stimulus depends on ROS production. Neutrophils treated with DPI and infected with *Ca. albicans* also showed a significant reduction in NETs (Fig. 2b). A similar significant reduction of NET formation was observed when neutrophils infected with *P. brasiliensis* yeast morphotype were treated with DPI. Interestingly, neutrophils stimulated with LPS or infected with *P. brasiliensis* conidia did not show a decrease in NET production when DPI was added (Fig. 2b).

Diminished expression of an AOX in *P. brasiliensis* is correlated with increased induction of NETs

An AOX of *P. brasiliensis* has been implicated in reduction of ROS, and is considered an important virulence factor in this fungus (Ruiz *et al.*, 2011). In order to determine the role of *P. brasiliensis* AOX in NET production, we used a mutant obtained by RNA antisense methodology as described elsewhere (Ruiz *et al.*, 2011). Of interest, we observed that neutrophils infected with *P. brasiliensis* yeast cells from the mutant strain (PbAOX) showed significantly higher NET formation ($P<0.05$) than those infected with the WT strain (ATCC 60855) or with fungal cells carrying the empty vector (PbEv60855) (Fig. 3). This result confirms that ROS may influence the formation of NETs against *P. brasiliensis* yeasts.

ROS production by PMN against *P. brasiliensis*

To determine whether *P. brasiliensis* yeasts activate ROS production by neutrophils, neutrophils were infected with *P. brasiliensis* yeasts. PMA induced high production of ROS by these cells. All the *P. brasililensis* strains (Pb60855, PbEV60855 and PbAOX) were able to induce increased ROS production. When co-cultures were treated with DPI, the production of ROS was reduced significantly to levels comparable with those observed in non-infected neutrophils (Fig. 4).

NETs do not exert a fungicidal mechanism against *P. brasiliensis*

To test the killing capacity of NETs against *P. brasiliensis*, neutrophils were co-incubated with *P. brasiliensis* yeast cells of strains Pb60855 and PbAOX. The fungicidal ability of NETs against *P. brasiliensis* was determined by counting c.f.u. No significant differences were found when c.f.u. numbers were compared between different strains (Fig. S2a).

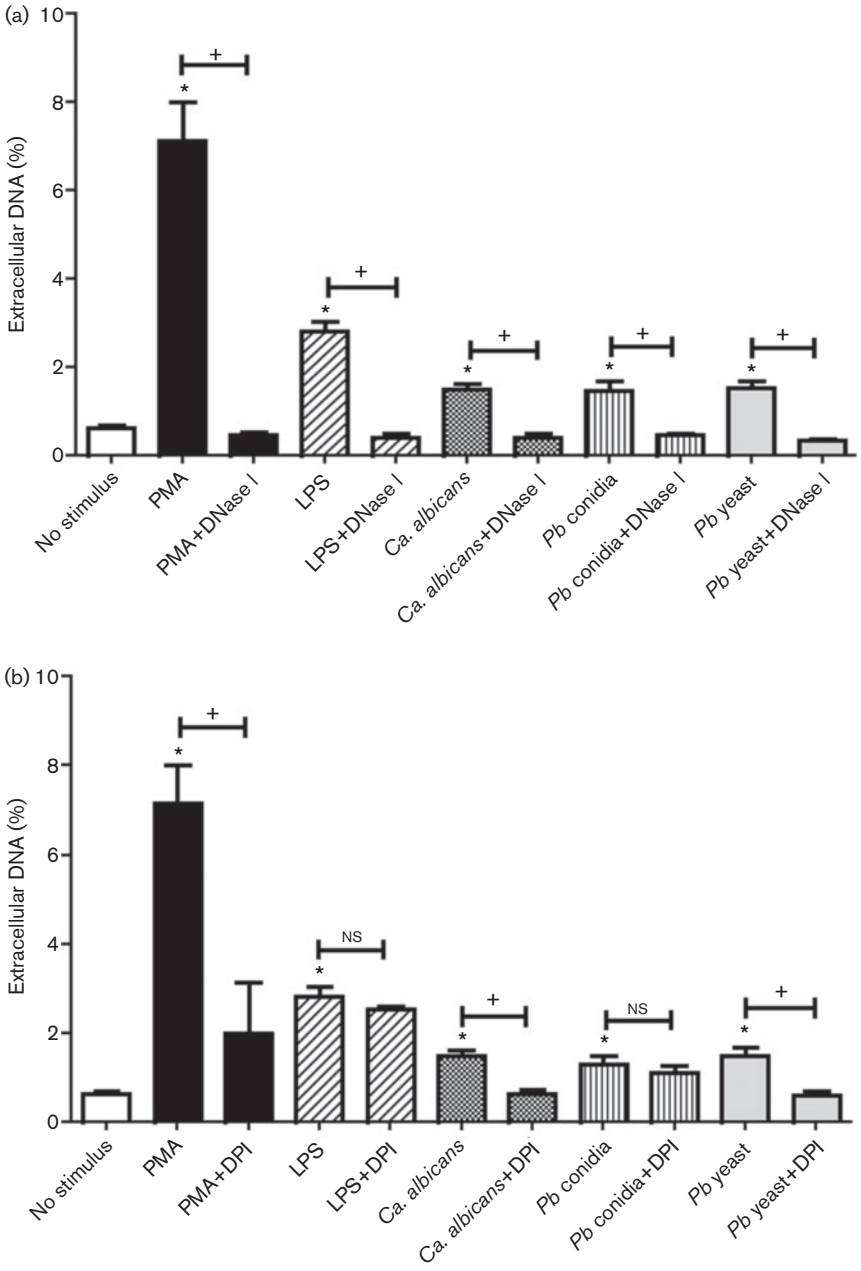

Fig. 2. NET formation by human neutrophils against *P. brasiliensis* is degraded by DNase I (a), and is either independent or partially dependent on ROS production according to the fungal morphotype (b). Neutrophils and fungus were prepared as described in Methods, treated with DNase I (a) or pre-treated with DPI (an NADPH oxidase inhibitor) for 30 min, stimulated with PMA and LPS, infected with *Ca. albicans* and *P. brasiliensis* morphotypes, and incubated for 3 h. Extracellular DNA was quantified using SYTOX Green by measuring its fluorescence intensity (RFU) as described in Methods. Data are presented as mean ± SE of three independent experiments. *Statistically significant difference in RFU levels compared with control ($P<0.05$). $^{+}$Statistically significant difference in RFU levels of treated co-cultures compared with untreated ones ($P<0.05$) by Mann–Whitney test. *Pb*, *P. brasiliensis*; NS, not significant.

In additional experiments, co-cultures of human neutrophils with *P. brasiliensis* yeasts (Pb60855 strain) were treated with DPI, DNase I or cytochalasin D. No significant differences were found when c.f.u. numbers were compared between different treatments (Fig. S2b). These results suggest that NETs do not exert a fungicidal effect against *P. brasiliensis*, as confirmed by the observation that NADPH activation was inhibited or DNA was degraded.

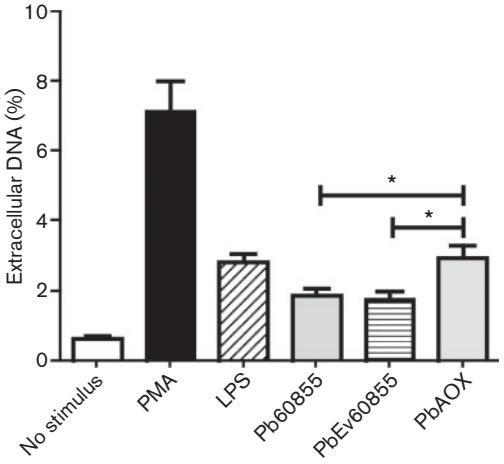

Fig. 3. A *P. brasiliensis* mutant with diminished expression of AOX induces higher NET formation. Neutrophils were co-incubated with yeast cells of a *P. brasiliensis* mutant strain with diminished expression of AOX (PbAOX), WT yeasts (ATCC 60855) or yeasts carrying the empty vector (PbEv60855) for 3 h. Extracellular DNA release was determined by measuring the fluorescence intensity using SYTOX Green stain as described in Methods. Data are presented as mean ± SE of three independent experiments. *Statistically significant differences in fluorescence levels of PbAOX compared with Pb60855 and PbEV60855 ($P<0.05$) by Mann–Whitney test. *Pb*, *P. brasiliensis*.

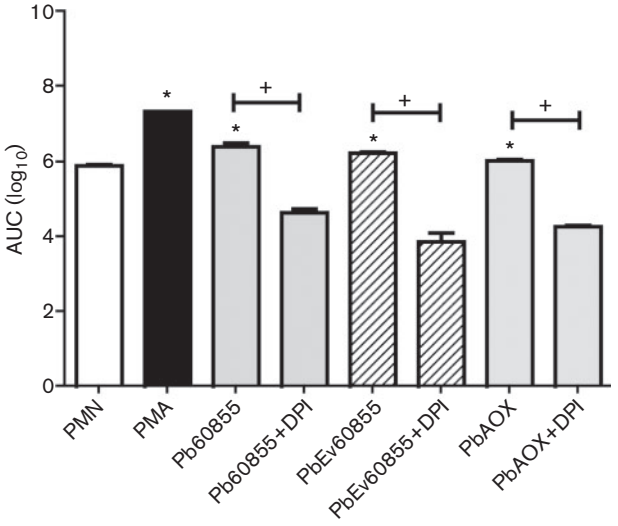

Fig. 4. *P. brasiliensis* yeasts induce ROS production by human neutrophils. Phagocytic cells were stimulated with PMA and infected with three strains of *P. brasiliensis* yeast cells comprising WT yeasts (Pb60855), yeast cells carrying the empty vector (PbEv60855) and the mutant strain with diminished expression of AOX (PbAOX). Neutrophils were pre-treated with DPI (an NADPH oxidase inhibitor) for 30 min. ROS production was measured using the luminol-enhanced chemiluminescence method as described in Methods. *Statistically significant difference in the area under the curve (AUC) compared with control ($P<0.05$). $^{+}$Statistically significant difference in AUC of DPI-treated co-cultures compared with untreated ones ($P<0.05$) by Mann–Whitney test. *Pb*, *P. brasiliensis*.

DISCUSSION

Neutrophils have been described as the major cells involved in the inflammatory response against *P. brasiliensis*. Neutrophils exhibit several microbicidal mechanisms, including both oxygen-dependent and -independent ones. A new microbicidal and particular mechanism has been described, that of NETs. This is characterized by extracellular release of DNA complexed with histones and other proteins stored in their granules (Brinkmann *et al.*, 2004). Various studies have shown that several medically important fungi, including *Ca. albicans*, *Aspergillus* spp. and *Cr. gattii*, have the ability to induce NETs (Urban *et al.*, 2006; Bruns *et al.*, 2010; Springer *et al.*, 2010). To our knowledge, the present report is the first to indicate that both the conidia and yeast morphotypes of *P. brasiliensis* induce NET formation by human neutrophils. However, no significant differences were found in NET production between *P. brasiliensis* morphotypes.

Interestingly, when we used DPI to inhibit NADPH oxidase activation, we found that NET production of neutrophils infected with *P. brasiliensis* conidia was not affected. By contrast, when yeast cells were used, we observed that addition of DPI to neutrophil/fungal co-cultures partially suppressed NET production. These results indicate that *P. brasiliensis* conidia induce NET formation through a ROS-independent mechanism, whereas yeasts depend on ROS production to a certain extent through activation of NADPH oxidase. Other reports have described the involvement of NADPH oxidase in NET formation. Thus, when NADPH oxidase activity was restored by gene transfer methodologies in neutrophils derived from the stem cells of patients suffering chronic granulomatous disease, re-establishment of NETosis and restoration of fungal defences against *Aspergillus nidulans* were observed (Yost *et al.*, 2009; Bianchi *et al.*, 2011). Additionally, neutrophils co-cultured with *A. fumigatus* and treated with DPI completely eliminated NET formation (Bruns *et al.*, 2010).

The cell wall is the first part of *P. brasiliensis* to come into contact with and be recognized by immune cells. It is one of the most variable structures between the different morphotypes of this fungus, especially with regard to its polysaccharide composition (Kanetsuna & Carbonell, 1970; Kanetsuna *et al.*, 1972). The yeast morphotype has higher proportions of α-1,3-glucan structures and less of those incorporating β-1-3-glucan, while mycelial and conidial morphotypes are composed mainly of the latter (Kanetsuna & Carbonell, 1970; Kanetsuna *et al.*, 1972). Studies on *Ca. albicans* have shown that the formation of NETs is induced after recognition of β-glucan by CR3 in neutrophils (Byrd *et al.*, 2013). In the present study, we found that conidia induce NET formation through a ROS-independent

mechanism; thus, it may be that recognition of this morphotype occurs via the β-glucan present on the conidial surface as well as the dectin-1 receptor, CR3 or other pattern-recognition receptors (PRRs) present on the surface of the phagocytic cells, whereas *P. brasiliensis* yeasts, whose cell walls are composed mainly of α-glucan, induce NET structures through a mechanism that is partially dependent on ROS production. In the latter case, recognition of yeast cells could be through a receptor other than dectin-1 or CR3; this differential recognition may or may not induce NADPH oxidase activation with subsequent ROS production and NET formation. In the ROS-independent NET production mechanism, the neutrophils rapidly void their nuclear content through vesicular secretion, yielding NETs and live intact cytoplasts that continue to crawl and digest microbes. Although there are currently no reports of α-glucan receptors, this molecule has been found to act as a decoy ligand for the β-glucan receptor (Rappleye *et al.*, 2007). More studies are needed in order to determine what components of *P. brasiliensis* morphotypes and which PRRs present on neutrophils are involved in the recognition of fungal cells and subsequent NET formation.

Several studies have shown that IFN-γ enhances the antifungal activity of murine and human neutrophils against *P. brasiliensis* (Kurita *et al.*, 2000). In the present study, we evaluated the effect of IFN-γ on NET production. However, we found that this cytokine did not induce or enhance the production of these structures.

On the other hand, one of the defence strategies exhibited by neutrophils against *P. brasiliensis* is the production of ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^\bullet$) (Kurita *et al.*, 1999b; Dias *et al.*, 2008). These oxidizing agents may ultimately alter the bioenergetic status of the cell and affect essential metabolic pathways that decrease fungal viability (Gessler *et al.*, 2007). As a counterpart, *P. brasiliensis* can also produce antioxidant molecules that may protect it from the oxidant molecules produced by the immune cells (Campos *et al.*, 2005; Dantas *et al.*, 2008; Maricato *et al.*, 2010). It is therefore noteworthy that an enzyme, AOX, recently described from *P. brasiliensis*, is involved in the detoxification mechanisms of this fungus (Ruiz *et al.*, 2011). Ruiz *et al.* (2011) used antisense technology to obtain a mutant strain of *P. brasiliensis* with reduced expression of AOX. They found that, in the presence of H_2O_2 *in vitro* the WT strain increased its AOX expression and presented a higher viability in comparison with the mutant PbAOX, whereas, *in vivo*, animals infected with the mutant strain showed lower fungal burdens and increased survival compared with animals infected with the WT strain. In the present study we evaluated the relevance of this enzyme to the production of NETs and found that neutrophils infected with the mutant strain (PbAOX) showed a higher level of NET formation than WT or the strain carrying the empty vector. Thus, we can hypothesize inhibition of NET formation in the presence of AOX as a mechanism that allows the fungus to evade the immune response.

In addition, in the present study, we demonstrated that neutrophils infected with *P. brasiliensis* yeasts were able to produce ROS. It was also confirmed that, when using DPI, inhibition of ROS production was observed. By the same token, we observed that this inhibitor also partially decreased NET formation induced by *P. brasiliensis* yeast cells, suggesting that NET activation is partially dependent on ROS.

Although it has been reported that NETs trap and efficiently kill microbial pathogens, it is also known that several bacteria, including *Staphyloccocus aureus*, *Streptococcus pneumoniae*, *Streptococcus suis* and *Neisseria meningitidis* can resist the antimicrobial effect of NET structures, and that this evasion mechanism could be attributed to the production of nucleases able to degrade these NETs, or alternatively to modification or expression of molecules on their membrane or cell wall that could interfere with them (Short *et al.*, 2014; Narayana Moorthy *et al.*, 2013; de Buhr *et al.*, 2014; Berends, *et al.*, 2010; Lappann *et al.*, 2013). Herein, we observed that the production of NETs was unable to kill *P. brasiliensis* yeasts efficiently. We did not find any difference in c.f.u. counts between different treatments that reduced or inhibited NET formation (DNase I and DPI). These findings indicate that *P. brasiliensis* yeasts are killed in a NET-independent fashion. More studies are needed in order to identify whether *P. brasiliensis* produces molecules capable of evading NET production.

In summary, our results show that NETs may be involved in the prevention of further spread by the fungus *P. brasiliensis*. Furthermore, we can hypothesize that the proteins present in the NET structures may have a fungistatic effect against *P. brasiliensis* yeasts, possibly by sequestration of iron or calcium (lactoferrin and calprotectin, respectively) (Brinkmann *et al.*, 2004; Urban *et al.*, 2009). More studies are needed in order to identify the molecules involved in such a mechanism.

ACKNOWLEDGEMENTS

This work was supported by the Departamento Administrativo de Ciencia, Tecnología e Innovación (Colciencias), Bogotá, Colombia, grant no. 2213-519-28621, and the Research Committee (CODI) of the Universidad de Antioquia through the Sustainability Strategy Program 2013–2014. Additional support was provided by the Corporación para Investigaciones Biológicas (CIB), Medellín, Colombia. The authors declare that there are no conflicts of interest.

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Edited by: M. Carol