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Leishmania donovani Promastigotes Evade the Antimicrobial Activity of Neutrophil Extracellular Traps

Christelle Gabriel,* † W. Robert McMaster, ‡,§ Denis Girard,* and Albert Descoteaux* †

Upon their recruitment to a site of infection and their subsequent activation, neutrophils release DNA and a subset of their granule content to form filamentous structures, known as neutrophil extracellular traps, which capture and kill microorganisms. In this study, we show that Leishmania promastigotes induced the rapid release of neutrophil extracellular traps from human neutrophils and were trapped by these structures. The use of Leishmania mutants defective in the biosynthesis of either lipophosphoglycan or GP63 revealed that these two major surface promastigote virulence determinants were not responsible for inducing the release of the surface protease neutrophil extracellular traps. We also demonstrate that this induction was independent of superoxide production by neutrophils. Finally, in contrast to wild-type Leishmania donovani promastigotes, mutants defective in lipophosphoglycan biosynthesis were highly susceptible to the antimicrobial activity of neutrophil extracellular traps. Altogether, our data suggest that neutrophil extracellular traps may contribute to the containment of L. donovani promastigotes at the site of inoculation, thereby facilitating their uptake by mononuclear phagocytes. The Journal of Immunology, 2010, 185: 4319–4327.

Neutrophils were isolated from venous blood of healthy volunteers by dextran sedimentation followed by centrifugation over Ficoll-Hypaque (Pharmacia Biotech, Quebec, Canada). Blood donations were obtained from informed and consenting individuals according to institutionally approved procedures. Cell viability was monitored by trypan blue exclusion, and the purity (>98%) was verified by cytology from cytocentrifuged preparations stained by Diff-Quick staining (Fisher Scientific, Ottawa, Ontario, Canada). (27).

Materials and Methods

Human neutrophils

Neutrophils were isolated from venous blood of healthy volunteers by dextran sedimentation followed by centrifugation over Ficoll-Hypaque (Pharmacia Biotech, Quebec, Canada). Blood donations were obtained from informed and consenting individuals according to institutionally approved procedures. Cell viability was monitored by trypan blue exclusion, and the purity (>98%) was verified by cytology from cytocentrifuged preparations stained by Diff-Quick staining (Fisher Scientific, Ottawa, Ontario, Canada). (27).
Parasites

Promastigotes of the *L. donovani* strains 1S and LV9 and the *L. major* strains LV39 and NIH S clone A2 were cultured at 26˚C in M199 supplemented with 10% heat-inactivated FBS, 100 μM adenine, HEPES, 5 μM hemin, 3 μM biotin, 1 μM biotin, and antibiotics. The *L. donovani* 1S isogenic mutants *lpg1*-KO and *lpg2*-KO were described previously (28). The *lpg1*-KO mutant secretes repeating Galβ1,4Manα1-PO4–containing molecules but lacks the ability to assemble a functional LPG glycan core (29), precluding synthesis of LPG. The rescued *lpg1*-KO add-back was generated by transfection of the pLeishZeo-LPG1 expression vector. The *lpg2*-KO mutant expresses the truncated LPG Gal(α1,6)Gal(β1,3)Glc(α1-P)Man(α1,3)Man(α1,4)Glu(α1,6)-PI and does not synthesize repeating Galβ1,4Manα1-PO4 units (30). The *L. major* NIH S clone A2 isogenic gp63-KO mutant and gp63-KO add-back were previously described (31).

Confocal immunofluorescence microscopy for the detection and quantification of NETs

Human neutrophils (10⁶ per well) were seeded on poly-L-lysine–coated glass coverslips (BD Biosciences, San Jose, CA) in RPMI 1640 supplemented with 10 mM HEPES, penicillin/streptomycin, and 2% heat-inactivated human serum and were allowed to adhere for 30 min at 37˚C in a humidified incubator with 5% CO₂. Adherent neutrophils were incubated in the absence or presence of either *Leishmania* promastigotes, zymosan, or LPG-coated zymosan during 10, 30, or 60 min at 37˚C. They were then fixed with 4% paraformaldehyde (Sigma-Aldrich), blocked overnight in blocking solution (PBS with 10% normal goat serum [Jackson ImmunoResearch Laboratories, West Grove, PA], 5% cold fish gelatin [Sigma-Aldrich], 1% BSA [Sigma-Aldrich], 0.05% Tween 20 [Sigma-Aldrich]), and incubated with primary Abs either directed against human neutrophil-elastase (Santa Cruz Biotechnology, Santa Cruz, CA) or against repeated subunits of LPG (CA7AE Ab). Secondary Abs coupled to Alexa Fluor 568 or 488 (Invitrogen/Molecular Probes, Eugene, OR) were used to detect primary Abs, and DRAQ5 (Biostatus Limited, Leicestershire, U.K.) was used for DNA labeling. Coverslips were mounted on slides with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). Specimens were analyzed with an oil immersion Nikon Plan Apo 100 (Toronto, Ontario, Canada) (numerical aperture 1.4) objective mounted on a Nikon Eclipse E800 microscope equipped with a Bio-Rad Radiance 2000 confocal imaging system (Bio-Rad, Hercules, CA). Percentages of cells producing NETs were determined by counting at least 100 cells per condition. Neutrophils that release DNA and elastase to form filamentous structures were considered as producing NETs.

Quantification of DNA released from neutrophils

Human neutrophils adhered to poly-L-lysine–coated glass coverslips were incubated with either *Leishmania* promastigotes or zymosan at a multiplicity of infection (MOI) of 10:1 for 30 min in RPMI 1640 supplemented with 10 mM HEPES, penicillin/streptomycin, and 2% heat-inactivated human serum. Neutrophils were opsonized by a 30-min incubation at 37˚C in the presence of 10% human C8-deficient serum (Sigma-Aldrich, St. Louis, MO).

**FIGURE 1.** Induction of NET by *L. donovani* promastigotes. A, B, Freshly isolated human neutrophils were adhered on poly-L-lysine–coated glass coverslips and incubated for 10 or 30 min with either serum-opsonized *L. donovani*-GFP promastigotes (A) or serum-opsonized zymosan (B). Samples were fixed and stained for neutrophil-elastase (red) and DNA (blue) (original magnification ×100). C, Human neutrophils adhered on poly-L-lysine–coated glass coverslips were incubated with either serum-opsonized or unopsonized *L. donovani* promastigotes at a parasite-to-cell ratio of 10:1. DNA release was quantified after 30 min of incubation. One representative experiment out of three performed in triplicate is presented as mean ± SD. *p* ≤ 0.01 compared with control neutrophils.
human serum at 37˚C in a humidified incubator with 5% CO2. For neutrophils in suspension, cells were distributed in a 48-well plate in the same culture medium (detailed above) and were incubated with either zymosan or L. donovani LV9 promastigotes at a MOI of 10:1 for 120 min at 37˚C in a humidified incubator with 5% CO2. Neutrophils in suspension were gently resuspended every 10 min. After incubation, 1 U/ml micrococcal nuclease (Worthington Biochemical, Lakewood, NJ) in the presence of 1 mM Ca2+ was added for 1 h at 37˚C. The nuclease activity was stopped with 5 mM EDTA, and samples were collected. Released DNA was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen/Molecular Probes). Samples were distributed into 96-well plates and were read in a spectrofluorometer reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA) with a filter setting of 480 nm (excitation)/520 nm (emission).

**Phagocytosis assay in suspension**

Human neutrophils (4 × 10^6 per well) were incubated with serum-opsonized zymosan or with serum-opsonized L. donovani LV9 promastigotes at a MOI of 3:1 in a 48-well plate in RPMI 1640 supplemented with 10 mM HEPES, penicillin/streptomycin, and 2% heat-inactivated human serum at 37˚C in a humidified incubator with 5% CO2. Cells were gently resuspended every 10 min. After 30 min of incubation, cells were washed with PBS and cytophased. Cytocentrifuged slides were stained with Diff-Quick, and phagocytosed particles were counted by light microscopy. Each condition was analyzed in triplicate, and at least 100 cells were scored per sample. Results were expressed as the number of ingested particles per 100 neutrophils.

**Effect of NETs on Leishmania promastigote survival**

Human neutrophils were adhered to poly-L-lysine–coated coverslips (BD Biosciences) in RPMI 1640 supplemented with 10 mM HEPES, penicillin/streptomycin, and 2% heat-inactivated human serum. Cells pretreated or not with DNase-1 (100 U/ml; Worthington Biochemical) were incubated with luciferase-expressing L. donovani 1S or L. major NIH S clone A2 promastigotes during 6 h in an incubator at 37˚C with 5% CO2. Controls consisted of promastigotes without neutrophils incubated in the absence or presence of DNase-I. Luciferase activity was measured using a luciferase assay system (Promega, Madison, WI). Briefly, after 6 h of incubation, cells were lysed with 1× Cell Culture Lysis Reagent (Promega, Madison, WI) containing 2× protease inhibitor (Roche, Laval, Canada). Luciferase activity was quantified using a Lumat LB 9507 luminometer (EG&G Berthold, Nashua, NH). Leishmania promastigote survival in the presence of human neutrophils was determined as the percentage of control values (luciferase-expressing Leishmania promastigotes incubated without neutrophils in presence or not of DNase-I).

**Influence of ROS on the induction of NETs by Leishmania promastigotes**

Human neutrophils adhered to poly-l-lysine-coated were incubated with the NADPH oxidase inhibitor diphenyleneiodonium (DPI) (5 or 10 μM) or catalase (1000 or 2000 U) for 30 min. Leishmania promastigotes were then added, and after 30 min DNA release was quantified.

**Incubation of neutrophils with Leishmania promastigote supernatant**

Human neutrophils adhered to poly-l-lysine-coated coverslips were incubated in the absence or presence of supernatants from L. donovani promastigotes in the stationary phase of growth, and after 30 min DNA release was quantified.

**Statistical analyses**

Each experiment was performed at least with three different blood donors. Comparisons of the means within and between groups were tested with the Student t test to determine statistical significance. A p value of ≤0.05 was considered significant.

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**FIGURE 2.** Different strains and species of *Leishmania* induce NET release in a time- and dose-dependent manner. Human neutrophils adhered on poly-L-lysine coverslips were incubated with either zymosan, L. donovani (strains 1S and LV9), or L. major (strain LV39) promastigotes at a MOI of 10:1 for A, B, and C or various ratios for D for the indicated time points. A, The percentage of neutrophils producing NETs was quantified for each time point. B–D, Release of DNA was quantified after 30 min. One representative experiment out of three to five performed in triplicates is presented as mean ± SD in each figure. *p* < 0.05; **p** ≤ 0.005 compared with control neutrophils and neutrophils incubated with zymosan.
were stained for the neutrophil-elastase (red) and DNA (blue). Triplicate presented as mean ± SD. B, C. Samples were fixed after 30 min and were stained for the neutrophil-elastase (red) and DNA (blue). L. donovani-GFP promastigotes are in green (original magnification ×100).

**FIGURE 3.** *Leishmania* promastigotes are trapped by NETs. Human neutrophils adhered on poly-l-lysine coverslips were incubated with *L. donovani* (1S and LV9 strains), *L. major*, or zymosan for 10 and 30 min at a particle-to-cell ratio of 10:1. A. Internalization of *Leishmania* promastigotes or zymosan was determined on at least 100 neutrophils for each condition. The data are the average of two independent experiments performed in triplicate presented as mean ± SD. B, C. Samples were fixed after 30 min and were stained for the neutrophil-elastase (red) and DNA (blue). L. donovani-GFP promastigotes are in green (original magnification ×100).

**Results**

Leishmania promastigotes induce the formation of NETs

To study one aspect of the interaction between *Leishmania* promastigotes and human neutrophils, we assessed the potential of these parasites to induce the formation of NETs. Human neutrophils were incubated for various time points with serum-opsonized *L. donovani*-GFP promastigotes (strain 1S), and the presence of NET was determined by confocal immunofluorescence microscopy. As early as 10 min after the addition of *L. donovani*-GFP promastigotes, neutrophils lost their typical rounded morphology and released NETs, which appear as filamentous structures identified by the presence of DNA and elastase (Fig. 1A). NET release appears to be contact-dependent, because we observed these structures only where neutrophils were associated with *L. donovani*-GFP promastigotes (Fig. 1A). In contrast, the levels of NET induced by serum-opsonized zymosan (Fig. 1B) were similar to the spontaneous baseline of NET release observed with resting neutrophils. We observed no differences between serum-opsonized and unopsonized promastigotes for their ability to induce the formation of NETs, as assessed by quantification of DNA released by neutrophils (Fig. 1C). NET formation was time-dependent, with >80% of neutrophils having released DNA and elastase after 1 h of contact with *L. donovani-GFP* promastigotes (Fig. 2A). We also assessed the ability of other *Leishmania* strains and species to induce the formation of NETs by quantifying the amount of DNA released by neutrophils 30 min after the initial contact with the parasites. As shown in Fig. 2B and 2C, promastigotes from two strains of *L. donovani* (Sudanese 1S and Ethiopian LV9) and the *L. major* strain LV39 induced comparable levels of DNA release. Confocal immunofluorescence microscopy confirmed that these *Leishmania* strains and species induced the formation of NETs (data not shown). Using various ratios of *L. donovani* LV9 promastigotes per neutrophil, we observed that the release of neutrophil DNA was dose-dependent, with a 4-fold increase above baseline levels for a MOI of 5:1 and a 10-fold increase above baseline levels at a MOI of 20:1 (Fig. 2D). Similar results were obtained with promastigotes of *L. donovani* 1S and *L. major* LV39 (data not shown).

Leishmania promastigotes are trapped by NETs

Because we observed rapid and massive formation of NETs in response to *Leishmania* promastigotes, we sought to determine the extent of parasite internalization by human neutrophils. Neutrophils adhered to poly-l-lysine–coated coverslips were incubated with either serum-opsonized promastigotes or serum-opsonized zymosan, and internalization was assessed by confocal immunofluorescence microscopy at 10 and 30 min after the addition of the particles. As expected, 8.5 and 50.5% of neutrophils had phagocytosed zymosan at 10 and 30 min, respectively (Fig. 3A, 3B). In contrast, very low levels of *L. donovani* (both 1S and LV9 strains) or *L. major* promastigotes were found to be internalized by human neutrophils (1%). Phagocytosis of *Leishmania* promastigotes by human neutrophils thus appears to be a rare event in our experimental system. Instead, as shown in Fig. 3C for *L. donovani*, promastigotes were found entrapped in NET fibers containing DNA and elastase. Because promastigote-induced NET formation occurred rapidly, it is possible that promastigotes get trapped before neutrophils can internalize them. Because previous studies revealed that *Leishmania* promastigotes are internalized by neutrophils in suspension (10, 18, 33, 34), it was important to assess the extent of *Leishmania* promastigote internalization and NET formation by human neutrophils in suspension. As shown in Fig. 4, we observed significant internalization of *L. donovani* promastigotes by human neutrophils in suspension, although to a lesser extent than zymosan. Importantly, we also found that *L. donovani* promastigotes induced DNA release from human neutrophils in suspension, indicating that NETs are produced under those conditions.

**NET release occurs in the absence of the parasite virulence factors LPG and GP63**

Given the prominent role of the surface glycolipid LPG in the establishment of *Leishmania* promastigotes inside mammalian hosts (2, 35, 36), we explored the possible role of LPG in the induction of NETs. To this end, we incubated human neutrophils with either wild-type (WT) *L. donovani* promastigotes, the LPG-defective *lpg1*-KO mutant, the Galβ1,4Manα1-PO4-defective *lpg2*-KO mutant, or the *lpg2*-KO add-back (*lpg2*-KO + *LPG2*). At various time points, NET formation was assessed by confocal immunofluorescence microscopy and by quantifying DNA release. Similar to WT promastigotes, both the *lpg1*-KO and the *lpg2*-KO mutants rapidly induced the formation of NETs and the release of DNA (Fig. 5), indicating that neither LPG nor other Galβ1,4Manα1-PO4-containing *Leishmania* glycoconjugates were responsible for the induction of NET release by *Leishmania* promastigotes. Of note, we observed morphological differences between NETs induced by WT and LPG-defective mutants at early time points (Fig. 5C). Hence, at 10 min, NETs...
induced by lpg2-KO promastigotes appeared to form clusters and looked more compact than those induced by WT promastigotes that were more filamentous (Fig. 5C). However, at 1 h after the addition of the promastigotes, NETs induced by both WT and lpg2-KO parasites were structurally similar. To confirm that LPG plays no role in the induction of NET formation, we incubated human neutrophils with either zymosan or zymosan coated with purified LPG and quantified DNA release. As shown in Fig. 6D, neither zymosan nor LPG-coated zymosan induced DNA release from neutrophils. Furthermore, in contrast to promastigotes, LPG-coated zymosan and

**FIGURE 4.** Leishmania promastigotes are internalized by human neutrophils in suspension and induce NET release. Human neutrophils in suspension were incubated with either serum-opsonized zymosan or serum-opsonized L. donovani (LV9) promastigotes at a MOI of 3:1 during 30 min (A, B) or at a MOI of 10:1 during 120 min (C). A, Diff-Quick-stained cytospin preparation showing internalized (white arrows) or noninternalized (black arrow) L. donovani promastigotes (original magnification ×100). B, Internalization of Leishmania promastigotes or zymosan was determined on at least 100 neutrophils. C, Release of DNA was quantified after 120 min. One representative experiment out of three to four performed in triplicate is presented as mean ± SD in each figure. *p ≤ 0.0002 compared with control neutrophils and neutrophils incubated with zymosan.

**FIGURE 5.** Induction of NET release is independent of LPG and other Galβ1,4Manβ1-PO4-containing L. donovani glycoconjugates. Human neutrophils adhered on poly-l-lysine coverslips were incubated for 10, 30, and 60 min with either WT, lpg1-KO, lpg2-KO, or lpg2-KO add-back L. donovani promastigotes. A, The percentage of neutrophils producing NETs was determined by confocal microscopy. The data are the average of three independent experiments performed in triplicate presented as mean ± SD. B, Release of DNA was quantified after 30 min. One representative experiment out of three performed in triplicate is presented as mean ± SD. *p < 0.05 compared with resting neutrophils. C, At the indicated time points, samples were fixed and stained for the neutrophil-elastase (red) and DNA (blue). L. donovani-GFP promastigotes are in green (original magnification ×100).
zymosan were equally internalized by human neutrophils (Fig. 6). The surface GPI-anchored metalloproteinase GP63 is another abundant promastigote surface molecule that contributes to the establishment of infection within mammals (31). To assess its possible role in the induction of NET release, we incubated human neutrophils with either WT L. major promastigotes, the gp63-KO mutant, or the rescued gp63-KO add-back. After 30 min of incubation, we assessed NET formation by confocal immunofluorescence, and we quantified the amount of DNA released by the neutrophils. As shown in Fig. 7A, WT, gp63-KO and gp63-KO add-back L. major promastigotes induced similar levels of NETs and DNA release from neutrophils, indicating that GP63 is not responsible for the induction of NET formation by Leishmania promastigotes. We also tested whether soluble factors released by promastigotes could induce the release of NET. As shown in Fig. 7B, addition of promastigote-conditioned medium that contains Leishmania exosomes (37) to neutrophils did not trigger the release of DNA.

**LPG confers L. donovani promastigote resistance to the antimicrobial activity of NETs**

Because NETs possess potent antimicrobial activity, we determined the impact of NET release on the viability of L. donovani promastigotes. We included lpg1-KO L. donovani promastigotes in this assessment, because LPG forms a dense glycocalyx at the promastigote surface that protects the parasite from microbicidal molecules (4). To this end, we incubated human neutrophils with luciferase-expressing L. donovani promastigotes (WT, lpg1-KO, and the rescued lpg1-KO add-back), and we measured luciferase activity after 6 h of incubation. As shown in Fig. 8A, lpg1-KO promastigote survival was significantly reduced compared with that of either wild-type or rescued lpg1-KO add-back promastigotes. The antimicrobial activity of NET can be abrogated with DNase-1 treatment of neutrophils (19, 38). We thus incubated human neutrophils with luciferase-expressing L. donovani WT and lpg1-KO promastigotes in the absence or presence of DNase-1, and we measured luciferase activity after 6 h of incubation. As shown in Fig. 8B, lpg1-KO promastigote survival was restored in the presence of DNase-1, indicating that LPG confers L. donovani promastigotes the ability to resist the microbicidal activity of NET. Of note, resistance to the antimicrobial activity of NET was also observed with L. major promastigotes (Fig. 8C).

**ROS are not involved in the induction of NETs by Leishmania promastigotes**

Previous studies revealed that ROS production is involved in the release of NETs (38, 39). To assess the contribution of ROS in the...
release of NETs, we incubated neutrophils in the absence or presence of either catalase or DPI before the addition of promastigotes. As shown in Fig. 9, we did not observe any significant effect of the ROS inhibition on the release of NETs induced by \textit{L. donovani} promastigotes, suggesting that a ROS-independent mechanism is involved in this process.

**Discussion**

In the current study, we report that although \textit{L. donovani} promastigotes induce and are trapped by NETs, the surface glycolipid LPG enables these parasites to resist the microbicidal activity of these structures. The observation that induction of NET release was independent of the promastigote opsonization status suggested that

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**FIGURE 7.** NET release is not mediated by GP63 or a secreted factor. Human neutrophils adhered on poly-L-lysine coverslips were incubated for 30 min with either WT, \textit{gp63}-KO, or \textit{gp63}-KO add-back \textit{L. major} promastigotes. A, Samples were fixed and stained for the neutrophil-elastase (green), DNA (blue), and \textit{L. major} promastigotes (anti-LPG, red) (upper panel). Release of DNA was quantified after 30 min (lower panel). One representative experiment out of three performed in triplicate is presented as mean ± SD (original magnification ×100). B, Adherent human neutrophils were incubated with either \textit{L. donovani} promastigotes or promastigote-conditioned medium. After 30 min, release of DNA was quantified. Results are the average of three independent experiments performed in triplicate presented as mean ± SD. *p < 0.05 compared with resting neutrophils.

**FIGURE 8.** \textit{L. donovani} promastigotes are resistant to the antimicrobial activity of NETs. A, Human neutrophils adhered on poly-l-lysine coverslips were incubated at a MOI of 10:1 with opsonized \textit{L. donovani} 1S promastigotes (WT, \textit{lpg1}-KO, and rescued \textit{lpg1}-KO add-back) expressing luciferase. After 6 h, promastigote survival was determined by dividing the luciferase activity relative light units (RLUs) in presence of neutrophils by the luciferase activity in the absence of neutrophils. One representative experiment out of four performed in triplicate is presented as mean ± SD. B, Adhered neutrophils were incubated in the absence or the presence of DNase-1 before the addition of \textit{L. donovani} promastigotes (WT and \textit{lpg1}-KO) expressing luciferase at a MOI of 10:1. After 6 h, promastigote survival was determined as described in A. One representative experiment out of two performed in triplicate is presented as mean ± SD. C, Adhered neutrophils were incubated at a MOI of 10:1 with either \textit{L. donovani} (1S) or \textit{L. major} (NIH S clone A2) promastigotes expressing luciferase. After 6 h, promastigote survival was determined as described in A. One representative experiment out of three performed in triplicate is presented as mean ± SD.
compared with resting neutrophils. and moniae killing by NETs. One such strategy used by some pathogens have evolved strategies to escape trapping and/or bactericidal/permeability-increasing protein (19, 23, 41). However, in these structures, which include histones, calprotectin, and the high local concentrations of antimicrobial molecules present in the existence of a ROS-independent mechanism. The release of NETs clearly remains to be elucidated, our data suggest the mechanism by which release may not involve secreted molecules or exosomes (37). Of to the bacterial surface (44). Another escape mechanism consists of avoiding NET induction. Hence, the hydrophobin RodA, the major surface component of Aspergillus fumigatus conidia, enables this form of the fungus to evade the triggering of NET formation (45). In the case of L. donovani promastigotes, our results indicate that LPG confers resistance to the microbialic activity of NETs. Previous studies revealed that the dense glycocalyx formed by LPG at the promastigote surface protects the parasite from hydrolytic enzymes present in the sand fly midgut and from the lytic components of complement (4, 35, 46). We propose that LPG acts as a physical barrier to protect promastigotes from the antimicrobial molecules present in NETs. Thus, similar to Mycobacterium tuberculosis (26), L. donovani promastigotes are trapped by NETs but evade the antimicrobial activity of these structures. It was recently reported that L. amazonensis promastigotes are killed by NETs (21). The discrepancy between those data and our results suggests that L. amazonensis, L. donovani, and L. major are differentially resistant to the antimicrobial activity of NETs. Alternatively, different experimental procedures may account for this discrepancy. Hence, Guimarães-Costa et al. (21) performed their killing assay by incubating L. amazonensis promastigotes with PMA-activated neutrophils or with supernatants from PMA-activated neutrophils, whereas we incubated L. donovani and L. major promastigotes with neutrophils adhered to poly-l-lysine–coated coverslips. Further studies will be required to address this issue.

Much attention has been recently devoted to the internalization of Leishmania promastigotes by neutrophils and the consequences of this process on infection (7, 47, 48). Under the experimental conditions used in this study to visualize NETs (neutrophils adhered to poly-l-lysine–coated coverslips), internalization of promastigotes by neutrophils was an unfrequent event. This does not exclude the fact that internalized promastigotes may survive in a compartment lacking appropriate microbicidal components (10) and thus enter macrophages according either to the Trojan horse strategy (12, 13, 49, 50) or the “Trojan rabbit” strategy (7, 11). However, consistent with previous reports, we found that human neutrophils in suspension significantly internalized L. donovani promastigotes, albeit to a lower extent than zymosan. More importantly, we found that neutrophils in suspension produced NET, as assessed by the quantification of DNA release. Interestingly, careful examination of a figure from an earlier study by Pearson and Steigbigel (33) showing Giemsa-stained cyt centrifuged neutrophils incubated for 3 h with L. donovani promastigotes strongly suggests that most neutrophils were dying, perhaps as a consequence of NET release.

NETs were reported in patients and in various experimental models of infection, where they may contribute to the confinement and/or killing of pathogens as well as the modulation of host immune responses (19, 21, 23, 42, 43, 45). In the context of Leishmania infection, NETs were observed in lesion biopsies of patients with active cutaneous leishmaniasis, and it was proposed that they could be relevant to control the parasite burden (21). The release and impact of NETs in vivo after inoculation of Leishmania promastigotes by the sand fly remain to be demonstrated, and the effect of sand fly saliva on the modulation of NET release and antimicrobial activity is an important issue that will need to be investigated. Whereas Guimarães-Costa et al. (21) proposed that NETs are an innate response that might contribute to diminish parasite burden in the Leishmania inoculation site, based on our findings, we propose that NETs might immobilize L. donovani promastigotes to facilitate their uptake by mononuclear phagocytes recruited at the site of inoculation.

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Disclosures

The authors have no financial conflicts of interest.

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