

Infection and Activation of Human Neutrophils with Fluorescent *Leishmania infantum*

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Abstract

Neutrophils (PMNs) are recruited in high numbers to sites of host infection by the protozoan parasites of the genus *Leishmania*. Although PMNs are capable of phagocytizing *Leishmania* parasites and are potent producers of antimicrobial compounds including reactive oxygen species (ROS), they are unable to control the establishment of infection. Prior studies document production of ROS in isolated PMNs incubated with *Leishmania* under conditions allowing phagocytosis, however without a measure of single cells' responses it cannot be discerned whether PMN activation and ROS production is suppressed or ineffective in the cells that internalize the parasite. To address these interactions, we engineered a strain of fluorescent, mCherry-expressing *Leishmania infantum* (mCherry-Li). By infecting isolated human PMNs *in vitro* with mCherry-Li, we observed ready association of parasites with PMNs in a time- and dose-dependent fashion. We also examined production of PMN ROS (using the fluorescent compound DHR123) and PMN activation (as evidence by loss of surface CD62L expression). Whereas many *Leishmania* associated (mCherry⁺) PMNs responded to parasites with ROS production and/or activation, a proportion exhibited neither response. Furthermore, a large proportion of mCherry - "bystander" PMNs displayed both ROS production and activation. The heterogeneous response of PMNs to *Leishmania* exposure leads us to hypothesize, first, that some PMNs exhibit decreased activation upon phagocytosis of *Leishmania*, and could support their maintenance. Second, responses of bystander PMNs may contribute to a local inflammatory environment that is ineffective at parasite clearance.

Keywords: *Leishmania*; Neutrophils; Flow cytometry

Introduction

The *Leishmania* spp. is vector-borne protozoa that cause a spectrum of highly prevalent diseases of humans, referred collectively as *leishmaniasis*. The most severe form of the disease is visceral *leishmaniasis* caused, in the New World, by *L. infantum* in endemic regions [1]. Within its phlebotomine sand fly vector the *Leishmania* spp. assume an extracellular promastigote state, but soon upon

inoculation into mammalian dermis the parasites are taken up by phagocytic cells wherein they transform to an obligate intracellular amastigote form. The mechanisms whereby the parasite is efficiently taken up by and survives within the mammalian phagocyte are critical to disease pathogenesis. Although parasites are found in macrophages through much of infection [2,3], it has become apparent that other cell types may perhaps act as the first haven for parasites within the host [4-8], but how these non-macrophage cell-types interact with *Leishmania* parasites is less-well understood. Many studies examine early *Leishmania*-host cell interactions by documenting in bulk culture [9]. However, methods examining the phenotypic responses of infected immune cells on a per-cell basis have been invaluable in increasing our understanding of early disease pathogenesis [5,10].

Neutrophils (PMNs), the most abundant circulating leukocyte in healthy humans, and are primary effector cells that provide host defense by migrating to sites of infection [11,12]. It is clear that PMNs play a role in *Leishmania* infections, but their effect on disease outcome remains unclear [5,6,8,13]. Neutrophils can internalize and kill at least some *Leishmania* promastigotes *in vitro* [14-18]. They are the first cell to migrate to, and take up parasites at the site of *in vivo* infection with either *L. major* [5,18-20] or with *L. infantum* [6] spp. Indeed, there appears to be distinct differences in the way various species of *Leishmania* result in activation and otherwise affect neutrophils [10,21-23]. In the context of disease progression, it has been suggested that, rather than control the infection, neutrophils exacerbate disease onset either by delaying apoptosis, by impairing activation of dendritic cells, or by serving as a vehicle route for silent entry of the parasite [4,24-26].

In order to investigate the interactions between *L. infantum* and infected mammalian cells, we engineered transgenic *L. infantum* expressing fluorescent proteins. We validated the use of mCherry-expressing *L. infantum* (mCherry-Li) to examine the infection of human PMNs *in vitro* by both fluorescent microscopy and flow cytometry. The detection of the mCherry signal also provided means to determine the temporal and dose-dependent uptake mCherry-Li by the PMNs. Furthermore, the ability to distinguish infected (mCherry⁺) from uninfected (mCherry⁻) PMNs allowed us to differentiate phenotypic responses based on infection status of individual.

Materials and Methods

Leishmania parasites

A Brazilian strain of wild type *L. infantum* (MHOM/BR/00/1669) was maintained in hamsters by serial intra cardiac injection of amastigotes. Parasites were grown as promastigotes at 26°C in liquid hemoflagellate-modified minimal essential medium (HOMEM) [27]. The gene encoding either mCherry or dTomato [28] was cloned into *Leishmania* expression vector pIRISAT (kindly provided by Stephen M. Beverly, Ph.D., Washington University at St. Louis). Wild-type parasite cultures were transfected with gel-purified integrating expression constructs by electroporation, and clones were selected from semisolid agar plates [29,30]. Transfectants, called mCherry-Li or dTomato-Li respectively, were grown to stationary phase in HOMEM. Genomic DNA was extracted from transfectants using a described procedure, subjected to restriction enzyme digestion with SmaI, and analyzed on a Southern blot [31]. Briefly, DNA digests were separated on a 0.8% agarose gel, transferred to Nylon (Roche, Mannheim,

Germany) and UV cross-linked. The gene encoding streptothricin acetyltransferase (SAT) was amplified by PCR from pIRISAT using the following primers: forward 5'-ATGAAGATTTCCGGTGATCCC-3'; reverse 5'-TTAGGCGTCATCCTGTGCTC-3'. The probe was [³²P]-labeled with a Random Primer Amplification Kit (Roche), incubated with the nylon filter in a roller bottle, and washed under high stringency conditions.

Human neutrophils

Protocols for all studies with human subjects were approved by the University of Iowa Institutional Review Board (protocol 201304709). Human neutrophils were isolated from venous blood of healthy adults after incubation in 3% Dextran (Pharmacosmos, Denmark) to sediment erythrocytes followed by density separation over a Histopaque Ficoll (Sigma-Aldrich, Missouri, USA) gradient, as previously described [32]. Purity and viability, assessed by Trypan blue (Invitrogen, California, USA) exclusion and microscopy, was consistently >95% viable neutrophils.

Neutrophil infections for fluorescence microscopy

For fluorescent microscopy, PMNs were labeled in 5 µm carboxy fluorescein succinimidyl ester (CFSE)(Molecular Probes-Invitrogen, California, USA) for 10 min, rinsed by centrifugation, and allowed to adhere for 30 minutes to 24-well plates containing coverslips that had previously been acid washed, flamed and coated with fibrinogen (10µg/ml of saline). Stationary phase mCherry-Li promastigotes were opsonized with 5% human serum for 15 minutes washed twice and cultured with PMNs at a multiplicity of infection (MOI) of 5:1. Infections were synchronized by centrifugation of plates (1400 rpm, 2 min), then incubated for varying times at 37°C prior to fixing in 2% paraformaldehyde. Images were generated using an Olympus IX-81 inverted fluorescence microscope. Images analyzed using Image J software.

Neutrophil infection for flow cytometry and cytospin

For flow cytometry, 2x10⁷ unlabeled PMNs/ml were incubated with 1:1, 5:1 or 10:1 serum opsonized mCherry-Li in RPMI with 10% fetal bovine serum, 2 mM L-glutamine, and 100 µg streptomycin/ml. Control PMNs were incubated in buffer without parasites. Cells were incubated at 37°C, 5% CO₂ for the time indicated in different experiments. To compare the detection of *Leishmania* parasite uptake as detected by flow cytometry vs. microscopy, Li parasites (either mCherry-expressing or CFSE stained) were incubated with PMNs in suspension as above for 30 minutes. PMN-parasites cultures were then divided into two portions, one analyzed by flow cytometry. The other portion was spun by cytospin onto a glass slide and cells stained with Hema 3 (modified Wright's stain) and analyzed by light microscopy. Slides were prepared in duplicate. The numbers of internalized and surface-bound parasites were quantified with at least 200 cells counted per slide.

Flow Cytometry

For detection of reactive oxygen species and surface CD62L by flow cytometry, the same protocol for PMN infection was followed as above. After 15 minutes at 37°C, 5% CO₂, DHR123 (Molecular Probes-Invitrogen, California, USA) was added to measure reactive oxygen species at a concentration of two micro molar (2µM). Five minutes later (20 minutes after adding parasites), neutrophils were put on ice

and stained with antibodies. PMNs were suspended in FACS staining buffer (1x PBS, 0.5% HI FBS, 0.01% sodium azide) with 10% vol/vol normal rat serum to block Fc receptors. After washing, samples were incubated at 4°C with fluorochrome-conjugated primary antibody to human CD62L PE-Cy7 (BD Bioscience, California, USA) for 30-60 minutes. Cells were then fixed in 2% PFA prior to flow cytometric analysis. Samples were analyzed on a FACS LSR II (Becton Dickinson, California, USA) and data were analyzed using FlowJo software (Treestar Inc., Oregon, USA).

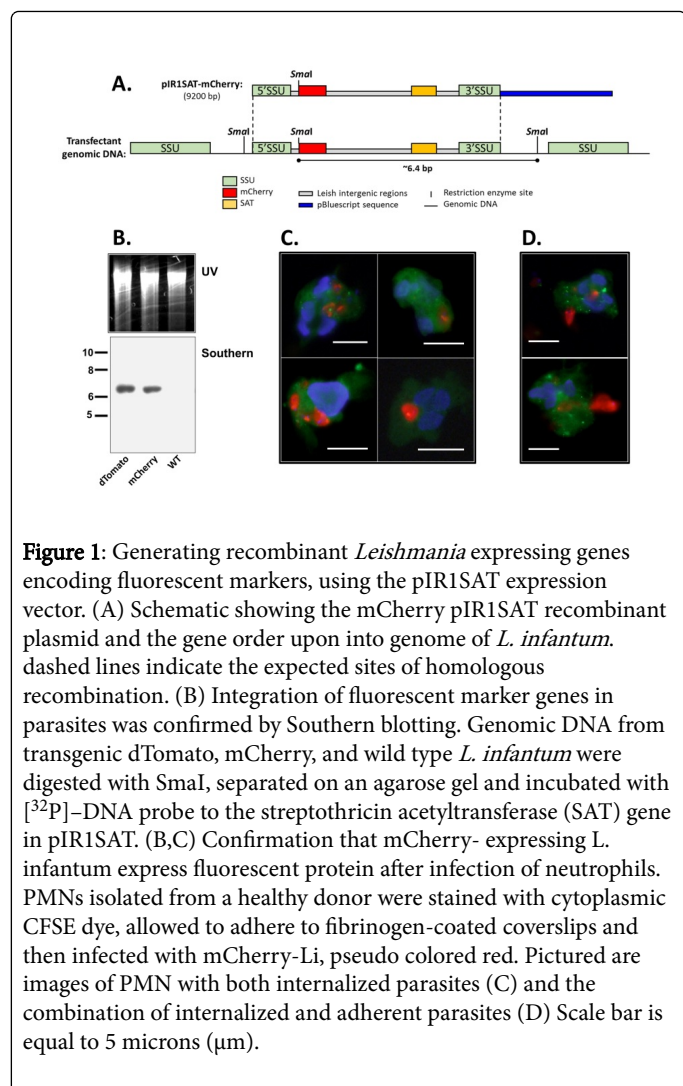
Results

Generation of recombinant mCherry-expressing *Leishmania infantum*

In order to accurately detect *L. infantum* harboring cells, we generated a panel of transgenic *L. infantum* lines constitutively expressing different fluorescent proteins. Genes encoding mCherry or dTomato were introduced into the *L. infantum* by homologous recombination at the SSU ribosomal DNA locus using the vector pIRISAT. Figure 1A illustrates the pIRISAT-mCherry construct and insertion site. To determine whether pIRISAT had been retained as an episome or had been integrated into the genome, Southern blots of *L. infantum* genomic DNA were probed with [³²P]-DNA corresponding to the full-length streptothricin acetyltransferase (SAT) gene sequence. If pIRISAT were not integrated, digestion of total promastigote DNA with the restriction enzyme SmaI should generate a 9.2 kb DNA fragment band due to linearizing the plasmid containing the SAT gene. In contrast, genomic integration of SAT and the fluorescence marker should generate a smaller fragment containing the SAT gene, digesting within the insertion and in the flanking SSU rDNA locus. Southern blot analysis revealed a 6.4 kb band in lanes containing genomic DNA from mCherry-Li and dTomato-Li transfectants not from wild type *L. infantum*, consistent with integration of transgenes into the parasite genome (Figure 1B). In contrast, a separate Southern blot of the pIRISAT-mCherry plasmid digested with SmaI yielded the expected 9.2 kb band corresponding to the linearized plasmid (not shown). Fluorescent protein expression was verified by fluorescence microscopy. Isolated human PMNs were stained with CFSE (to simplify cytoplasmic visualization), allowed to adhere to a fibrinogen-coated coverslip, and infected with mCherry-Li for 20 minutes. The majority of mCherry-Li associated with PMNs were engulfed by PMNs (Figure 1C). Internalized parasites could be differentiated from surface-adherent parasites microscopically (Figure 1D). These experiments suggest that mCherry-Li stably expressed the fluorescent marker and could, at least during short-term PMN-infections, continue to be detected even following phagocytic uptake of the parasites.

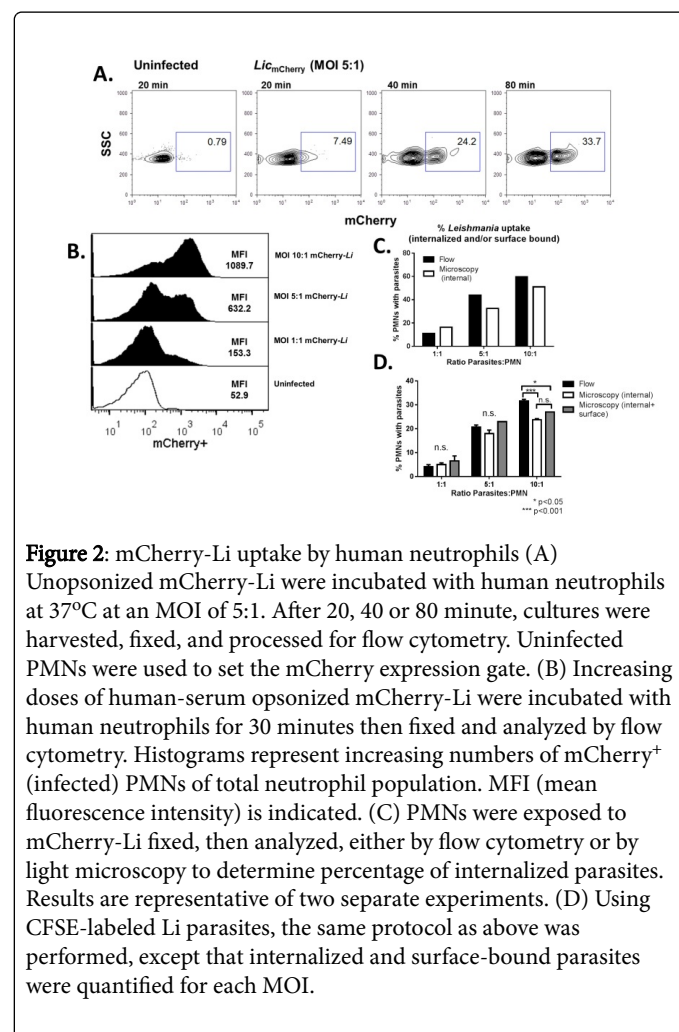
L. infantum uptake by human neutrophils

We examined the kinetics of attachment and uptake of *L. infantum* parasites by human neutrophils using flow cytometry. PMNs in suspension were incubated with mCherry-*L. infantum* at an MOI of 5:1, and we measured the uptake of the mCherry signal by flow cytometry (Figure 2A) with uninfected PMNs as the control. The proportion of mCherry⁺ PMNs increased over the first 80 minutes of incubation, indicating engulfment or surface-bound parasites associated with the PMNs.



PMNs were exposed for twenty minutes with varying MOIs of mCherry-Li. Higher MOIs resulted not only in higher percentages of mCherry⁺ PMNs, but an increased mCherry signal (MFI) indicating a higher parasite burden on a per-cell basis (Figure 2B). To determine whether mCherry⁺ PMNs detected by flow cytometry corresponded to PMNs with surface bound or internalized parasites, PMNs were cultured with mCherry-Li in increasing MOIs, fixed in 2% PFA, and divided into two portions. The first part was analyzed by flow cytometry (Figure 2C, black bars). The second part was applied to glass slides using a cytospin, stained and analyzed by light microscopy for the percentage of PMNs that clearly contained intracellular parasites (Figure 2C, white bars). We consistently detected similar but slightly higher numbers of infected PMNs by flow cytometry than microscopy at each MOI. To discern whether the difference was due to the numbers of surface bound, as opposed to internalized parasites, we analyzed the proportions of fluorescent CFSE-labeled parasites that were attached or internalized by microscopy, in parallel with flow cytometry (Figure 2D). At MOIs of 1:1 and 5:1 there was an insignificant trend, but at an MOI of 10:1 there was significantly more association of Li with PMNs measured by flow cytometry compared to microscopy. Again, there was a trend toward increased numbers of total (internalized plus surface-bound) PMN-associated parasites, but

the difference did not reach statistical significance. This could indicate either those microscopic counts are underestimates, or that some parasites lysed and deposited their fluorescent dye in the recently infected neutrophils, as we previously reported [33]. With either scenario, these data suggest that the *L. infantum*: PMN ratio influences parasite burden in both individual cells as well as in the entire neutrophil population. Additionally, these results confirmed that the majority of PMNs marked with the mCherry signal contain internalized parasites, and that a minority have either attached parasites or recently killed intracellular Li parasites in our *in vitro* culture.



PMN production of ROS and activation

Studies examining interactions between PMNs and *Leishmania* species show that PMNs take up but do not eliminate all internalized parasites [4,25]. We questioned how internalization of *L. infantum* would alter the PMNs' activation status and production of microbicidal compounds such as ROS. To address this question, we exposed human PMNs to mCherry-Li in suspension and used flow cytometry to distinguish between Li-associated or non-associated PMNs and to examine PMN ROS-production and activation. As a control for the independent effects of isolated procedures on PMNs, an unexposed group of PMNs that was derived from the same preparation was exposed to incubation conditions and buffer additions exactly in

parallel with neutrophils infected with parasites or zymosan particles. Thus art factual effects of isolation procedures should be evident by inspecting this control.

We measured oxidative burst with the fluorescent indicator 1,2,3-dihydrorhodamine (DHR123) [11]. After culturing mCherry-Li and PMNs at increasing MOIs, we compared DRH123 fluorescence in both infected (mCherry⁺) and “bystander (mCherry⁻) PMNs (Figure 3A). Parasite-containing (mCherry⁺) PMNs at each MOI showed increased ROS activity compared to uninfected bystanders from the same conditions. However, we noted that bystander neutrophils in both MOI 5:1 and MOI 10:1 both showed a substantial amount of DHR123 fluorescence. To test whether the oxidative response of bystander neutrophils was caused by a soluble mediator, we incubated neutrophils with supernatant from a previous neutrophil *L. infantum* infection (Figure 3B, bottom histogram) or from *Leishmania*-conditioned media (not shown). Neither supernatant induced ROS production from neutrophils. This suggests that stimulation of bystander neutrophils could require direct contact with parasites or with infected PMNs.

The activation status of infected neutrophils was further explored by staining for surface CD62L (L-selectin), an integrin important for neutrophil migration through host endothelium, which is cleaved from the PMN cell surface upon activation [34,35]. Neutrophils were cultured with different ratios of mCherry-Li. The magnitude of activation, according to the loss of surface CD62L, and ROS generation was measured in mCherry-associated neutrophils within the culture (mCherry⁺ in top row of Figure 3C) or in bystander neutrophils (mCherry⁻; bottom row of Figure 3C). Both infected and bystander PMNs in the culture responded with ROS generation and CD62L loss, and the magnitude was impacted by the multiplicity of infection (Figure 3C). At MOIs of 5:1 and 10:1, the majority of mCherry⁺ PMNs produced DHR123 and was CD62L low. However, the bystander cells in these conditions showed large numbers of CD62L low and DHR123⁺ cells, indicating large amounts of activation. Interestingly, the markers were not fully correlated: substantial numbers of CD62L low cells remained DHR123 negative in all conditions, both in mCherry⁺ and mCherry⁻ PMNs. Furthermore, at the 1:1 MOI, a group of mCherry⁺ neutrophils remained CD62L high DHR123⁻. This requires additional validation, but could suggest that a subset of neutrophils is able to bind or internalize *L. infantum* without triggering either marker of neutrophil activation.

Discussion

It is well established that protozoan *Leishmania* spp. survive inside macrophages throughout chronic infection of their mammalian hosts [3]. Reports over the past 5 years have made it clear that neutrophils are among first cells to arrive at the site of a sand fly bite and to take up the parasite [5,6]. An essential step in our understanding of the role of PMNs in *leishmaniasis* is the bidirectional effect of parasites on neutrophils and vice versa. Prior reports document generation of PMN ROS by the collective population of neutrophils in response to *Leishmania* phagocytosis *in vitro* [14,34]. Generation of transgenic parasites expressing fluorescent markers enabled us to examine these questions on a single cell basis. We were thus able to study the interactions between human PMNs from healthy human donors and *L. infantum* promastigotes.

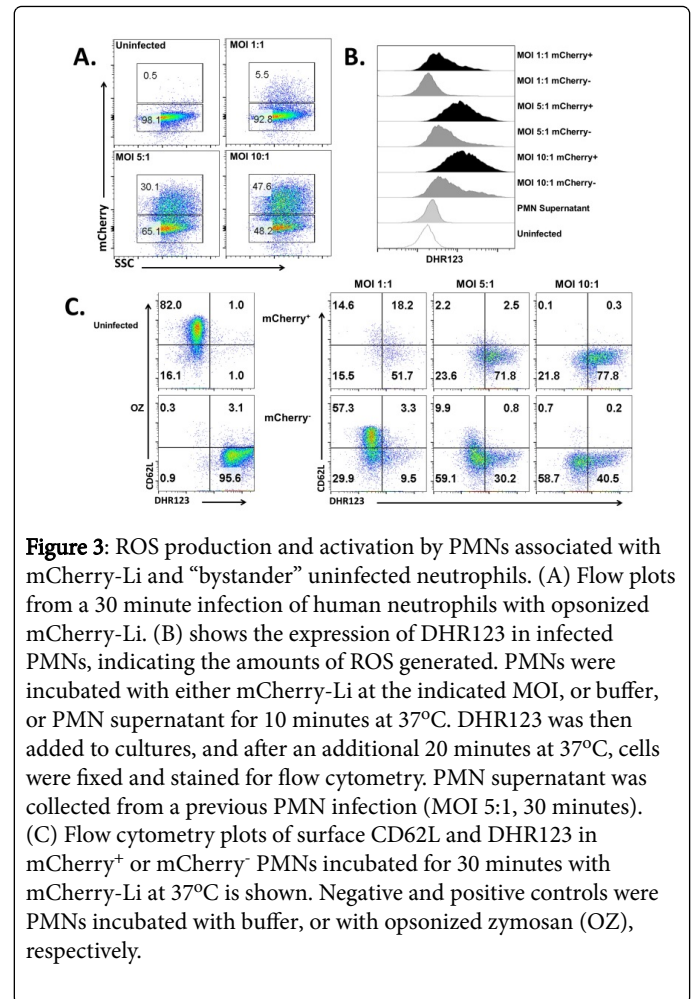


Figure 3: ROS production and activation by PMNs associated with mCherry-Li and “bystander” uninfected neutrophils. (A) Flow plots from a 30 minute infection of human neutrophils with opsonized mCherry-Li. (B) shows the expression of DHR123 in infected PMNs, indicating the amounts of ROS generated. PMNs were incubated with either mCherry-Li at the indicated MOI, or buffer, or PMN supernatant for 10 minutes at 37°C. DHR123 was then added to cultures, and after an additional 20 minutes at 37°C, cells were fixed and stained for flow cytometry. PMN supernatant was collected from a previous PMN infection (MOI 5:1, 30 minutes). (C) Flow cytometry plots of surface CD62L and DHR123 in mCherry⁺ or mCherry⁻ PMNs incubated for 30 minutes with mCherry-Li at 37°C is shown. Negative and positive controls were PMNs incubated with buffer, or with opsonized zymosan (OZ), respectively.

Our investigations confirmed that human PMNs take up serum-opsonized mCherry-Li promastigotes *in vitro*, as detected by both microscopy and flow cytometry. Parasite burden in neutrophils was increased by longer incubation times, peaking at 80 minutes, and also by incubation with greater ratios (MOIs) of parasites. Using the mCherry marker to distinguish between infected and uninfected neutrophils in the culture, we found that infected neutrophils showed greater levels of ROS and enhanced activation (evidence by loss of surface CD62L) compared to unexposed neutrophils. However, this response was heterogeneous, and this was particularly evident at low infection rates. Subsets of infected (mCherry⁺) neutrophils avoided acquisition of both markers of activation (ROS production and loss of CD62L). This finding contrasts with early reports homogeneous activation of PMNs in response to parasites *in vitro* [14].

Previous reports were not able to differentiate whether ROS production corresponded to cells harboring intracellular parasites, and thus allows us to distinguish the responses of individual infected or bystander cells. Our observation that neutrophil populations exhibit heterogeneous responses to parasites in culture is consistent with observations *in vitro* and *in vivo* that PMNs take up, and though they may kill some parasites early in infection [13], they are unable to control *Leishmania* infection [5,22,23,25]. Our data raise the curious possibility of “silent” parasite uptake by a neutrophil subset. It is possible that there could be a threshold of infection level required to stimulate ROS production by PMNs [14,34,36], and/or that parasites

may prevent ROS generation by some PMNs, similar to their inhibitory effects on some macrophage functions [37]. It has long been established that *Leishmania* parasites can bind and utilize surface receptors on macrophages (including CR3, CR1 and mannose receptor) as a means of “safe entry” [38-40]. Of these receptors, only CR3 (a.k.a. CD11b/CD18) is highly expressed on PMNs [38]. CR3 on macrophages does not trigger activation, and it is possible that neutrophil CR3 is also involved in suppression of neutrophil responses in a subset of cells.

Despite observations of murine neutrophils to the contrary [21,23], our data also showed activation of “bystander” human PMNs in cultures containing *L. infantum* infected neutrophils. Bystander activation was not observed when fresh PMNs were incubated in conditioned medium from either *L. infantum* or *L. infantum* infected neutrophils. This leads us to speculate that transient physical interactions with parasites, or with parasite-infected or activated neutrophils, may trigger activation of uninfected bystander neutrophils. Finally, in both infected and uninfected PMNs the loss of CD62L and production of ROS do not always occur in concert, suggesting differences between the triggers of both these activation associated events.

The contribution of neutrophils to the control or exacerbation of chronic *leishmaniasis* remains controversial, in part due to different systems of study and lack of specificity of depletion methods [13,41,42]. One thing that is clear is that neutrophils are abundant and interact extensively with the parasite during the first hours of infection [5,6,19]. Many other diseases such as those causing lung inflammation [43] or autoimmune disorders [44] document a role for highly activated neutrophils contributing to off-target cellular pathology. Our findings suggest that the concentration of parasites during the infection of infiltrating PMNs influences not only infected, but also bystander PMNs. We hypothesize that the infected PMNs responding to *L. infantum* phagocytosis with low or absent ROS production and/or activation could promote the maintenance of a parasite subset that establishes infection. Second, responses of some infected and of bystander PMNs may contribute to a local inflammatory environment that is ineffective at parasite clearance. There may be a larger role for PMNs in establishing infection and modulating the local immune environment at the inoculation site than has previously been appreciated.

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