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Neutrophil interactions with *Leishmania* amastigotes: implications for chronic leishmaniasis

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Neutrophil interactions with *Leishmania* amastigotes: implications for chronic leishmaniasis

by

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Dedication

To my wonderful wife, Rachel, for her constant love and support. This would have been a much tougher 4 years without you. Thank you for everything!

To my mother and father, for teaching me perseverance.

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Neutrophil interactions with *Leishmania* amastigotes: implications for chronic leishmaniasis

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Leishmaniasis is a neglected tropical disease responsible for considerable morbidity and mortality in the developing world. The immune response against *Leishmania* parasites must be fine-tuned to promote pathogen clearance without triggering excess inflammation, and this requires the concerted efforts of numerous innate and adaptive immune cell types. Neutrophils are a prevalent component of the inflammatory infiltrate during the acute phase (caused by promastigotes) and chronic phase (caused by amastigotes) of leishmaniasis, but the role of these cells in the anti-parasite immune response and disease pathogenesis is incompletely characterized at this time. The work presented in this dissertation is intended to bridge a gap in our understanding of interactions between *Leishmania* parasites (especially the amastigote stage) and neutrophils. First, we found that neutrophils readily internalize *Leishmania amazonensis* promastigotes and amastigotes and respond to infection through CD11b upregulation and oxidative burst. However, cytokine release and the ability of neutrophils to clear internalized parasites differed depending on the infecting parasite stage. Specifically, neutrophils efficiently killed promastigotes and responded to infection by releasing pro-inflammatory TNF-α. In contrast, neutrophils were unable to effectively kill *L. amazonensis* amastigotes and responded to infection by releasing anti-inflammatory IL-10. To determine whether amastigote resistance to neutrophil microbicidal mechanisms is conserved among *Leishmania* species, we compared neutrophil responses to amastigotes of *L. amazonensis* and *L. braziliensis*. We found that *L. braziliensis* is a significantly more potent trigger for neutrophil activation, oxidative burst, degranulation, and cytokine release when compared to *L. amazonensis*. Heightened neutrophil activation in response to *L. braziliensis* infection strongly corresponded to an enhanced ability of neutrophils to kill this parasite species. Finally, to better understand the role of neutrophils in a model of chronic cutaneous leishmaniasis, we treated *L. amazonensis*-infected mice with anti-neutrophil antibodies between weeks 4 and 10 post-infection. We observed that anti-neutrophil treatment exacerbated lesion progression in infected mice. Collectively, these findings suggest that neutrophils may play a previously-underappreciated role in the chronic phase of cutaneous leishmaniasis.

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Pm	Promastigote
DC	Dendritic cell
Am	Amastigote
DALY	Disability-adjusted life year
CL	Cutaneous leishmaniasis
ML	Mucocutaneous leishmaniasis
DCL	Diffuse cutaneous leishmaniasis
VL	Visceral leishmaniasis
APC	Antigen presenting cell
MHC II	Major histocompatibility complex type II
IL-12	Interleukin-12
Th1	T helper 1
IFN-γ	Interferon-γ
TNF-α	Tumor necrosis factor-α
iNOS	Inducible nitric oxide synthase
NO	Nitric oxide
IL-10	Interleukin-10
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
CFSE	Carboxyfluorescein succinimidyl ester
MOI	Multiplicity of infection
LPS	Lipopolysaccharide
GM-CSF	Granulocyte-macrophage colony-stimulating factor
EM	Electron microscopy
DHR 123	Dihydrorhodamine 123
Rho 123	Rhodamine 123
fMLP	Formyl-Methionyl-Leucyl-Phenylalanine
ROS	Reactive oxygen species
MFI	Mean fluorescence intensity
OD	Optical density
PI	Propidium iodide
PS	Phosphatidylserine
NET	Neutrophil extracellular trap
IgG	Immunoglobulin G
Mac-1	Macrophage-1 antigen
PICD	Phagocytosis-induced cell death
CCL3	Chemokine (C-C motif) ligand 3
IL-17	Interleukin 17
IL-18	Interleukin 18
IL-22	Interleukin 22
IL-4	Interleukin 4
PBMC	Peripheral blood mononuclear cell
TLR	Toll-like receptor

Chapter 1: Introduction

LEISHMANIA BIOLOGY AND CO-EXISTENCE WITH HUMANITY

Leishmania spp. are a group of obligate intracellular protozoa belonging to the Trypanosomitida order. They are widely distributed in the tropics and subtropics and are the causative agents of a cluster of clinical diseases known as leishmaniases. Parasites alternate between two life cycle stages that are highly adapted to the distinct environments of the parasite's life cycle. Flagellated promastigotes (**Pm**) reside in the midgut of infected female sandflies and gain access to the skin of vertebrate hosts when sandflies take a bloodmeal. In the skin, promastigotes are rapidly internalized by residential and recruited cell populations, including macrophages, dendritic cells (**DCs**), and neutrophils. Within macrophages, promastigotes convert into amastigotes (**Am**), which actively replicate and cause disease in mammalian hosts. Amastigotes undergo several rounds of replication in macrophages before escaping into the extracellular space and seeking new host phagocytes. The parasite's life cycle is completed when naïve sandflies consume parasitized blood containing amastigotes. Subsequently, sandfly-ingested amastigotes convert back into promastigotes (Fig. 1.1).

Archeological evidence indicates that leishmaniasis has plagued humanity for millennia. The presence of *L. donovani* DNA in bone fragments from Upper Nubia (in present-day Sudan) and Egypt suggests parasite-human coexistence in the Old World for at least 4,000 years [1]. Human leishmaniasis in the New World also appears to have ancient origins, as samples of pre-Incan Moche pottery (dating between 100 and 800 AD)

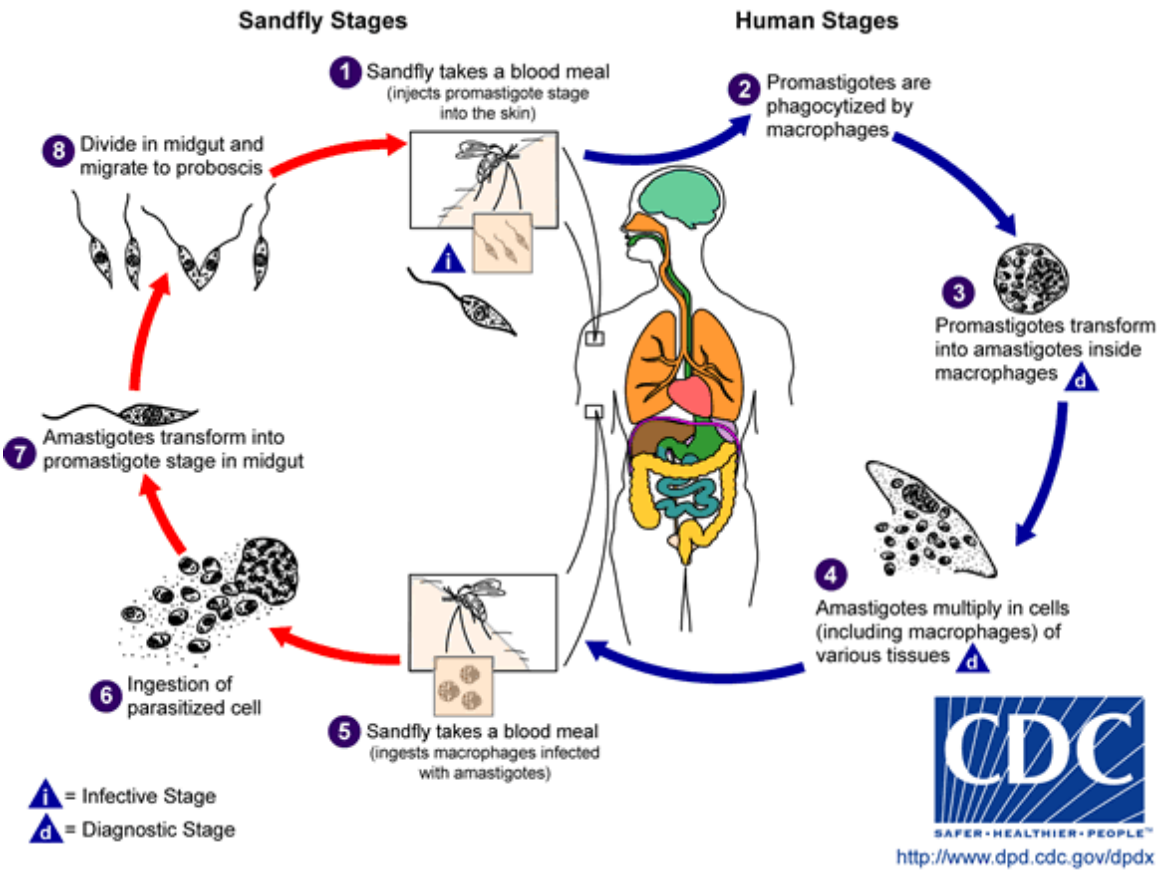

Figure 1.1: Life cycle of *Leishmania* parasites

(1) Promastigotes are deposited into the skin when infected female sandflies take a bloodmeal. **(2)** Promastigotes are rapidly internalized by host phagocyte populations, including macrophages. **(3)** Inside macrophages, promastigotes convert into amastigotes. **(4)** Amastigotes replicate and ultimately escape from macrophages into the extracellular space to infect new phagocytes. **(5)** Naïve sandflies ingest blood containing amastigote-infected cells. **(6)** Amastigotes are liberated into the sandfly gut as parasitized cells are degraded. **(7)** In the sandfly midgut, amastigotes convert back into flagellated promastigotes. **(8)** Promastigotes divide and mature in the midgut before being delivered to a new host.

NB: In areas where human visceral leishmaniasis is common, infected individuals can serve as reservoir hosts, as depicted in this image. In areas where visceral leishmaniasis is not prevalent, reservoir hosts are typically dogs, foxes, or rodents [2]. Image source: Leishmaniasis page, DPDx, Centers for Disease Control and Prevention [3].

depict figures with nasopharyngeal tissue damage indicative of mucocutaneous leishmaniasis [4, 5].

The earliest publication to link clinical manifestations of leishmaniasis to a causative agent of infection was written by Sir William Boog Leishman in 1903, although it is believed that a number of other physicians had observed parasites in tissue samples from infected patients prior to the publication of Leishman's article [6]. While conducting an autopsy, Leishman identified small "oval bodies" in the spleen of a soldier that had recently died of a mysterious febrile illness endemic to the Indian subcontinent (locally known as kala-azar). Although originally unable to identify these bodies, Leishman went on to describe the parasite's morphological similarity to the recently-described pathogen responsible for nagana (now known as *Trypanosoma brucei*), and ventured to state that these two pathogens may be closely related [7]. Soon after, Charles Donovan independently reported similar oval bodies in the spleen and blood of indigent patients that had died of kala-azar, and Donovan later went to great lengths to convince the scientific community that these bodies (and not other agents such as *Plasmodium*) were responsible for the disease [8]. To honor their contributions to medicine, the parasite species responsible for this disease, *Leishmania donovani*, carries the namesake of these two men.

LEISHMANIASIS IN HUMANS: DISEASE BURDEN, IMPACT, AND CLINICAL MANIFESTATIONS

The most recent epidemiological studies estimate that there are approximately 12 million people with active leishmaniasis and 51,000 disease-related deaths per year [9].

However, it is important to consider that these approximations may be a gross underestimate of the true burden of infection for several reasons. Firstly, infection is prevalent in many developing areas of the world that lack adequate measures to estimate disease burden. Critically, the governments of many endemic countries do not keep accurate records of disease prevalence because leishmaniasis has not been deemed a reportable infection [10]. Finally, many infections fail to result in symptomatic cutaneous disease, and in some endemic areas, asymptomatic carriers may outnumber symptomatic patients by 50:1 [11]. The importance of asymptomatic individuals (clinically and immunologically) will be further explored later in this chapter.

Leishmaniasis is considered one of the thirteen core neglected tropical diseases by the World Health Organization. Diseases given this classification are considered major factors in perpetuating poverty in developing areas of the world. Although leishmaniasis is considerably less deadly than many other tropical diseases (such as malaria), the developmental impact of leishmaniasis is staggering. For this reason, the burden of leishmaniasis (and the burden of many other low-mortality neglected diseases) is typically expressed using the disability-adjusted life year (**DALY**) metric [12]. The DALY is a practical method for measuring how a disease limits the social contributions of afflicted persons by reducing lifespan and/or inducing disability. For example, if 20 people in a community acquire a febrile illness and are bedridden for a year, the impact of the illness was 20 DALYs. The most recent estimates indicate that leishmaniasis is responsible for 2.1 million DALYs [9]. Additionally, leishmaniasis can take a severe financial toll on afflicted families, as traveling to a health center and receiving prolonged treatment for a single case of leishmaniasis can cost a family its entire annual income [9].

In humans, symptomatic leishmaniasis is highly variable in its clinical presentation, and it is likely that both host and pathogen factors contribute to the development of distinct disease manifestations [13]. The vast majority of symptomatic patients present with localized cutaneous leishmaniasis (**CL**), which is characterized by singular or multiple well-demarcated ulcerations of the skin that correspond to sandfly bite sites (Fig. 1.2, left panel). Skin ulceration is commonly preceded by a period of localized swelling at the site of parasite infection, and this may be accompanied by lymphadenopathy [14]. However, due to the lack of overt symptoms, patients rarely present during the pre-ulcerative phase of disease. The time between parasite inoculation and the development of ulcerated lesions is highly variable, but typically takes weeks to months [10]. In many cases, localized CL self-resolves after patients acquire protective adaptive immunity, and lesion healing is frequently associated with appreciable deposition of scar tissue at the site of ulceration (Fig. 1.2, right panel). However, disease control may be hindered in immunocompromised patients and in those infected with particular parasite species (such as members of the *Leishmania mexicana* complex), necessitating the use of harsh pharmacotherapy to encourage healing of ulcerated skin areas [15]. It is important to emphasize that in the absence of drug treatment, protective immunity and lesion resolution are not synonymous with sterile cure (i.e. the healing of ulcers does not correlate with complete parasite elimination from the site of infection) [16]. Therefore, factors that alter host immune status (such as corticosteroids or HIV) can trigger disease reactivation in clinically-resolved patients [17, 18].

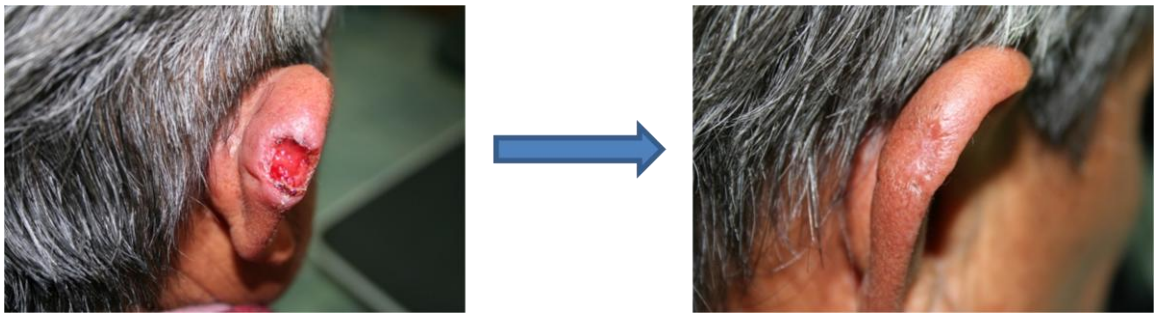

Figure 1.2: Typical progression of cutaneous leishmaniasis (CL) in humans

Left panel: Localized cutaneous leishmaniasis on a patient's ear. Right panel: Lesion resolution after several weeks of treatment. Frequently, CL is self-limiting, but non-healing ulcers typically respond well to sodium stibogluconate or paromomycin. Images are used with patient and caretaker permission from the La Unidad de Investigación en Leishmaniasis del Instituto de Medicina Tropical "Alexander von Humboldt" de la Universidad Peruana Cayetano Heredia, Lima, Perú.

Months to decades after infection, patients may develop one of several leishmaniasis sequelae, which can be severely disfiguring, highly refractory to treatment, and potentially fatal. It is worth mentioning that the risk of acquiring one of these secondary forms of disease is not limited to patients with active CL; asymptomatic individuals and patients with resolved lesions are also at risk [10, 19]. Three major forms of secondary leishmaniasis are widely acknowledged and will be discussed in this chapter.

Mucocutaneous leishmaniasis (**ML**; also known as espundia) is a potential complication for patients infected with members of the *Leishmania Vianna* subgenus (including *L. braziliensis, L. panamensis, L. peruviana,* and *L. guayensis*). ML is due to immune hypersensitivity to parasites, resulting in extensive inflammation-mediated nasopharyngeal tissue damage (Fig. 1.3). In most cases, it is suspected that parasites travel hematogenously from a noncontiguous cutaneous site to the nasopharynx. However, direct parasite dissemination from localized CL lesions on the lip, nose, or face have also been implicated in initiating ML [20]. As many as 10% of ML patients have no prior history of CL [10, 21]. Initial symptoms may include rhinorrhea and chronic nasal congestion, and much of the initial tissue damage may involve inconspicuous internal structures such as the nasal septum. This, coupled by the fact that many patients lack access to adequate medical care, frequently means that mucosal tissue damage can be quite extensive by the time patients seek medical attention. Late ML can be associated with laryngitis and obliteration of the nose, palate, and underlying bone [22]. In endemic areas with suitable medical facilities, CL patients may undergo routine examinations (e.g. ensuring the nasal septum remains unperforated) so that early ML is detected and treated

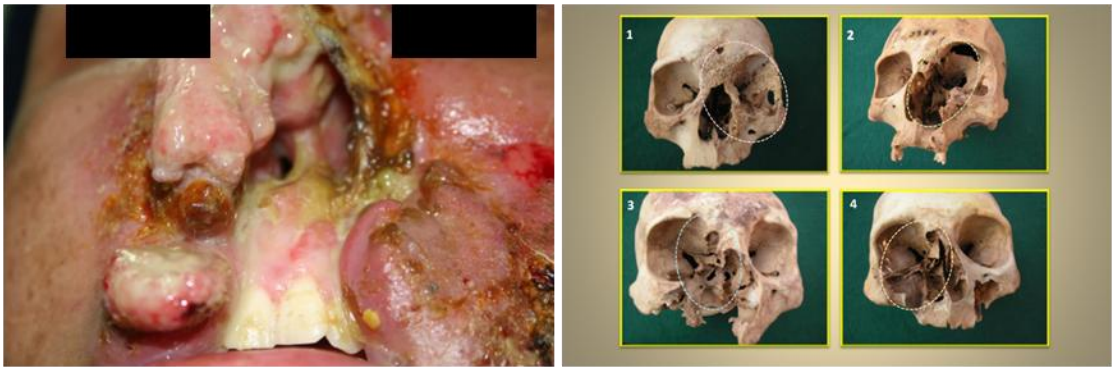

Figure 1.3: Features of severe mucocutaneous leishmaniasis (ML)

Left panel: Severe nasopharyngeal damage characteristic of untreated mucocutaneous leishmaniasis. Image is used with patient and caretaker permission from the La Unidad de Investigación en Leishmaniasis del Instituto de Medicina Tropical "Alexander von Humboldt" de la Universidad Peruana Cayetano Heredia, Lima, Perú. Right panel: Skulls found in an archeological site in Chile (dated 500-1000 years ago) displaying extensive bone resorption. PCR confirmed the presence of *Leishmania* DNA in damaged areas [22]. Image is used with publisher's permission (See Appendix A).

prior to the onset of extensive tissue damage. However, in its later stages, ML may be highly refractory to treatment [23].

Diffuse cutaneous leishmaniasis (**DCL**) is a rare form of leishmaniasis that manifest in a small subset of patients infected with members of the *Leishmania mexicana* complex (including *L. mexicana*, *L. amazonensis*, and *L. pifanoi*). DCL is characterized by parasite dissemination away from areas of primary infection, resulting in the development of additional nodular or plaque-like lesions in skin areas that were not initially infected (Fig. 1.4). In the latter stages of DCL, the majority of a patient's skin may be compromised with secondary lesions [24]. Patients with DCL commonly exhibit selective T cell anergy to parasites (i.e. parasite antigens to not induce a delayed-type hypersensitivity response), while their T cell responses to other antigens remain intact [25]. Notably, DCL is highly refractory to therapy [15], and although harsh pharmacotherapy may yield temporary resolution, lesion relapse is extremely common [26].

Visceral leishmaniasis (**VL**; also referred to as kala-azar) is the most severe form of disease, and occurs when *L. donovani* or *L. infantum* parasites invade the spleen, liver, and bone marrow. As a result, patients typically experience fever, cachexia, pancytopenia, and hepatosplenomegaly (Fig. 1.5). In the absence of pharmacotherapy, VL is 100% fatal due to secondary bacterial infection or coagulopathy [27, 28]. Patients with immunodeficiency (e.g. individuals with AIDS or those taking immunosuppressive drugs) are especially at risk [29], but VL is also observed in patients with apparently-normal immune systems. In some areas where VL is prevalent, parasite resistance to conventional drug therapy has made successful treatment more difficult [30].

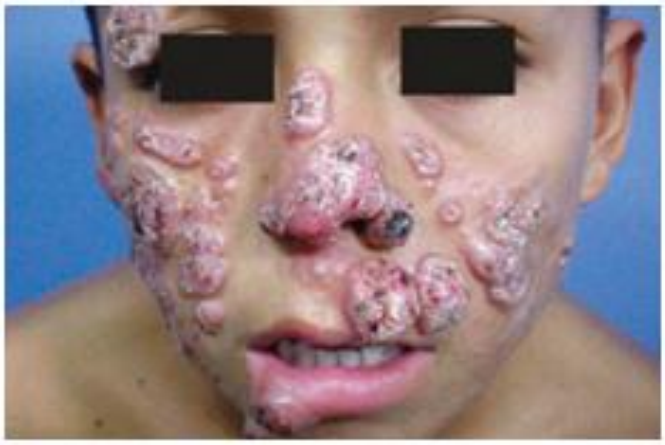

Figure 1.4: Clinical presentation of diffuse cutaneous leishmaniasis (DCL)

Patients previously exposed to parasites of the *Leishmania mexicana* complex may develop DCL, which is characterized by dissemination of parasites throughout the skin. In general, DCL is highly refractory to therapy and may require repeated treatments with toxic agents (such as amphotericin B) to facilitate lesion resolution [26]. Image is used with publisher's permission (See Appendix B).

Figure 1.5: Manifestations of visceral leishmaniasis (VL)

Features of VL include cachexia, pancytopenia, and organomegaly. Without treatment, VL is 100% fatal. Children and immunosuppressed individuals are especially at risk. Image source: Leishmaniasis page, World Health Organization [31].

FUNDAMENTALS OF ANTI-PARASITE IMMUNITY AND PARASITE IMMUNOEVASION: GENERAL FEATURES OF HOST-PATHOGEN INTERACTIONS

In the host, *Leishmania* parasites spend a majority of their time inside macrophages, and for this reason, anti-parasite immune strategies and parasite immunoevasion tactics prominently feature the modification of macrophage functions (Fig. 1.6). Importantly, successful anti-*Leishmania* immunity critically relies on antigen presenting cells (**APC**) such as dendritic cells, which respond to *Leishmania* infection by exposing parasite peptides on major histocompatibility complex class II (**MHC II**), increasing their surface expression of co-stimulatory molecules, and by releasing IL-12 [32]. IL-12 release, in combination with direct interactions between APCs and naïve $CD4^+$ T cells, drives type 1 helper T cell (**Th1**) differentiation. Protective Th1 cells, in turn, release IFN-γ (along with several other critical factors, such as TNF-α) upon interacting with infected macrophages [33], and binding of these cytokines to macrophage receptors drives expression of inducible nitric oxide synthase (**iNOS**). iNOS-generated nitric oxide (**NO**) then exerts direct toxic effects against internalized parasites [34]. In the context of some infections (such as infection with *L. major*), a potent Th1 response may be sufficient for limiting parasite growth and promoting lesion resolution [35]. However, immune correlates of protection for more immunosuppressive parasites (such as *L. amazonensis*) are less clear, as T effector cells are actually pathogenic in mice infected with this species [36].

As discussed above, parasite survival strategies also center (directly or indirectly) on modifying macrophage functions and resisting macrophage microbicidal activity. Principally, many *Leishmania* species possess advanced detoxification networks to limit

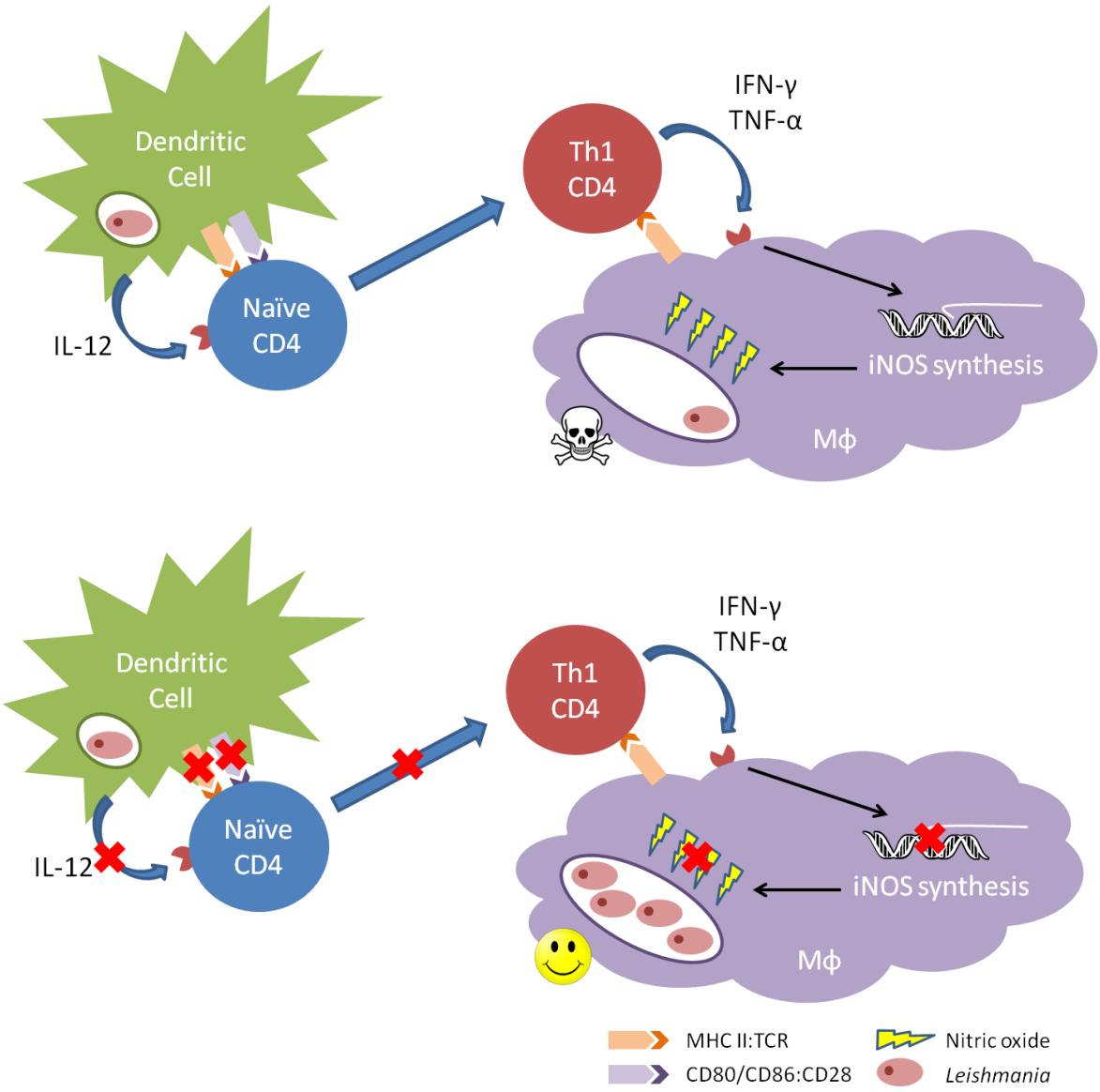

Figure 1.6: General features of anti-parasite immunity and parasite immunoevasion

Top group: Recognition of parasites by dendritic cells drives surface exposure of peptide-loaded MHC II molecules, expression of co-stimulatory molecules, and IL-12 release. Interactions between activated dendritic cells and naïve $CD4^+$ T cells facilitate Th1 differentiation. Communication between infected macrophages and Th1 cells promotes IFN-γ and TNF-α release, resulting in macrophage activation and iNOS expression. iNOS-derived NO is directly toxic to parasites. Bottom group: Immune processes shown to be disrupted by parasites are indicated with a red X. Parasite immunoevasive strategies promote parasite survival and persistence.

NB: This is a generalized diagram; different parasite species may disrupt the processes above with variable efficiency.

cellular damage done by NO [37]. Additionally, parasites can disrupt macrophage signaling to limit cell activation and iNOS expression, resulting in reduced NO production [38]. Finally, parasites can weaken or delay the development of a Th1 response by disrupting important APC functions, including inhibiting IL-12 production [39], reducing co-stimulatory molecule expression [40], and blocking peptide-loaded MHC II molecules from reaching the cell surface [41].

NEUTROPHILS IN LEISHMANIASIS: LITERATURE OVERVIEW AND GAPS

The high frequency of individuals with asymptomatic parasite infection, combined with the observation that these persons actually have a weaker parasite-specific adaptive immune response than patients with active disease [42], has prompted considerable inquiry into whether the innate immune response is sufficient for parasite control in subclinical cases. Since neutrophils rapidly and abundantly respond to the site of parasite infection [43-45], the role of these cells in the promastigote-mediated phase of leishmaniasis has been an intense area of research.

We and others have demonstrated that neutrophils possess some direct leishmanicidal activity *in vitro*, suggesting that these cells may represent an important component of the early immune response against incoming promastigotes [46, 47]. However, several studies report that a subset of promastigotes can survive for prolonged periods in neutrophils without sustaining lethal damage [48-50]. Subsequently, infected neutrophils may act as Trojan Horses or Trojan Rabbits [51], whereby parasite-laden cells (or free parasites that have escaped from neutrophils) can be readily internalized by

neighboring phagocytes in a manner that enhances parasite infectivity and persistence [43, 52, 53]. Therefore, it is likely that neutrophils play a dual protective and permissive role shortly after promastigote infection by reducing incoming parasite burden and subsequently facilitating safe passage of surviving parasites to naïve host cells.

Although neutrophils are frequently the first-responders during acute infection or tissue damage, it is important to consider that these cells are commonly encountered in chronic inflammatory and infectious foci as well [54-57]. Critically, neutrophils have been identified as a consistent component of the inflammatory infiltrate in the lesions of chronic cutaneous [58, 59], diffuse cutaneous [60], and mucocutaneous leishmaniasis [61]. However, it is unclear how these cells contribute to amastigote clearance and the maintenance of a low-grade inflammatory response. Presently, there is a scarcity of studies examining neutrophil-amastigote interactions, and anti-neutrophil studies assessing the impact of these cells during the chronic phase of leishmaniasis are largely absent from the literature.

OBJECTIVES OF THIS DISSERTATION

As mentioned above, there is a relative abundance of studies investigating the role of neutrophils during leishmaniasis. However, because neutrophils have been traditionally viewed as early responders that are gradually replaced by more specialized cell types as infection progresses, many of these studies focus on neutrophil activity during the acute (i.e. promastigote-mediated) phase of infection. Despite prevalent observations that neutrophils are present at the site of chronic infection and internalize amastigotes *in vivo* [58, 59, 62], there is a clear paucity of studies investigating the

immunological ramifications of neutrophil-amastigote interactions. Therefore, this dissertation is intended to help bridge the gap in our understanding of neutrophil responses to *Leishamnia* amastigotes. The rationale for conducting and reporting this work is that improving our knowledge of neutrophil function against amastigotes will greatly aid in dissecting the mechanisms responsible for chronic parasite persistence and disease pathogenesis. Additionally, learning more about neutrophil functions during leishmaniasis may shed light on the role of these cells in the context of other chronic infectious and inflammatory disorders.

Chapter 2: *Leishmania amazonensis* amastigotes trigger neutrophil activation, but resist neutrophil microbicidal mechanisms[1]

INTRODUCTION

Leishmania parasites are obligate intracellular protozoa that cause leishmaniasis, a neglected tropical disease responsible for extensive morbidity and mortality in the developing world. Infection is initiated when metacyclic promastigotes are deposited into the skin by the bite of a female sandfly, and parasitism of host neutrophils, dendritic cells (DCs), and macrophages rapidly ensues. In macrophages, promastigotes convert into amastigotes, the parasite stage that replicates in mammalian hosts. *Leishmania* amastigotes are able to modify macrophage functions and resist macrophage microbicidal activity, resulting in the establishment of an environment that is permissive for parasite growth [63-65]. Parasite-mediated manipulation of multiple signaling pathways in other cell types, such as DCs, is also well established, and disruption of innate immune cell function ultimately hinders the formation of a potent, effective T helper cell response. Consequently, amastigote replication continues unabated in the context of low-grade inflammation and tissue damage [66, 67].

Neutrophils rapidly recruit to the site of infection after metacyclic promastigotes are delivered into the skin, either via their natural sandfly vector or by needle injection [43, 53]. After contacting each other, *Leishmania* promastigotes and neutrophils may each undergo one of several fates. For example, *L. major* promastigotes can survive inside neutrophils and ultimately use these cells as Trojan Horses to facilitate silent

[1] Published in *Infection and Immunity*, 81(11):3966-74, 2013, with minor modifications.

infection of macrophages [52]. In contrast, neutrophils respond to *L. amazonensis* promastigotes by undergoing several forms of cell death; many cells encountering parasites rapidly die by NETosis, a specialized form of death that results in parasite entrapment and degradation [47], while the remaining neutrophils largely die by apoptosis [68].

In numerous mouse models, antibody-mediated neutrophil depletion has been extensively used to determine how these cells contribute to the pathogenesis of various infectious diseases [69-71]. However, there is currently a lack of consensus regarding the function of neutrophils during *Leishmania* promastigote infection, as these cells have been implicated in both promoting and inhibiting disease progression in different studies [72, 73]. Despite reporting contradictory roles for neutrophils in controlling infection, depletion studies nevertheless emphasize the importance of these cells in the early disease process of cutaneous leishmaniasis.

According to several clinical reports, neutrophil recruitment to the site of infection is not limited to the promastigote-mediated phase of disease, but continues throughout the course of chronic leishmaniasis as well. In *L. tropica*-infected patients, neutrophils were recovered from lesions ranging from 1-36 months in duration [58]. Neutrophils were also observed in the ulcerated lesions of patients chronically infected with *L. major*, and in some patients, these cells were the predominant immune cell type at the site of infection [59]. Interestingly, BALB/c mice infected with *L. major* display a progressive increase in the number of intralesional neutrophils throughout the first 6 weeks of infection [74], suggesting that persistent neutrophil recruitment may be a characteristic feature of chronic cutaneous leishmaniasis.

Amastigote-laden neutrophils have been isolated from numerous infected hosts, including experimentally-infected macaques and naturally-infected humans, dogs, and foxes [59, 62, 75, 76]. However, the immunological ramifications of amastigote-neutrophil interactions remain largely uncharacterized. We have recently demonstrated that *L. amazonensis* amastigotes are highly resistant to the antimicrobial effects of purified human histone proteins [77], which are known to be released together with other microbicidal agents when neutrophils undergo NETosis [78]. Currently, it is unclear whether neutrophils recognize amastigotes and influence amastigote clearance or persistence [79].

In this study, we aimed to examine the interaction between *L. amazonensis* amastigotes and peritoneal neutrophils obtained from C57BL/6 mice. We demonstrate that neutrophils efficiently internalized both the amastigote and promastigote forms of the parasite, particularly when parasites are opsonized with *Leishmania*-specific antibodies. Parasite uptake resulted in neutrophil activation and oxidative burst, but neutrophils differed in their response to amastigotes and promastigotes in several ways, including cytokine secretion and pathogen clearance. Specifically, neutrophils responded to promastigotes by releasing TNF-α and by killing the majority of parasites. In contrast, neutrophils failed to efficiently kill amastigotes and preferentially released IL-10 in response to this stage of parasite. Therefore, the role of neutrophils during leishmaniasis may differ depending on the stage of parasite encountered. These findings have important implications for understanding the pathogenic mechanisms of immune system dysfunction and chronic parasite persistence during experimental cutaneous leishmaniasis.

MATERIALS AND METHODS

Mice: Female C57BL/6 and BALB/c mice were purchased from Taconic Farms (Germantown, NY). C57BL/6 mice were the source of the majority of neutrophils in this study, while BALB/c mice were predominately used for the maintenance of parasite infectivity and for isolating lesion-derived amastigotes. Neutrophils from BALB/c mice were used as a control for the clearance of lesion-derived amastigotes (data not shown). B6(Cg)-Ncf1m1J/J mice deficient in the gp47 subunit of NADPH oxidase were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred on campus. Mice were maintained under specific pathogen-free conditions and used at 6 to 12 weeks of age, according to protocols approved by the Animal Care and Use Committee of the University of Texas Medical Branch (Galveston, TX).

Parasite cultivation: The infectivity of *L. amazonensis* (RAT/BA/74/LV78 and MHOM/BR/77/LTB0016 strains) was maintained by regular passage through BALB/c mice. Strain RAT/BA/74/LV78 was used for all experiments containing promastigotes and amastigotes. Strain MHOM/BR/77/LTB0016 was used in the infection of mice to generate immune serum. Promastigotes were cultured at 26°C in M199 containing 40 mM HEPES, 10% heat-inactivated FBS, 0.1% hemin in 50/50 H_2O and triethanolamine (Frontier Scientific, Logan, UT), 0.1 mM adenine, pH 7.5, 5 mM L-glutamine, and 50 µg/mL gentamicin. Metacyclic promastigotes were purified as described previously [80] by using the monoclonal antibody 3A1, which was generously provided by Dr. Norma Andrews (University of Maryland). All experiments containing promastigote groups utilized metacyclic promastigotes purified in this way. Axenic amastigotes were cultured at 32°C in Grace's insect cell culture medium (Invitrogen, Carlsbad, CA), pH 5.2,

supplemented with 20% FBS and 25 μg/mL gentamicin. Lesion-derived amastigotes were collected from the footpads of infected BALB/c mice through mechanical tissue disruption, followed by 3 washes and incubation in amastigote medium. Lesion-derived amastigotes were used within 48 h of isolation from infected footpads. Prior to use, lesion-derived amastigotes were washed an additional 3 times to remove any residual tissue components. Fresh parasite lysates were prepared through 2 freeze-thaw cycles followed by sonication for 15 min.

Production of luciferase-expressing parasites: Circular pSP72-YNEO-αIR-LUC1.2 was generously provided by Dr. Barbara Papadopoulou (Laval University, Quebec, Canada). Logarithmic phase RAT/BA/74/LV78 promastigotes were transfected with 35 μg plasmid, as reported previously [81], resulting in episomal expression of firefly luciferase. Following a 24 h rest period, selection for luciferase-expressing promastigotes was performed via titration of G418 (Invitrogen). Luciferase-expressing amastigotes were derived from logarithmic promastigote cultures. To maintain selective pressure, luciferase-expressing promastigotes and amastigotes were grown in normal parasite medium containing G418 (50 μg/mL).

Generation of immune serum and parasite opsonization: C57BL/6 mice were infected with *L. amazonensis* MHOM/BR/77/LTB0016 promastigotes in the rear footpads for 12 weeks. Infected mice and age and sex-matched naïve mice were subsequently sacrificed, and serum was collected, heat-inactivated, and stored at -20°C. To ensure suitable anti-*Leishmania* antibody concentrations, antibody titers were determined via direct enzyme-linked immunosorbent assay (**ELISA**). In experiments

utilizing opsonized amastigotes, parasites were incubated in naïve or immune serum (10%) for 20 min at room temperature prior to infection.

Neutrophil collection: Peritoneal exudate cells were obtained from mice 5 h after injection with 3% thioglycollate (Sigma-Aldrich, St. Louis, MO). Thioglycollate was removed and neutrophils were purified via density gradient centrifugation with Percoll (Sigma-Aldrich). Neutrophil purity (>95%) was validated by fluorescence-activated cell sorting (**FACS**) and examination of morphology after staining; cell viability was routinely >95%, as monitored by trypan blue exclusion. Prior to treatment or co-culture with parasites, neutrophils were plated in tissue culture-treated polystyrene. Because *L. amazonensis* poorly tolerates high temperatures, all neutrophil-parasite co-cultures were maintained at 32°C.

Neutrophil phagocytosis of parasites. Parasites were labeled with carboxyfluorescein succinimidyl ester (**CFSE**, Sigma-Aldrich), as described previously [82]. Neutrophils were co-cultured with CFSE-labeled amastigotes or promastigotes at a multiplicity of infection (**MOI**) of 5 for 4 h at 32°C and 5% CO2. Cells were collected, stained with APC-conjugated anti-Ly6G (BD Biosciences, San Jose, CA) and analyzed by FACS. Neutrophils were identified based on forward/side scatter characteristics and Ly6G positivity. Parasite-carrying neutrophils were identified based on CFSE positivity. In some experiments, neutrophils were treated with lipopolysaccharide (**LPS**), cytochalasin D (Sigma-Aldrich), or granulocyte-macrophage colony-stimulating factor (**GM-CSF**, PeproTech, Oak Park, CA), and parasites were opsonized in heat-inactivated naïve or immune serum prior to co-culture. Data were collected using an Accuri C6 flow cytometer (Accuri Cytometers Inc., Ann Arbor, MI). Flow cytometry data were

subsequently analyzed using CFlow version 1.0.227.4 (Accuri Cytometers Inc.) or FlowJo version 7.6.1 (Tree Star, Ashland, OR).

Electron microscopy (EM): Following 4 h of co-culture with amastigotes, neutrophils were fixed in Ito's fixative (2.5% formaldehyde prepared from paraformaldehyde, 0.1% glutaraldehyde, 0.03% CaCl2, and 0.03% trinitrophenol in 0.05 M cacodylate buffer, pH 7.3) at room temperature for 15 min and then overnight at 4°C. After washing in 0.1M cacodylate buffer, samples were post-fixed in 1% osmium tetroxide in the same buffer for 1 h and en bloc stained with 1% aqueous uranyl acetate for 20 min at 60°C. After dehydration in a graded series of ethanol, samples were embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on a Leica EM UC7 ultramicrotome (Leica Microsystems, Buffalo Grove, IL), stained with lead citrate, and examined using a Philips 201 transmission electron microscope (Philips Electron Optics, Eindhoven, The Netherlands) at 60 kV.

Measurement of neutrophil activation and oxidative burst: Neutrophil-parasite co-cultures were incubated for 4 h at 32°C and 5% CO2. After 4 h, some neutrophils were blocked with anti-CD16/CD32, and stained with PerCP-Cy5.5-conjugated anti-CD11b (eBioscience, San Diego, CA) and APC-conjugated anti-Ly6G, and samples were analyzed by FACS. Separate cell groups were stained with APC-conjugated anti-Ly6G and dihydrorhodamine 123 (**DHR 123**, 1 μM, Sigma-Aldrich), which converts to fluorescent rhodamine 123 (**Rho 123**) when oxidized. In some experiments, 1 μM N-formyl-methionyl-leucyl-phenylalanine (**fMLP**; Sigma-Aldrich) was added for the last 5 min of incubation prior to measurement of oxidative burst. The oxidation reaction was stopped on ice and neutrophil production of reactive oxygen species (**ROS**) was analyzed

by gating on $Ly6G^+$ cells and measuring the mean fluorescence intensity (**MFI**) of Rho 123 by FACS. To determine whether parasite-mediated oxidative burst was restricted to infected cells, amastigotes were labeled with PKH26 (Sigma-Aldrich), according to the manufacturer's instructions. MFI of Rho 123 was then compared in $PKH26^+$ (infected) and $PKH26^-$ (bystander) neutrophils.

Neutrophil cytokine detection: To minimize protease activity, neutrophils were treated with 50 μg/mL aprotinin (Sigma Aldrich) prior to treatment with parasites at a MOI of 5. Supernatants were collected after 24 h and cytokine concentration was measured via ELISA (eBioscience). After treatment with tetramethylbenzidine substrate and stop solution, optical density (**OD**) values at 450 nm were measured with a Multiskan Ascent ELISA Reader (Labsystems, Helsinki, Finland).

Measurement of neutrophil apoptosis: Neutrophils were co-cultured with amastigotes in the presence or absence of GM-CSF (20 ng/mL). After 18 h, neutrophils were collected and stained with APC-conjugated anti-Ly6G and the Annexin V:FITC apoptosis detection kit I (BD Biosciences). Early apoptosis in $Ly6G^+$ neutrophils was quantified by FACS based on positive staining for Annexin V and negative staining for propidium iodide (**PI**). To determine whether changes in apoptosis were restricted to infected cells, CFSE-labeled amastigotes were co-cultured with neutrophils followed by PE-conjugated Annexin V staining.

Parasite killing by neutrophils: Luciferase-expressing amastigotes or promastigotes were co-cultured with neutrophils at a MOI of 0.1. In some experiments, to better simulate lesion-derived amastigotes, axenic parasites were pre-coated with heat inactivated serum from infected mice prior to co-culture with neutrophils. At 0, 6, and 18

h post-infection, co-cultures were lysed and frozen at -80°C prior to analysis. Parasite burdens were estimated by mixing lysates with Luciferase Assay Substrate (Promega Corporation, Madison, WI) and measuring photon emission on a Veritas Microplate Luminometer (Turner BioSystems Inc., Sunnyvale, CA). Parasite survival was estimated by comparing baseline photon emission at 0 h to signal intensity at subsequent time points.

Statistical analysis: Differences between two groups were determined by using two-tailed Student's *t* test. Graphs were prepared by using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). The difference between two groups was considered significant when the *p* value was ≤ 0.05.

RESULTS

Neutrophils internalize *L. amazonensis* promastigotes and amastigotes

To investigate the interaction between neutrophils and *Leishmania* amastigotes, we opted to use thioglycollate-elicited neutrophils from C57BL/6 mice and *L. amazonensis* amastigotes. We selected this particular system to dissect amastigote-neutrophil interactions for several reasons. Firstly, C57BL/6 mice are traditionally viewed as a resistant strain in regards to *Leishmania* infection [83] and C57BL/6 neutrophils have been shown to respond to *L. major* by secreting biologically-active IL-12p70 [84]. Thioglycollate-elicited neutrophils can be isolated with high yield and purity, which is advantageous for conducting a detailed analysis of neutrophil function [85]. Finally, we opted to examine neutrophil responses to *L. amazonensis* because these parasites are easily propagated as amastigotes *in vitro*, induce a non-healing disease

phenotype in both C57BL/6 and BALB/c mice, and have been shown to have potent immunosuppressive effects on numerous cell types [40, 86].

Neutrophil uptake of metacyclic promastigotes is a critical feature of the initial phase of *Leishmania* infection. As infection progresses, neutrophils may also encounter amastigotes liberated from ruptured macrophages. However, despite the popularity of murine models of cutaneous and visceral leishmaniasis, reports of amastigote uptake by mouse neutrophils are largely absent from the literature. We compared neutrophil phagocytosis of CFSE-labeled axenic amastigotes and metacyclic promastigotes. After 4 h of co-culture, we observed that approximately 8.6% of neutrophils engulfed amastigotes, while 7.9% of neutrophils internalized promastigotes. Phagocytosis of parasites was inhibited in neutrophils that were pretreated with cytochalasin D (20 μM), confirming that parasite uptake was mediated via an actin polymerization-dependent mechanism. Of note, parasite opsonization in heat-inactivated serum collected from *L. amazonensis*-infected mice markedly enhanced neutrophil phagocytosis of both promastigotes and amastigotes (Fig. 2.1A). Opsonization with naïve mouse serum also enhanced parasite uptake, but to a lesser extent when compared to serum from infected animals (data not shown). Phagocytosis of amastigotes was also significantly enhanced in neutrophils treated with LPS (100 ng/mL) or GM-CSF (200 ng/mL) (Fig. 2.1B). Electron microscopy was used to confirm amastigote internalization. Ultrastructural analysis indicated that internalized amastigotes were housed within tight, membrane-bound vacuoles (Fig. 2.2). The presence of intact flagellar remnants in some internalized amastigotes (Fig. 2.2 arrow) suggested that parasites were not damaged by neutrophils

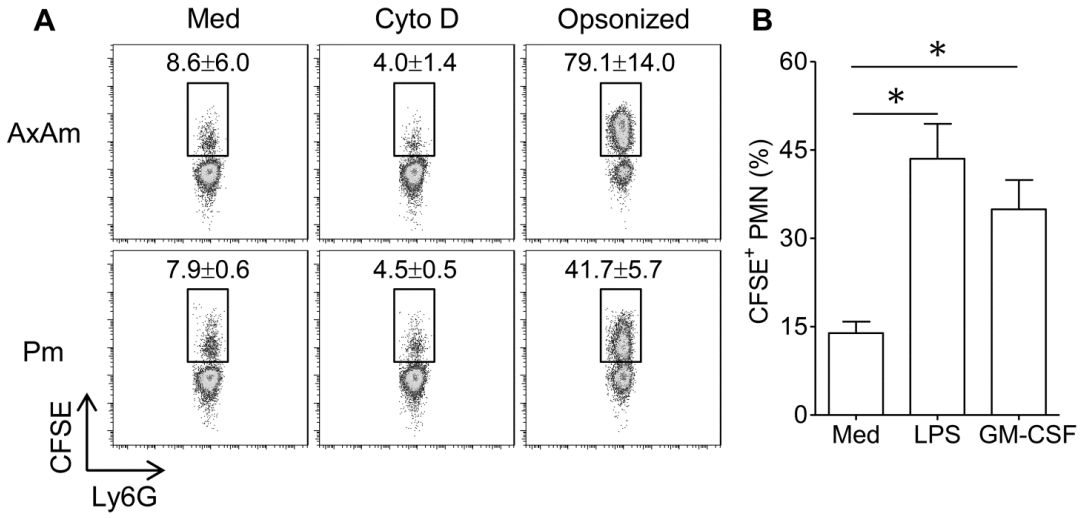

Figure 2.1: Neutrophil phagocytosis of *L. amazonensis* parasites

(A) Thioglycollate-elicited peritoneal neutrophils were co-cultured with CFSE-labeled axenic amastigotes (AxAm) or metacyclic promastigotes (Pm) for 4 h. In some groups, neutrophils were pretreated with cytochalasin D (Cyto D, 20 μM) or parasites were opsonized in serum from infected mice. Neutrophils were identified by forward/side scatter characteristics and by Ly6G positivity. CFSE positivity in the boxes shown represents $Ly6G^+$ neutrophils carrying parasites. Values are mean percentages of $CFSE^+$ cells ± 1 standard deviation (SD). (B) Percentages of $CFSE^+$ neutrophils (PMN) carrying amastigotes after 4 h in medium alone (Med) or in the presence of LPS (100 ng/mL) or GM-CSF (200 ng/mL). Data are pooled from 3 independent repeats and are shown as means ± standard errors. * ($p < 0.05$) indicates statistically significant differences between the groups.

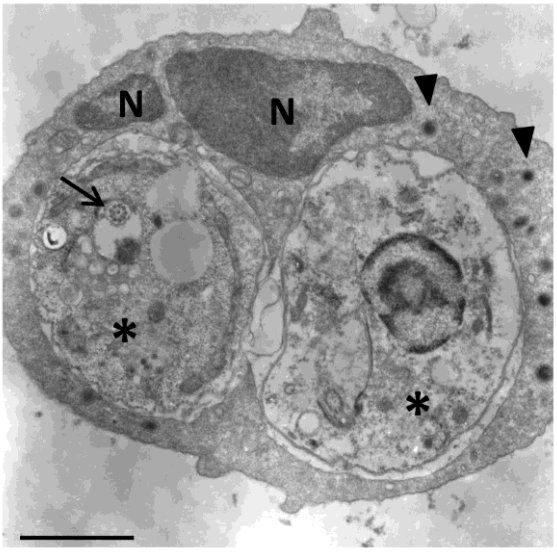

Figure 2.2: Ultrastructural analysis of amastigote uptake by neutrophils

Neutrophils were co-cultured with serum-coated amastigotes for 4 h, fixed, and prepared for analysis via electron microscopy. A characteristic neutrophil is depicted, exhibiting a multilobular nucleus (N), electron-dense granules (arrowheads), and 2 intracellular amastigotes (asterisks). A flagellar remnant is also clearly visible in the amastigote on the left (arrow). Bar = 2 μm.

during phagocytosis. In some instances, neutrophils carrying 4 or more parasites were also observed (image not shown).

Amastigote infection triggers neutrophil activation and oxidative burst.

Activated neutrophils characteristically upregulate CD11b on their surface, which reflects their ability to execute a number of important functions, including phagocytosis, degranulation, apoptosis, and oxidative burst [87-90]. As shown in Fig. 2.3A, neutrophil co-culture with axenic amastigotes or promastigotes resulted in an appreciable upregulation of surface CD11b on infected neutrophils. However, the extent of CD11b upregulation did not differ between neutrophils co-cultured with promastigotes or amastigotes. In contrast, neutrophil co-culture with lesion-derived amastigotes resulted in a significant increase in CD11b upregulation over that of axenic amastigotes (Fig. 2.3A)

Because ROS are a critical component of the microbicidal armament of neutrophils, and because promastigote-induced ROS production in neutrophils has been reported [91], we investigated whether amastigotes also trigger neutrophil oxidative burst. To do this, we labeled resting and parasite-laden neutrophils with DHR 123, a cell-permeable dye that converts into fluorescent rhodamine 123 (Rho 123) when oxidized [92]. As shown in Fig. 2.3B, neutrophil co-culture with amastigotes or promastigotes for 4 h resulted in a significant increase in Rho 123 fluorescence when compared to cells resting in medium. While promastigotes tended to elicit more ROS, the difference between amastigote and promastigote-mediated oxidative burst was not significant, regardless of whether the entire neutrophil population (Fig. 2.3B) or the Rho 123^{+}

population (Fig. 2.3C) was examined. In contrast, lesion-derived amastigotes did elicit significantly more neutrophil oxidative burst than their axenically-cultured counterparts (Fig. 2.3B). DHR 123-labeled parasites had no detectable dye oxidation, confirming that the ROS measured in our assay was neutrophil-derived (data not shown).

We examined whether oxidative burst was occurring in infected or bystander neutrophils by co-culturing cells with PKH26-labeled amastigotes. After 4 h of infection, neutrophils could be clearly gated based on PKH26 positivity, and we observed that amastigote-laden ($PKH26^{hi}$) cells were the major producers of ROS (Fig. 2.3D). Neutrophil oxidative burst in response to parasites was dependent upon the presence of intact parasites, as parasite lysates failed to increase ROS production above control levels (data not shown). These findings collectively indicate that parasite internalization is required for neutrophil oxidative burst in response to *L. amazonensis*.

To determine whether parasites were able to alter oxidative burst elicited by an external signal such as fMLP, we compared neutrophil production of ROS in response to amastigotes, fMLP, or both stimuli combined. Co-culture with parasites plus fMLP treatment resulted in substantially greater ROS production when compared to amastigote or fMLP treatment alone (data not shown). To ensure that the observed increase in ROS was due to phagocyte NADPH oxidase rather than mitochondrial damage or other ROS sources, we compared ROS production in neutrophils from wild type (WT) mice and mice deficient in the gp47 subunit of the NADPH oxidase complex. $gp47^{-/-}$ neutrophils produced appreciably less ROS in response to fMLP or amastigotes when compared to WT neutrophils, confirming that the majority of the ROS detected in our assay was derived from the NADPH oxidase (data not shown).

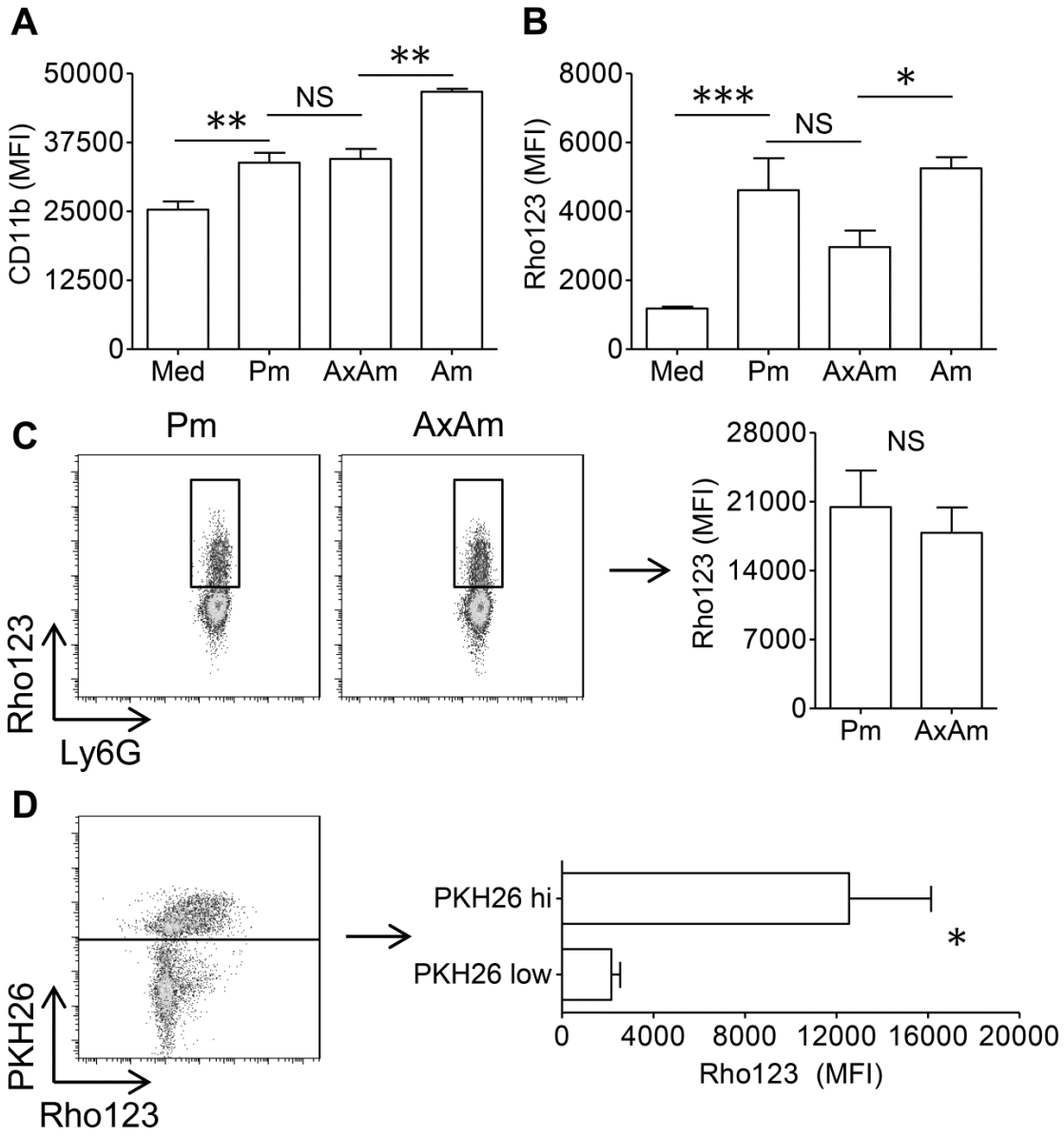

Figure 2.3: Neutrophil activation and oxidative burst after contact with parasites

(A) Mean fluorescence intensity (MFI) of CD11b on neutrophils resting in medium (Med) or co-cultured with metacyclic promastigotes (Pm), axenic amastigotes (AxAm), or lesion-derived amastigotes (Am). (B) MFI of rhodamine 123 (Rho 123) in dihydrorhodamine 123-labeled $Ly6G^{+}$ cells after 4 h of co-culture with parasites. (C) Oxidative burst in $Ly6G^{+}$ Rho 123^{+} neutrophils, indicating that the extent of burst on a per-cell basis does not differ for neutrophils co-cultured with promastigotes or amastigotes. (D) ROS production in neutrophils infected with PKH26-labeled axenic amastigotes for 4 h, showing that the majority of ROS was generated in $PKH26^{hi}$ (amastigote-laden) cells. All data are pooled from at least 2 independent experiments and shown as means ± standard errors. * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$) indicate statistically significant differences between groups. NS, not significant.

Promastigotes and amastigotes trigger differential cytokine release from neutrophils.

Although neutrophils release only small quantities of cytokines, neutrophil-derived mediators have been shown to play an important role in the pathogenesis of multiple conditions, including arthritis [93], cancer [94], and HIV infection [95]. We measured cytokine release by neutrophils after 24 h of co-culture with axenic amastigotes or metacyclic promastigotes. When compared to baseline cytokine secretion of resting neutrophils, we observed that promastigotes promoted TNF-α release, but failed to induce IL-10 secretion. In contrast, amastigotes induced considerably less TNF-α and preferentially induced the release of IL-10 (Fig. 2.4A and 2.4B). While a previous study reported some IL-12p40 and IL-12p70 release from *L. major*-infected murine neutrophils [84], the concentration of IL-12 in the supernatants of *L. amazonensis*-infected neutrophils was below the level of detection, regardless of whether axenic amastigotes or metacyclic promastigotes were utilized (data not shown).

Amastigote infection accelerates neutrophil apoptosis.

The lifespan of circulating neutrophils is typically short (8-20 h), but neutrophil survival can be altered by recruitment to a site of inflammation, contact with pro-survival signals, or phagocytosis of infectious cargo [96, 97]. Infection with promastigotes from several *Leishmania* species has been shown to alter neutrophil lifespan, but the outcome (prolonged lifespan vs. accelerated cell death) may be largely context-dependent. For example, *L. major* promastigotes have been shown to inhibit apoptosis of human peripheral blood neutrophils in vitro (30). In contrast, the majority of *L. major*-laden

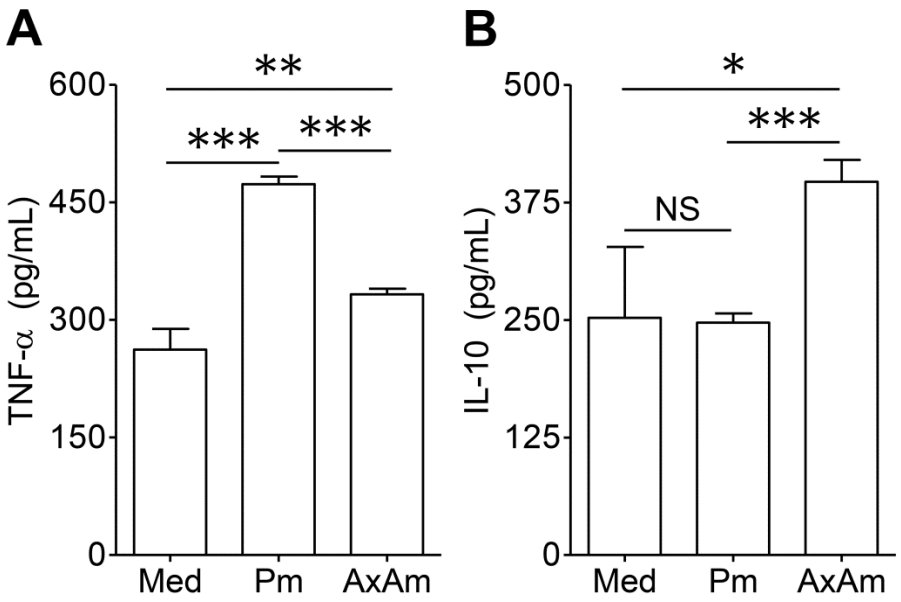

Figure 2.4: Neutrophil cytokine production in response to parasites

Neutrophils were co-cultured with axenic amastigotes (AxAm) or metacyclic promastigotes (Pm) for 24 h. Production of TNF-α (A) and IL-10 (B) was analyzed by ELISA. Results are pooled from 2 independent repeats and shown as means ± standard errors. * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$) indicate statistically significant differences between groups. NS, not significant.

neutrophils isolated from recently-infected mice were apoptotic [53]. To determine how amastigotes influence neutrophil longevity, we co-cultured neutrophils with axenic or lesion-derived amastigotes at a MOI of 5 for 18 h. Cells were then stained with FITC-conjugated Annexin V to measure phosphatidylserine (**PS**) surface exposure, an indicator of apoptosis. A significantly greater percentage of cells co-cultured with amastigotes exposed PS when compared to uninfected neutrophils, suggesting that amastigote infection accelerated neutrophil apoptosis. The percentage of apoptotic neutrophils did not differ between co-cultures containing axenic or lesion-derived amastigotes (Fig. 2.5A). Infecting neutrophils with amastigotes at a lower dose (a MOI of 2) resulted in similar increases in neutrophil apoptosis (data not shown). We also examined neutrophil death in response to promastigotes and observed that promastigote-infected neutrophils remaining after 18 h exhibited a similar acceleration in apoptosis (data not shown). However, we noted that the majority of promastigote-infected neutrophils were not recoverable after 18 h, suggesting that apoptosis was not the primary form of neutrophil death (data not shown). These findings support a previous report that *L. amazonensis* promastigotes potently trigger early neutrophil NETosis prior to the onset of apoptosis [47]. We also noted that GM-CSF, which prolongs neutrophil lifespan by activating the PI3K and extracellular signal-regulated kinase pathways [98], partially reversed axenic amastigote-mediated apoptosis (Fig. 2.5B).

To determine whether amastigote-mediated apoptosis was restricted to infected cells, amastigotes were labeled with CFSE prior to co-culture with neutrophils. As shown in Fig. 2.5C, resting cells in cultures lacking amastigotes and $CFSE^-$ cells from neutrophil-amastigote co-cultures had similar percentages of Annexin V^+ cells at 18 h

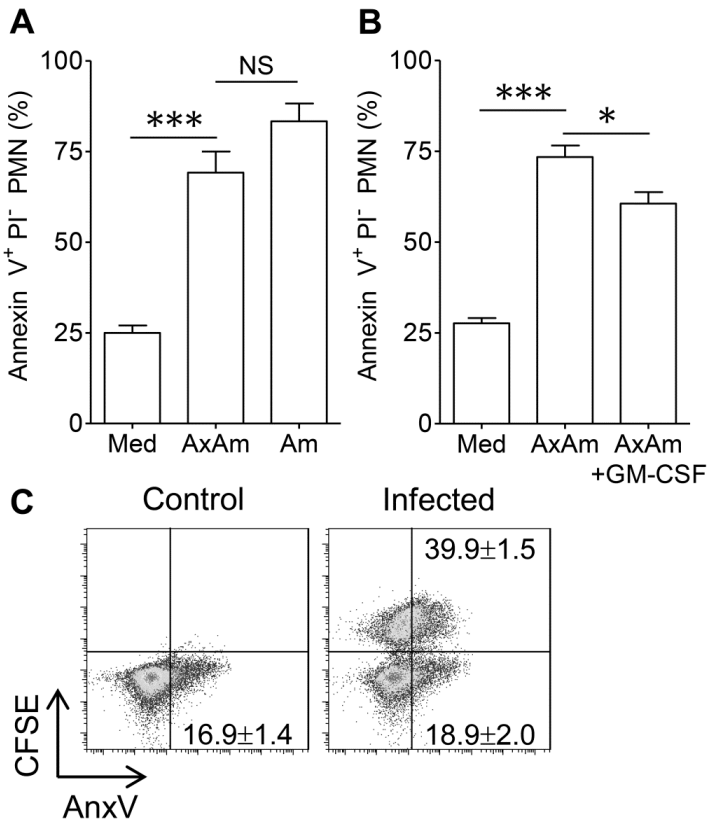

Figure 2.5: Accelerated neutrophil apoptosis after amastigote uptake

(A) Percentages of PS^+ PI^- neutrophils after 18 h of culture in medium (Med) or after co-culture with axenic amastigotes (AxAm) or lesion-derived amastigotes (Am). (B) Neutrophil apoptosis in medium alone or in response to axenic amastigotes in the presence or absence of GM-CSF (20 ng/mL). All data in panels A and B are pooled from at least 2 independent repeats and shown as means ± standard errors. * ($p < 0.05$) and *** ($p < 0.001$) indicate statistically significant differences between the groups. NS, not significant. (C) Comparison of apoptosis in resting neutrophils, $CFSE^+$ (parasite-carrying) neutrophils, and $CFSE^-$ (bystander) neutrophils. PS surface exposure in neutrophils was measured via binding of annexin V (AnxV). The percentages of apoptotic cells were calculated by dividing the number of PS^+ cells by the total number of cells for each group. Values are mean percentages of apoptotic cells ± 1 SD. Shown are representative results of one of three independent repeats.

(comparing 16.9% vs. 18.9%). In contrast, nearly 40% of $CFSE^+$ neutrophils carrying parasites were apoptotic. In all of our experiments, fewer than 3% of neutrophils stained positively for propidium iodide (**PI**, a marker of necrosis) at 18 h, implying that neutrophil necrosis was unaffected by parasite infection during our observation period (data not shown). Therefore, amastigote infection decreased the lifespan of parasite-carrying neutrophils, rather than that of bystander neutrophils, via accelerated neutrophil apoptosis.

Amastigotes are more resistant to neutrophil microbicidal mechanisms than promastigotes.

Neutrophil uptake and elimination of *L. amazonensis* promastigotes has been previously documented [47], but it is unclear whether neutrophils can destroy amastigotes in a similar manner. To address this issue, we co-cultured luciferase-expressing promastigotes or amastigotes with neutrophils and tracked the loss of luciferase activity in serial samples over time (Fig. 2.6A). To ensure efficient parasite internalization by neutrophils, we used a very low infection dose (MOI of 0.1). Consistent with previous reports [47], we found that neutrophils killed approximately 55% of metacyclic promastigotes within 6 h of co-culture, and that more than 65% of promastigotes were killed by 18 h. Importantly, the luciferase activity in co-cultures containing axenic amastigotes failed to decline over the 18 h period assayed (Fig. 2.6B). Axenic amastigote-dependent luciferase activity remained nearly constant even when parasites and neutrophils were co-cultured for as long as 40 h (data not shown), indicating a remarkable resistance of axenic amastigotes against neutrophil microbicidal defenses. To

validate and expand upon these findings, we also examined the survival of lesion-derived amastigotes following co-culture with neutrophils. While we noted some killing of lesion-derived amastigotes after 18 h, the extent of amastigote survival still surpassed that of metacyclic promastigotes (Fig. 2.6C). Clearance of lesion-derived amastigotes was comparable between neutrophils obtained from C57BL/6 and BALB/c mice (data not shown). We also noted that coating axenic amastigotes in immune serum prior to co-culture with neutrophils had a small, but appreciable effect on parasite clearance at 18 h, suggesting that host tissue components may aid in the partial elimination of amastigotes (Fig. 2.6C). Taken together, these results indicate that amastigotes, regardless of their source, demonstrated a clear survival advantage during interaction with neutrophils when compared to metacyclic promastigotes.

DISCUSSION

During the early stages of infection, neutrophils may aid in the elimination of many promastigotes, but surviving parasites may acquire a distinct advantage in the subsequent infection of macrophages [43, 52]. The complicated role of neutrophils during promastigote infection is exemplified by the presence of several contradicting studies that followed disease progression in neutrophil-depleted mice. In response to neutrophil depletion with the monoclonal antibody RB6-8C5, Chen et al. noted no difference in the progression of *L. major* infection in resistant C3H/HeJ mice, while depleted BALB/c mice were less able to control infection than non-depleted animals [72]. In contrast, Tacchini-Cottier et al. observed that depletion of neutrophils with the monoclonal

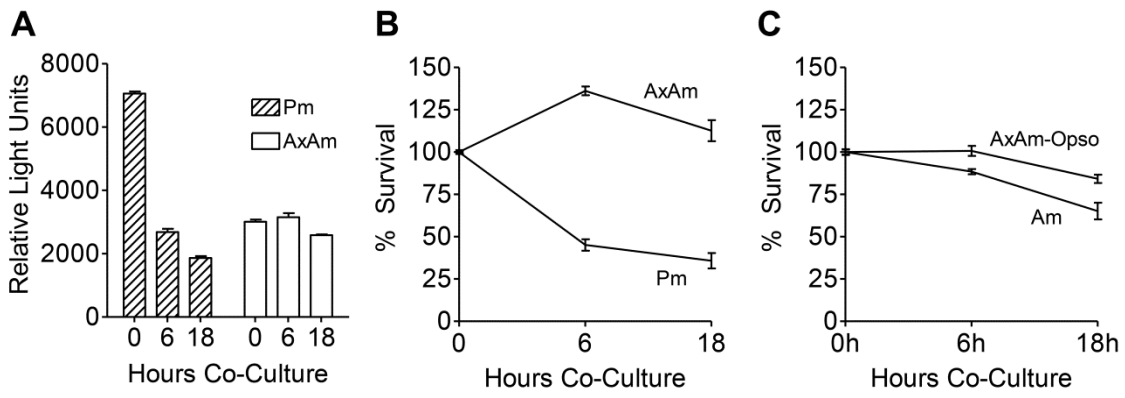

Figure 2.6: Neutrophil killing of promastigotes and amastigotes

Luciferase-expressing metacyclic promastigotes (Pm) and axenic amastigotes (AxAm) were co-cultured with neutrophils (at an MOI of 0.1) for 0, 6, or 18 h. Cells were lysed and treated with luciferin substrate to elicit photon emission. Photon emission of 0 h samples was used as a reference for 100% parasite survival, and the subsequent decay in signal at 6 h and 18 h was used to estimate the extent of parasite killing. (A) Representative graph showing photon intensity in relative light units. (B) Survival of axenic amastigotes and metacyclic promastigotes co-cultured with neutrophils for 6 h and 18 h. (C) Survival of lesion-derived amastigotes (Am) and axenic amastigotes opsonized in fresh serum from infected mice (AxAm-Opso) after 6 h and 18 h of co-culture with neutrophils. The data in panels B and C are pooled from at least 2 independent experiments and shown as means ± the errors.

antibody NIMP-R14 reduced the severity of *L. major* infection in BALB/c mice, while depletion in resistant C57BL/6 mice failed to alter the time required for lesion resolution [73]. The discrepancies between these two studies may be due in part to the use of antibodies with varying neutrophil specificity (RB6-8C5 also depletes eosinophils, inflammatory monocytes, and several other immune cell populations, while NIMP-R14 also depletes inflammatory monocytes) and different *L. major* strains [99].

Given that promastigotes predominantly encounter neutrophils prior to contacting monocytes or macrophages, it is somewhat surprising that this stage of parasite is relatively susceptible to neutrophil microbicidal mechanisms in our *in vitro* studies. It is possible that the neutrophil-promastigote interaction *in vivo* is complicated by additional vector, host, or parasite components that improve parasite resistance against killing by neutrophils [43, 100]. It is also possible that the purification of metacyclic promastigotes from parasite cultures may weaken the natural defenses of this parasite against neutrophils *in vitro*. Alternatively, some promastigote killing by neutrophils may favorably alter the inflammatory milieu at the site of infection, resulting in improved survival conditions for the remaining promastigotes. For example, neutrophil extracellular traps (**NETs**) have been shown to be a potent stimulus for type I interferon release from plasmacytoid DCs [101], and we have previously demonstrated that type I interferon signaling can promote parasite survival and disease pathology [68]. Regardless of the underlying mechanisms, it is evident that promastigotes are relatively susceptible to neutrophil microbicidal defense in our study.

Despite a handful of observations documenting contact between neutrophils and *Leishmania* amastigotes in several mammalian hosts, the outcome of this interaction is

unclear. Herein we report that amastigotes of *L. amazonensis* successfully survive within murine neutrophils despite triggering neutrophil activation and apoptosis. *L. amazonensis* is particularly adept at modifying the mammalian immune response to establish chronic persistence. This is best exemplified by the uncommon clinical manifestation of infection known as diffuse cutaneous leishmaniasis (DCL). DCL patients exhibit selective anergy against *Leishmania* antigens and cannot control parasite replication and dissemination throughout the skin, resulting in the appearance of multiple, disfiguring lesions that are often refractory to treatment [86]. *L. amazonensis* infection in mice is similarly characterized by progressively growing lesions, poor T helper cell responses, and unchecked parasite growth, even in mouse strains (e.g. C57BL/6 and C3H) that are genetically resistant to *Leishmania major* [102]. Unlike many other species, *L. amazonensis* amastigotes thrive under axenic conditions, permitting extensive opportunities to study this stage of parasite *in vitro*. These characteristics make *L. amazonensis* an excellent tool to study pathogenesis and immunoevasion in the context of chronic infection [86].

We have previously demonstrated a pathogenic role for antibodies and B cells during *L. amazonensis* infection [103]. It has also been reported that macrophage uptake of antibody-coated *L. mexicana* amastigotes can result in the robust secretion of IL-10, contributing to parasite immunoevasion [104]. Similarly, mice lacking IgG (due to a deletion of the Ig heavy chain) are more resistant *to L. major* infection, and passive transfer of parasite-specific antibodies to IgG-deficient animals resulted in increased IL-10 production, larger lesions, and increased parasite burden [105]. Herein, we demonstrate that coating of *L. amazonensis* amastigotes in serum from infected mice

greatly enhanced parasite uptake by neutrophils (Fig. 2.1A), but serum coating had only marginal effects on neutrophil-mediated killing of axenic amastigotes *in vitro* (Fig. 2.6C). These results provide further evidence to support the notion that parasite-specific antibodies are not a major protective component of the immune response during *Leishmania* amastigote infection.

Phagocytosis of certain pathogens and subsequent respiratory burst can result in a Mac-1-dependent acceleration in neutrophil apoptosis through a process known as phagocytosis-induced cell death (**PICD**) [106]. In contrast, many pathogens such as *Francisella tularensis*, *Mycobacterium tuberculosis*, and *Chlamydia pneumoniae* can prolong neutrophil lifespan as a part of their immunoevasion strategy [107-109]. In this study, we observed that *L. amazonensis* amastigotes do not utilize an anti-apoptotic strategy when infecting neutrophils (Fig. 2.5A). The mechanism that *L. amazonensis* parasites employ to accelerate neutrophil apoptosis remains undetermined. We are currently investigating whether parasites trigger neutrophil apoptosis through a PICD-like mechanism by utilizing anti-CD11b and anti-CD18 antibodies and mice deficient in phagocyte respiratory burst (such as $gp47^{-/-}$ mice).

The ability of neutrophils to modify the functions of other immune cell types is an essential area for future investigation, particularly in the context of *Leishmania* infection. Importantly, neutrophils can display antigen-presenting functions and prime T cells [110, 111]. *L. amazonensis*-infected DCs are particularly poor at priming and activating T cells and fail to trigger a strong adaptive immune response [67]. However, parasite infection does ultimately result in the generation of antigen-specific T cells and B cells, possibly indicating that antigen presentation may proceed through alternative or atypical

mechanisms. The ability of neutrophils to present parasite antigens and prime T cells directly is largely unexplored at this time.

There is ample evidence that neutrophils can modulate DC function during leishmaniasis. For example, early DC recruitment during *L. major* infection was dependent upon CCL3 secretion by neutrophils, and CCL3 blockade delayed the development of a protective adaptive response [112]. Additionally, *L. major*-loaded neutrophils isolated from infected mice were efficiently internalized by dermal DCs, and parasites delivered through this mechanism were less efficient in activating DCs and priming T cells than free parasites [53].

Neutrophils can also aid or hinder macrophages in clearing *Leishmania* infection. Murine macrophages infected with *L. amazonensis* displayed enhanced microbicidal activity when co-cultured with neutrophils [113]. In contrast, delivery of *L. major* to macrophages via apoptotic human neutrophils can result in anti-inflammatory cytokine production, favoring parasite growth [52]. These findings suggest that neutrophils can positively or negatively affect the function of other immune cell types, and that the outcome of this interaction may vary greatly depending upon neutrophil activation and life status.

It is interesting to note some discrepancies between our findings and those from several previous reports. Specifically, the newly-described method for culturing axenic *L. major* amastigotes yields parasites that are not readily internalized by human neutrophils [114]. Additionally, it has been shown that *L. donovani* amastigotes derived from infected hamsters are degraded by human neutrophils [79]. These disparities suggest that differences in host and parasite species, as well the methods used to isolate

parasites, may contribute to dramatic differences in the interaction between *Leishmania* amastigotes and host neutrophils.

It was surprising to observe that lesion-derived amastigotes triggered more neutrophil activation and were more susceptible to neutrophil-mediated killing than their axenically-cultured counterparts (Fig. 2.6C). We suspect that these differences are largely due to host components that remain associated with lesion-derived amastigotes (such as anti-*Leishmania* antibodies and complement components). However, it is currently unclear which host components are responsible for the improvement in lesion-derived amastigote clearance that we observed. Additionally, the mechanical process of isolating amastigotes from the footpads of infected mice is relatively vigorous, and lesion-derived parasites may require additional time to fully recover and prime their anti-neutrophil defenses.

This study, for the first time, examines in detail how murine neutrophils respond to *Leishmania* amastigotes and promastigotes. Herein, we provide evidence that both promastigotes and amastigotes are efficiently internalized by mouse neutrophils and similarly trigger neutrophil activation and oxidative burst. However, we observed that amastigotes are highly resistant to neutrophil microbicidal mechanisms and induce anti-inflammatory IL-10 release, while promastigotes trigger more TNF-α secretion and are more susceptible to killing by neutrophils. Collectively, this study supports and expands upon our previous understanding of the role of neutrophils during leishmaniasis [68, 77], and highlights the possible cross-talk between neutrophils and other immune cells involved in parasite recognition and clearance.

	Amastigotes	Promastigotes
Uptake by Neutrophils	Yes	Yes
Neutrophil Activation	Yes	Yes
Cytokine Production	↑ IL-10 ↓ TNF-α	↓ IL-10 ↑ TNF-α
Major Form of Neutrophil Death	Apoptosis	NETosis
Killing by Neutrophils	Resistant	Susceptible

Table 2.1: Summary of results discussed in Chapter 2

Chapter 3: Distinct neutrophil responses to amastigotes of *Leishmania amazonensis* and *Leishmania braziliensis*

INTRODUCTION

Leishmania braziliensis and *L. amazonensis* are causative agents of cutaneous leishmaniasis in overlapping endemic areas of South America. Rarely, infection with these parasites can cause severe secondary forms of leishmaniasis in patients. Cellular hypersensitivity against *L. braziliensis* can induce mucocutaneous leishmaniasis, which is characterized by extensive nasopharyngeal tissue destruction [10]. In contrast, cellular hyposensitivity against *L. amazonensis* can result in diffuse cutaneous leishmaniasis, which is characterized by uncontrolled parasite dissemination throughout the skin [15]. Importantly, a complete understanding of the mechanisms responsible for the opposing immune responses to these 2 parasites remains unclear.

Previous work from our laboratory indicates that amastigotes of *L. braziliensis* and *L. amazonensis* differ considerably in their ability to induce murine DC activation, and this highly linked with the acquisition of T cell-mediated immunity and infection control [82]. Specifically, pronounced DC activation in response to *L. braziliensis* is associated with rapid T effector cell differentiation and production of IFN-γ and IL-17. In contrast, *L. amazonensis* infection induces minimal DC activation, and is associated with delayed acquisition of a Th1 response and failure to control parasite growth [82].

To better understand amastigote interactions with other innate immune cells, we recently assessed neutrophil activation and microbicidal activity against *L. amazonensis* amastigotes. We found that amastigotes are a poor stimulus for neutrophil activation and are highly resistant to neutrophil microbicidal activity [46]. These findings supported an

earlier report from our laboratory that *L. amazonensis* amastigotes are also highly resistant to purified human histones [77], which are important microbicidal components of neutrophil extracellular traps (NETs).

Currently, there is little known about neutrophil interactions with *L. braziliensis* amastigotes, despite a recent report that neutrophils are a prevalent component of the inflammatory infiltrate in mucocutaneous leishmaniasis [61]. Therefore, we examined the ability of *L. braziliensis* amastigotes to trigger neutrophil activation and tested whether they are susceptible to neutrophil microbicidal mechanisms. We found that in comparison to *L. amazonensis*, *L. braziliensis* amastigotes were more efficiently internalized by neutrophils and induced significantly more neutrophil activation, oxidative burst, degranulation, and cytokine production. Potent neutrophil activation by *L. braziliensis* amastigotes corresponded with efficient parasite clearance by these cells, especially in the presence of PMA. However, killing of *L. amazonensis* was consistently poor, even in PMA-activated neutrophils. Because macrophages play a central role in parasite replication and persistence, we also examined whether the addition of neutrophils to infected macrophages could promote amastigote clearance. We observed that macrophage microbicidal activity against both parasite species was similarly enhanced in the presence of neutrophils. Our results suggest that *L. amazonensis* and *L. braziliensis* amastigotes differ in their ability to trigger neutrophil activation and degranulation, and that these differences correspond with the capacity of neutrophils to directly clear these parasites. However, despite poor direct microbicidal activity against *L. amazonensis*, neutrophils may still be able to enhance parasite clearance through favorable interactions with infected macrophages. Our findings of neutrophil-mediated leishmanicidal activity

against both parasite species suggest that persistent neutrophil recruitment to the site of infection may play a role in limiting parasite growth during leishmaniasis.

MATERIALS AND METHODS

Reagents. All chemical reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise specified.

Mice. Female C57BL/6 and BALB/c mice were purchased from Taconic Farms (Germantown, NY). Syrian Golden hamsters were purchased from Harlan Sprague Dawley (Indianapolis, IN). All animals were maintained under specific pathogen-free conditions and used in accordance with protocols approved by the Animal Care and Use Committee of the University of Texas Medical Branch (Galveston, TX).

Parasite Cultivation. Infectivity of *L. amazonensis* (strain RAT/BA/74/LV78) and *L. braziliensis* (strain LC1418) was maintained by regular passage through BALB/c mice and Syrian Golden hamsters, respectively. Promastigotes of both species were cultured at 26°C in Schneider's *Drosophila* medium (Crescent Chemical Company, Islandia, NY), pH 7.0, supplemented with 20% heat-inactivated fetal bovine serum and gentamicin (50 μg/mL). Axenic amastigotes of both species were generated by culturing stationary-phase promastigotes at 32°C in Grace's insect cell culture medium (Invitrogen, Carlsbad, CA), pH 5.2, supplemented with 20% heat-inactivated FBS and gentamicin (25 μg/mL).

Neutrophil collection. Peritoneal exudate cells were obtained from mice 5 h after injection with 3% thioglycollate broth. Thioglycollate was removed and neutrophils were purified via density gradient centrifugation with Percoll. Neutrophil purity (~95%) was routinely assessed by fluorescence-activated cell sorting (FACS) and examination of

morphology; cell viability was routinely >95% as monitored by trypan blue exclusion. All neutrophil experiments were carried out in tissue culture-treated polystyrene. All neutrophil-parasite co-cultures were carried out at 32°C with 5% CO_2.

Quantifying parasite uptake by neutrophils. *Leishmania* amastigotes were labeled with carboxyfluorescein succinimidyl ester (CFSE) and co-cultured with neutrophils at a multiplicity of infection (MOI) of 5 for 4 h as described previously [46]. Cells were washed, blocked with anti-CD16/CD32, and stained with anti-Ly6G-AlexaFluor 647 (BioLegend, San Diego, CA) and anti-CD11b-PE-Cy7 (BD Bioscience, San Jose, CA). Cells were analyzed using a LSRII FACSFortessa (BD Bioscience) and ImageStreamx Mark II Imaging Flow Cytometer (Amnis Corporation, Seattle, WA). Neutrophils were identified based on forward/side scatter characteristics and Ly6G/CD11b positivity. Phagocytosis of labeled parasites was quantified by flow cytometry based on CFSE positivity of $Ly6G^+CD11b^+$ neutrophils, and parasite uptake was further verified using the Internalization feature on the imaging flow cytometer, which excludes cells making close contact with extracellular parasites (see Fig. 3.1B, leftmost panel). Images obtained from the imaging flow cytometer were captured using 60X magnification.

Assessment of neutrophil activation and oxidative burst. Neutrophil activation was assessed by surface upregulation of CD11b, which was examined after cells were blocked with anti-CD16/CD32 and stained with anti-Ly6G-APC (BD Bioscience) and anti-CD11b-PerCP-Cy5.5 (eBioscience, San Diego, CA). Oxidative burst was assessed by staining cells with anti-Ly6G-APC and dihydrorhodamine 123 (DHR 123, 1 μM), which converts to the fluorescent product rhodamine 123 (Rho 123) upon oxidation. The oxidation reaction was stopped on ice and neutrophil oxidative burst was quantified by

gating on $Ly6G^+$ cells and measuring mean fluorescence intensity (MFI) of Rho 123 by FACS. Data were collected using an Accuri C6 flow cytometer (Accuri Cytometers Inc., Ann Arbor MI). Activation and oxidative burst flow cytometry data were analyzed using CFlow version 1.0.227.4 (Accuri Cytometers Inc.) and FlowJo version 7.6.1 (Tree Star, Ashland, OR).

Measurement of neutrophil degranulation. Neutrophil degranulation in response to parasite infection was assessed by measuring reductions in myeloperoxidase (MPO) mean fluorescence intensity (representing MPO loss from the cell interior) via flow cytometry. Specifically, neutrophils were co-cultured with amastigotes in the presence or absence of LPS (100 ng/mL) for 4 h followed by cell staining with anti-Ly6G-APC, anti-CD11b-PerCP-Cy5.5, and anti-MPO-FITC (Abcam, Cambridge, UK). As a positive control, neutrophils were pre-treated with cytochalasin B (5 μg/mL) for 10 min followed by treatment of fMLP (10 μM) as described previously [115]. MPO values for samples are shown as a percentage of the mean fluorescence intensity of MPO compared to MPO intensity of resting neutrophils. Corresponding MPO activity in culture supernatants was assessed by adding tetramethylbenzidine and measuring OD values at 450 nm on a Multiskan Ascent ELSA reader (Labsystems, Helsinki, Finland) as previously described [116].

Assessing cytokine release in neutrophil-amastigote co-cultures. To minimize protease activity, neutrophils were treated with aprotinin (50 μg/mL) prior to treatment with parasites (MOI of 5) for 24 h. Cytokine levels were then assessed by multiplex (eBioscience) and samples were analyzed with a Bio-Plex 200 multiplex reader equipped with Bio-Plex Manager 6.0 software (Bio-Rad Laboratories, Hercules, CA). Neutrophil

production of IL-10 and IL-22 after 4 h of co-culture with parasites was confirmed by flow cytometry by staining $Ly6G^+$ $CD11b^+$ neutrophils with anti-IL-10-PE or anti-IL-22-PE (eBioscience).

Quantifying parasite clearance by neutrophils. To ensure efficient parasite internalization, amastigotes were co-cultured with neutrophils at a ratio of 1 parasite per 10 neutrophils (MOI of 0.1) for 6 h. In some experiments, co-cultures were incubated with PMA (100 nM) and/or DNase 1 (100 U/mL, Thermo Scientific, Waltham, MA). At the end of incubation, co-cultures were adjusted to 5 mM EDTA and incubated for 30 min to liberate adherent cells. Subsequently, parasite survival was assessed by limiting dilution in Schneider's *Drosophila* medium as previously described [117].

Macrophage-neutrophil co-cultures. Bone marrow-derived macrophages were generated from 6-8 week C57BL/6 mice as described previously [77]. Briefly, bone marrow cells were flushed from the femurs of mice and cultured in Iscove's modified Dulbecco's medium (IMDM, Invitrogen) supplemented with 10% FBS, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 50 μg/mL gentamicin, 100 U/mL penicillin, and 20 ng/mL recombinant murine macrophage colony-stimulating factor (rM-CSF, eBioscience) for 10 days at 37°C and 5% CO_2. Cells received fresh medium containing rM-CSF on Day 5 and were collected for use on Day 10. Macrophages were infected with *L. amazonensis* or *L. braziliensis* amastigotes at a ratio of 3:1 for 24 h. Infected macrophages were then washed to remove free amastigotes and co-cultured with neutrophils (at a 3:1 neutrophil-to-macrophage ratio) in fresh IMDM. After 24 h of macrophage-neutrophil co-culture, IMDM was replaced with complete Schneider's

medium, and live parasites were counted on a hemacytometer after incubating for 3-4 days.

Statistical analysis. Differences between two groups were determined by two-tailed Student's *t* test. Graphs were prepared by using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA). The differences between groups were considered significant when the *p* value was < 0.05.

RESULTS

Neutrophils competently engulf *L. braziliensis* amastigotes. Using a combination of conventional FACS and imaging flow cytometry, we compared the ability of neutrophils to engulf CFSE-labeled amastigotes of *L. amazonensis* and *L. braziliensis* after 4 h of co-culture. Surprisingly, we observed that *L. braziliensis* uptake by $CD11b^{+}Ly6G^{+}$ neutrophils was 7 times more efficient than that of *L. amazonensis* (84.7% of *L. braziliensis*-exposed cells vs. 11.7% of *L. amazonensis*-exposed cells, Fig. 3.1A). Imaging flow cytometric analysis was used to confirm our findings and enabled us to compare the number of internalized parasites per infected cell while excluding potentially confounding anomalies such as extracellular parasite-neutrophil doublets (Fig. 3.1B, leftmost panel). We observed that nearly 70% of *L. braziliensis*-infected cells carried only 1 parasite, while approximately 60% of *L. amazonensis*-infected cells carried multiple parasites (Fig. 3.1C). These findings demonstrate that murine neutrophils differ substantially in their ability to internalize amastigotes of *L. amazonensis* and *L. braziliensis*.

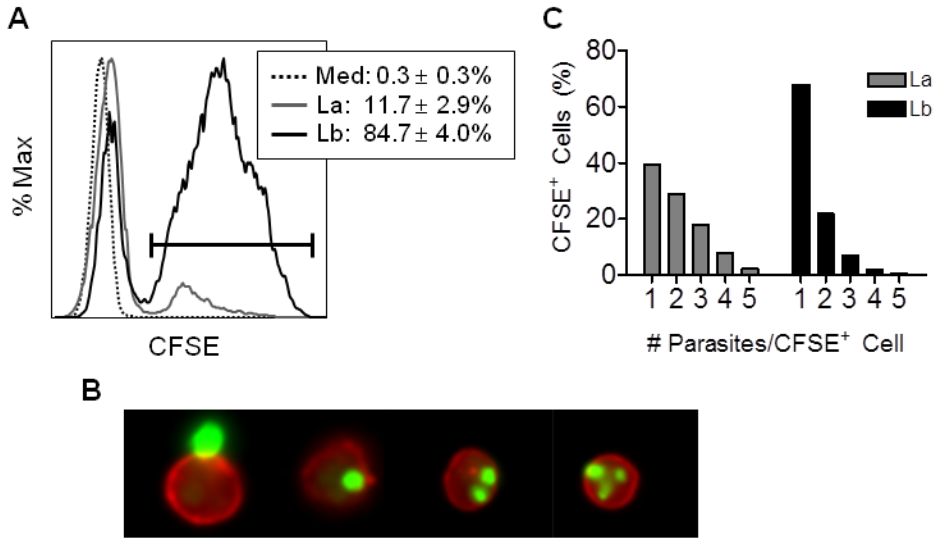

Figure 3.1: Neutrophil uptake of *L. amazonensis* and *L. braziliensis* amastigotes

Thioglycollate-induced peritoneal neutrophils were co-cultured with CFSE-labeled amastigotes for 4 h. Cells were then analyzed by both FACS and imaging flow cytometry to assess parasite uptake. (A) Histogram showing $CD11b^{+}Ly6G^{+}$ neutrophil uptake of CFSE-labeled parasites. Dotted line = uninfected neutrophils. Solid gray line = *L. amazonensis*-infected neutrophils. Solid black line = *L. braziliensis*-infected neutrophils. (B) Representative events from imaging flow cytometric analysis showing (from left to right) an uninfected neutrophil (red) in close contact with an amastigote (green), and neutrophils infected with 1, 2, and 3 parasites. (C) Graphic representation of imaging flow cytometric analysis showing the percentage of infected cells carrying the designated number of *L. amazonensis* (La) or *L. braziliensis* (Lb) parasites.

***L. braziliensis* amastigotes potently induce neutrophil activation and oxidative burst.** Surface CD11b upregulation on neutrophils is typically associated with an activated phenotype [90], and we previously noted a small, but significant, increase in neutrophil surface CD11b expression after infection with *L. amazonensis* amastigotes [46]. Surprisingly, when we compared CD11b upregulation in response to *L. amazonensis* and *L. braziliensis* by FACS, we observed that *L. braziliensis* was a significantly greater trigger for CD11b exposure than *L. amazonensis* (Fig. 3.2A, $p < 0.001$). Interestingly, *L. braziliensis* metacyclic promastigotes were a weaker inducer of CD11b upregulation than amastigotes (data not shown), which is consistent with our previous observations that *L. braziliensis* amastigotes are a stronger inducer of DC activation than promastigotes [82].

Imaging flow cytometric analysis suggested that the extent of CD11b upregulation corresponded with the number of internalized parasites for both parasite species (Fig. 3.2B and 3.2C, comparing cells carrying 1 parasite with cells carrying 3 or more parasites). We also noted that CD11b upregulation occurred in bystander ($CFSE^-$) cells, as uninfected cells from neutrophil-amastigote co-cultures had higher levels of CD11b than medium control neutrophils (Fig. 3.2B and 3.2C, comparing white Med bar and dark 0 bars). The amount of CD11b upregulation in bystander cells was approximately 2-fold higher in neutrophil-*L. braziliensis* co-cultures (mean intensity of $12.5x10^3$) as compared to neutrophil-*L. amazonensis* co-cultures (mean intensity of $6.3x10^3$). These results suggest that amastigotes can facilitate neutrophil activation in both infected and uninfected cells, with neutrophils carrying multiple parasites exhibiting the most activated phenotype.

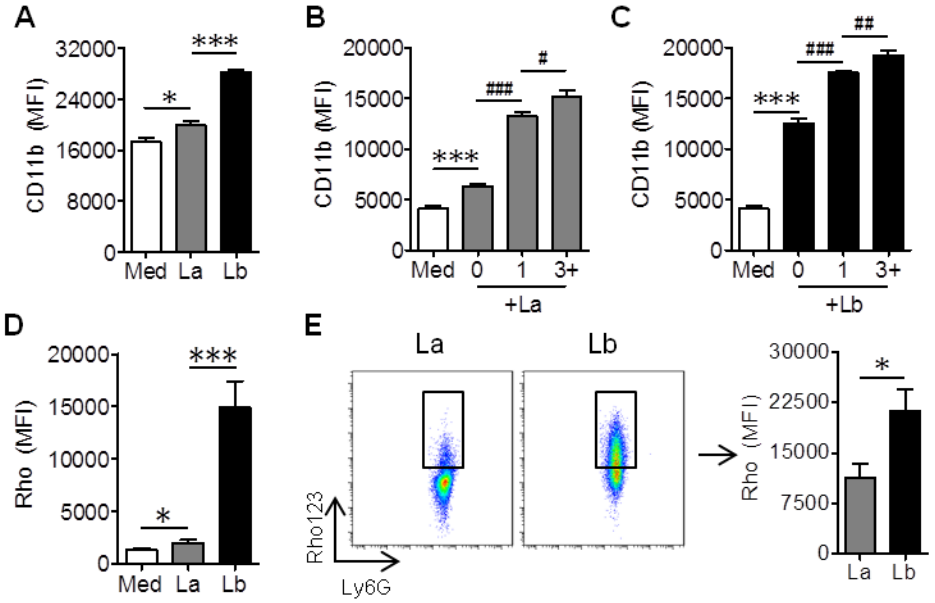

Figure 3.2: Amastigote-induced neutrophil activation and oxidative burst

Neutrophils were co-cultured with amastigotes for 4 h, followed by flow cytometric analysis. (A) FACS analysis of CD11b surface expression on resting neutrophils (Med) and neutrophils exposed to unlabeled *L. amazonensis* (La) or *L. braziliensis* (Lb) amastigotes. (B and C) Imaging flow cytometric analysis of CD11b expression on resting neutrophils, uninfected cells from neutrophil-amastigote co-cultures (0), and infected cells carrying 1 parasite or 3 or more parasites. *** ($p < 0.001$) between medium control and bystander neutrophils. # ($p < 0.05$), ## ($p < 0.01$), and ### ($p < 0.001$) between groups in neutrophil-amastigote co-cultures. (D) Oxidative burst in total $Ly6G^+$ neutrophils in medium alone or in response to amastigotes. (E) Oxidative burst in $Ly6G^+$ Rho 123^+ neutrophils, demonstrating that the intensity of oxidative burst was significantly greater in *L. braziliensis*-infected cells compared to *L. amazonensis*-infected cells. * ($p < 0.05$) and *** ($p < 0.001$) between indicated groups.

In addition to CD11b upregulation, activated neutrophils typically undergo oxidative burst, resulting in the release of noxious reactive oxygen species (ROS) [118]. To determine neutrophil oxidative burst after parasite infection, we treated cells with DHR 123, a cell-permeable probe that converts into fluorescent Rho 123 upon oxidation [119]. As reported previously, we observed an appreciable oxidative burst response in *L. amazonensis*-infected cells. However, we were surprised to find that *L. braziliensis* infection was a significantly greater stimulus for oxidative burst (Fig. 3.2D). By gating on Rho 123^{+} (ROS-producing) neutrophils and comparing the intensity of neutrophil oxidative burst on a per cell basis, we found that Rho 123 intensity in *L. braziliensis*-infected cells was nearly twice that of *L. amazonensis*-infected neutrophils (Fig. 3.2E). Curiously, oxidative burst in response to *L. braziliensis* metacyclic promastigotes was much weaker than amastigote-mediated burst (data not shown). Collectively, these data suggest that *L. braziliensis* amastigotes are a significantly stronger trigger for neutrophil activation and oxidative burst when compared to *L. amazonensis* amastigotes or *L. braziliensis* promastigotes.

***L. braziliensis* amastigotes independently induce neutrophil degranulation.** Neutrophils can respond to pathogens by releasing a number of pre-synthesized antimicrobial components into the phagosome and extracellular space (termed degranulation), and this process can play an important role in both pathogen clearance and tissue damage [120]. Degranulation is commonly assessed by measuring supernatant levels (or enzyme activity) of established granule components, such as myeloperoxidase (**MPO**) [115] or β-glucosaminidase [121]. However, these assays require relatively high

cell concentrations to achieve appropriate sensitivity. To overcome this limitation, we developed a degranulation assay that can quantify MPO loss from the cell interior via flow cytometry. To validate this assay, we compared MPO mean fluorescence intensity of resting neutrophils (containing 100% of their MPO in intracellular granules) and neutrophils treated with cytochalasin B and fMLP, a combination of signals that potently induces neutrophil degranulation as previously described [115]. As shown in Figure 3.3A, a significant decrease in MPO mean fluorescence intensity occurred in response to cytochalasin B and fMLP. Importantly, MPO loss from the interior of treated cells correlated with an increase in corresponding MPO activity in culture supernatants (data not shown). To quantify amastigote-induced neutrophil degranulation, we examined MPO loss in infected neutrophils after 4 h of co-culture. We observed that *L. amazonensis* amastigotes failed to trigger degranulation in the absence of a secondary signal, such as LPS. In contrast, *L. braziliensis* amastigotes induced degranulation independently of a secondary signal, and LPS treatment further enhanced amastigote-induced degranulation (Fig. 3.3B). Interestingly, we noted that *L. braziliensis* metacyclic promastigotes induced a degranulation response similarly to *L. braziliensis* amastigotes (data not shown). These data demonstrate that *L. braziliensis* infection alone is a sufficient trigger for neutrophil degranulation, whereas infection with *L. amazonensis* amastigotes neither induces nor inhibits neutrophil degranulation.

Amastigotes induce pro-inflammatory and anti-inflammatory cytokine secretion. Neutrophil activation is typically accompanied by cytokine release, and we recently

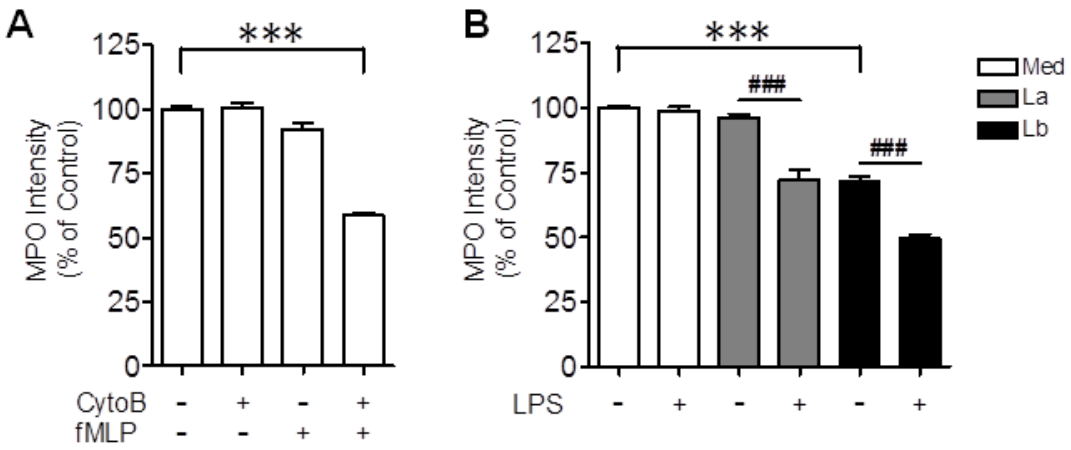

Figure 3.3: Amastigote-induced neutrophil degranulation

(A) Validation of a FACS-based degranulation assay. Neutrophil MPO loss occurs in cells treated with cytochalasin B followed by fMLP. *** ($p < 0.001$) between medium control and cytochalasin B/fMLP treatment. (B) Neutrophil degranulation in response to amastigotes (MOI 5) and/or LPS. MPO values for each treatment are expressed as a percentage compared to the MFI of medium control. *** ($p < 0.001$) between medium control and Lb-infected neutrophils. ### ($p < 0.001$) between parasite alone and parasite + LPS.

reported that *L. amazonensis* amastigotes and promastigotes preferentially trigger IL-10 and TNF-α release from neutrophils, respectively [46]. In an effort to better understand the neutrophil anti-amastigote response, we further characterized neutrophil cytokine release in response to amastigotes via multiplex. We found that neutrophils respond to both species by releasing appreciable amounts of several cytokines, including IL-22, IL-10, TNF-α, and IL-18 (Fig. 3.4 A-D, respectively). Importantly, *L. braziliensis* amastigotes induced greater levels of all of these cytokines. Because there are few reports of IL-22 release from neutrophils [122], and to exclude the possibility that rare non-neutrophil peritoneal exudate cells were the source of IL-22 in our experiments, we confirmed neutrophil production of this cytokine by flow cytometry (Fig. 3.4E). Interestingly, neutrophil production of IL-22 occurred within 4 h of co-culture with amastigotes of both parasite species, particularly in response to *L. braziliensis* infection. Neutrophil production of IL-10 was similarly triggered shortly after infection with amastigotes, with *L. braziliensis* being a more potent signal for cytokine synthesis (Fig. 3.4F).

***L. braziliensis* amastigotes are more susceptible to direct neutrophil killing than *L. amazonensis* amastigotes.** We and others have reported that *L. amazonensis* promastigotes can be efficiently killed by neutrophils and select neutrophil components [46, 47, 77]; in contrast, *L. amazonensis* amastigotes are highly resistant to neutrophil microbicidal mechanisms [46]. To determine if amastigote resistance to neutrophil killing is a conserved phenomenon, we added *L. amazonensis* and *L. braziliensis*

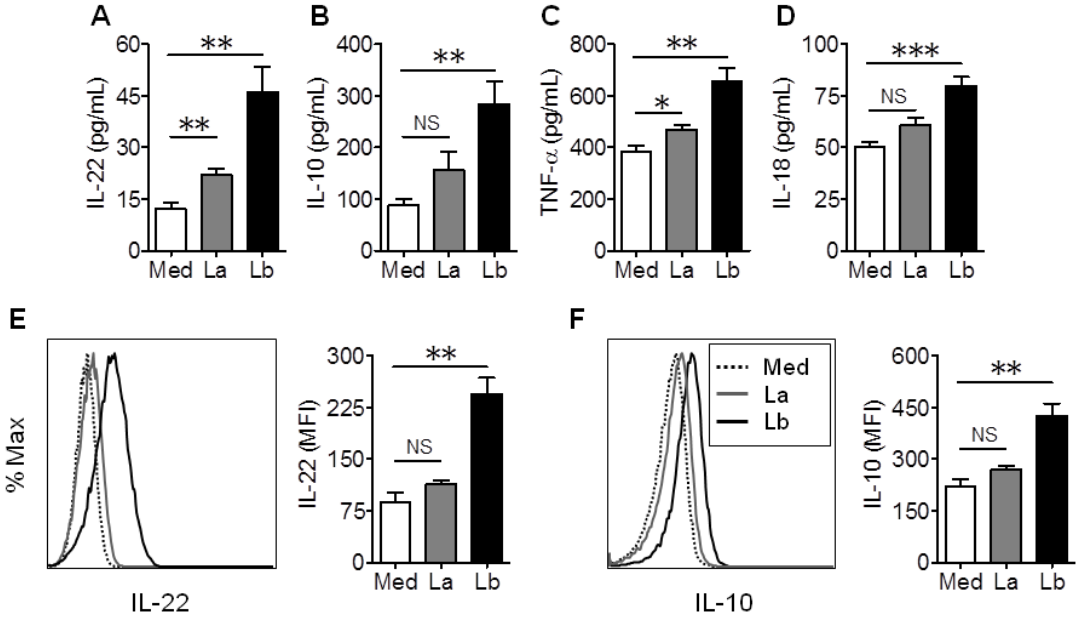

Figure 3.4: Amastigote-induced cytokine production

(A-D) Neutrophil release of IL-22, IL-10, TNF-α, and IL-18 was assessed by multiplex after 24 h of co-culture with amastigotes of *L. amazonensis* or *L. braziliensis*. (E and F) Neutrophil production of IL-22 and IL-10 was confirmed by flow cytometry after 4 h of co-culture with amastigotes. * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$) between medium control and amastigote-infected neutrophils. NS, not significant.

amastigotes to medium alone or medium containing neutrophils. At the end of 6 h of incubation, parasite survival was assessed by limiting dilution, as previously described [117]. Importantly, we observed that resting neutrophils were able to kill approximately 60% of *L. braziliensis* amastigotes after 6 h, while *L. amazonensis* survival was not significantly altered by the presence of neutrophils (Fig. 3.5A and B). Activating neutrophils with PMA enhanced *L. braziliensis* clearance by an additional 15%, and adding DNase to PMA-treated cells significantly improved *L. braziliensis* survival (Fig. 3.5B), suggesting that neutrophil NETosis may play an important role in neutrophil leishmanicidal activity. In contrast, survival of *L. amazonensis* amastigotes was not significantly altered by the presence of PMA-activated neutrophils (Fig. 3.5A).

Neutrophil-mediated clearance of *L. amazonensis* and *L. braziliensis* in infected macrophages. Because of the central role of macrophages during *Leishmania* infection, several studies have assessed the ability of neutrophils to reduce *Leishmania* burden in infected macrophages [113, 123, 124]. Interestingly, although neutrophils have been shown to increase macrophage microbicidal activity against *L. amazonensis* amastigotes [113], the effect of neutrophils on *L. braziliensis* amastigote-infected macrophages remains unexplored.

We observed that the presence of neutrophils potently decreased intramacrophagic survival of both *L. amazonensis* and *L. braziliensis* after 24h of co-culture (Fig. 3.5C). These results suggest that successful neutrophil-mediated clearance of amastigotes from infected macrophages may occur even when direct neutrophil

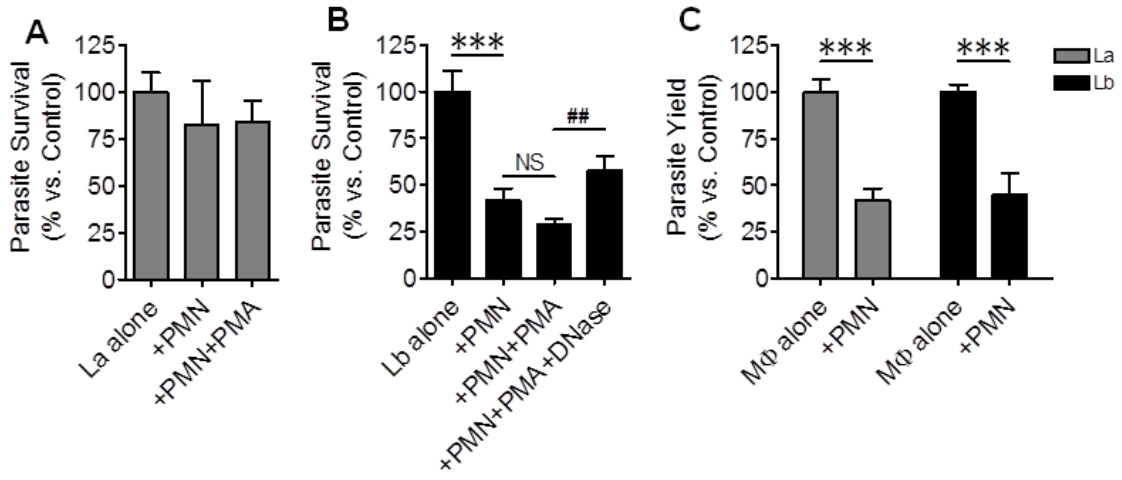

Figure 3.5: Neutrophil leishmanicidal activity against *L. amazonensis* and *L. braziliensis* amastigotes

(A and B) Survival of parasites in medium alone vs. parasites co-cultured with resting neutrophils (+PMN), PMA-activated neutrophils (+PMN+PMA), and PMA-activated neutrophils treated with DNase (+PMN+PMA+DNase). *** ($p < 0.001$) between parasites alone and parasite + resting neutrophil groups. ## ($p < 0.01$) between PMA-activated neutrophils and PMA-activated neutrophils + DNase. NS, not significant. (C) Neutrophil-mediated clearance of amastigotes from infected macrophages after 24 h of co-culture. *** ($p < 0.001$) between indicated groups.

microbicidal mechanisms are insufficient for parasite killing (as is the case for *L. amazonensis*).

DISCUSSION

Multiple species of *Leishmania* can induce leishmaniasis in mice and humans. However, despite belonging to the same genus, some parasite species have been genetically distinct for 40-80 million years [125]. Therefore, it is likely that individual species have developed unique strategies to establish infection in mammalian hosts and maintain chronic persistence. Two such species, *L. braziliensis* and *L. amazonensis*, share endemic areas in South America, but are responsible for clinically-distinct spectra of disease [20]. We have previously demonstrated that *L. amazonensis* and *L. braziliensis* amastigotes differ in their ability to induce DC activation and cytokine production [82]. However, recent suggestions that neutrophils may play an important role in maintaining the chronic inflammatory process in leishmaniasis [126], and the identification of neutrophils in the lesions of chronic cutaneous [58, 59], diffuse cutaneous [60], and mucocutaneous leishmaniasis [61] prompted us to compare neutrophil responses to these two parasites as well. We were surprised to find distinct differences in the interaction between amastigotes and neutrophils depending on the parasite species in question. Overall, we found that *L. braziliensis* induced significantly more neutrophil activation, oxidative burst, degranulation, and cytokine release than *L. amazonensis*, and this highly associated with direct leishmanicidal activity against *L. braziliensis*, but not *L. amazonensis.*

Currently, it is unclear why *L. braziliensis* and *L. amazonensis* display distinct differences in susceptibility to neutrophil microbicidal mechanisms. *L. braziliensis* clearance appears to be partially NETosis-dependent, as adding DNase to *L. braziliensis*-neutrophil co-cultures improved parasite survival (Fig. 3.5B). However, because neutrophils utilize a diverse antimicrobial arsenal, it is likely that other mechanisms contribute to *L. braziliensis* clearance by these cells. We believe that poor neutrophil killing of *L. amazonensis* amastigotes is associated with increased resistance of these parasites to the microbicidal activity of NETs. This hypothesis is supported by the observation that PMA-activated neutrophils failed to adequately kill *L. amazonensis* (Fig. 3.5A), and is further substantiated by our previous work demonstrating that *L. amazonensis* amastigotes are highly resistant to purified histones, which are an important antimicrobial element of NETs [77].

IL-22 is an IL-10 family member that is tightly linked to neutrophils and Th17-mediated inflammation. Although a number of cell types can synthesize IL-22 (including Th17 cells), receptor expression is limited to stromal cells, including epithelial cells and keratinocytes [127]. When appropriately regulated, IL-22 can contribute to barrier immunity by signaling through non-hematoiepoetic cells to increase expression of G-CSF, IL-1β, IL-6, CXCL1, and antimicrobial peptides such as defensins [127]. Therefore, IL-22 can improve neutrophil-mediated immunity at sites of infection by increasing granulopoiesis, promoting neutrophil homing, and increasing neutrophil activation. However, sustained IL-22 production may play a pathogenic role in chronic or highly dysregulated inflammatory foci, such as psoriasis [128] and rheumatoid arthritis [129].

The finding that *L. braziliensis* amastigotes can induce a significant increase in neutrophil IL-22 production is interesting for several reasons. Firstly, although neutrophil-derived IL-22 has recently been reported in the context of experimental dextran sodium sulfate-mediated colitis [122], we were unable to find any published reports of IL-22 production by neutrophils in response to a specific pathogen. At this time, it is unclear whether amastigote-mediated neutrophil IL-22 production is a unique feature of *Leishmania* infection or whether infection-induced IL-22 production by neutrophils is a common (but previously-undocumented) phenomenon.

Importantly, reports of IL-22 involvement in *Leishmania* control and pathogenesis (regardless of cellular source) are sparse. Hezarjaribi *et al*. demonstrated that exogenous IL-22 enhanced the protective effects of vaccination in BALB/c mice infected with *L. major*, resulting in increased IFN-γ and reduced IL-4 expression [130]. Additionally, Pitta *et al*. demonstrated that *Leishmania* antigen-induced IL-22 production in human peripheral blood mononuclear cells (**PBMCs**) correlated with protection against visceral leishmaniasis [131]. However, it is also possible that IL-22 plays a pathogenic role in chronic leishmaniasis; given that mucocutaneous leishmaniasis in humans is associated with a highly dysregulated Th17-like response and prominent neutrophil recruitment [61], it is tempting to speculate that IL-22 plays an important role in maintaining granulocyte recruitment and perpetuating tissue damage in this disease manifestation.

Although elegant flow cytometric methods for quantifying degranulation have been established for human neutrophils (including individual markers to quantify the release of distinct granule types) [132], we were unable to find a reliable FACS-based method for measuring murine neutrophil degranulation. Current quantification methods

require relatively high cell concentrations in order to detect extracellular MPO or other products released during degranulation, and many commercially-available MPO detection kits are incompatible with culture medium containing serum. Herein, we present a new method for measuring neutrophil degranulation by quantifying MPO loss from the cell interior. This assay can easily be incorporated into a multi-color flow cytometry panel, which is ideal for analyses of mixed cell populations (e.g. infected vs. uninfected cells, neutrophil-macrophage co-cultures) or for precious samples.

It is interesting to consider that neutrophils can limit amastigote replication in macrophages even when potent activators of traditional macrophage microbicidal activity, such as IFN-γ and LPS, fail to control the growth of *L. amazonensis* amastigotes (Henard et al., *PLoS NTD*, in press). A previous report found that neutrophil-mediated amastigote killing in macrophages was dependent on TNF-α, neutrophil elastase, and plasminogen activating factor, but was not dependent on reactive oxygen or reactive nitrogen species [113]. These findings indicate that nonconventional macrophage microbicidal mechanisms (which can be bolstered by innate immune cells such as neutrophils) may have a previously underappreciated role in *Leishmania* control.

Collectively, this study is an important first step in identifying novel cellular and molecular mechanisms that drive differential immune recognition and control of *Leishmania spp.* Importantly, anti-parasite immunity is a complex process involving multiple innate and adaptive immune cell types. As a consequence, successful vaccine and immunotherapeutic development critically rely on a clear understanding of the role of numerous immune cell populations. Although neutrophils are short-lived, their ability to

kill pathogens and influence the inflammatory milieu should be important considerations for future studies.

	L. amazonensis	*L. braziliensis*
Uptake by Neutrophils	Poor	Efficient
Neutrophil Activation/Oxidative Burst	Limited	Pronounced
Degranulation	Insufficient	Sufficient
Cytokine Production	Limited	Pronounced
Killing by Neutrophils	Resistant	Susceptible

Table 3.1: Summary of results discussed in Chapter 3

Chapter 4: Data Summary, Study Limitations, and Future Directions

Much of the original research and data assembled for this dissertation have been presented in the form of manuscripts in Chapters 2 and 3. In Chapter 4, I intend to summarize my findings, address some study limitations, and present additional data to aid in the discussion of some potential future directions. **Portions of this chapter (including data and tables) are being prepared for submission as part of a literature review.**

DISTINCT DIFFERENCES IN THE NEUTROPHIL RESPONSE AGAINST *LEISHMANIA* PROMASTIGOTES AND AMASTIGOTES

As discussed in Chapter 2, the majority of studies exploring the role of neutrophils in leishmaniasis focus on how these cells interact with promastigotes. However, a growing body of literature indicates that neutrophil recruitment is not restricted to the promastigote-mediated acute phase of infection. **Prior to this study, the ability of neutrophils to affect the anti-amastigote immune response and to influence amastigote survival and growth was largely unexplored.** We found that murine neutrophils internalize *L. amazonensis* amastigotes and promastigotes with similar efficiency, and phagocytosis of parasites triggered comparable levels of neutrophil activation and oxidative burst. However, neutrophil release of cytokines differed depending on the stage of parasite encountered, as promastigote infection preferentially induced TNF-α release while amastigote infection was a more potent signal for IL-10 release. Additionally, amastigotes were significantly more resistant to neutrophil microbicidal mechanisms than promastigotes. Our findings in Chapter 2 demonstrate that

neutrophils may play dissimilar roles during the acute and chronic phases of leishmaniasis because they interact with promastigotes and amastigotes differently.

While this study is an important first step in improving our understanding of neutrophil-amastigote interactions, one noteworthy limitation should be addressed. Specifically, the *in vitro* nature of our experiments makes it difficult to extrapolate the role of neutrophils at the site of parasite infection. It is well established that neutrophils are highly sensitive and responsive to the inflammatory environment around them [96]. However, the relatively low number of neutrophils that are obtainable from the site of infection in mice prevents the use of these cells in conducting detailed functional studies *ex vivo*. To partially address the limitations of our *in vitro* analysis, a discussion of preliminary *in vivo* data will be discussed later in this chapter.

L. BRAZILIENSIS AMASTIGOTES ARE SUSCEPTIBLE TO NEUTROPHIL MICROBICIDAL MECHANISMS

After completing the work presented in Chapter 2, we wondered whether amastigote resistance to neutrophil microbicidal mechanisms was a conserved trait among *Leishmania spp*. Importantly, many laboratory parasite strains do not undergo amastigogenesis under cell-free culture conditions, making it challenging to study amastigote interactions with immune cells *in vitro*. Fortunately, the Soong laboratory utilizes two *Leishmania* strains (*L. amazonensis* LV78 and *L. braziliensis* LC1418) that reliably grow as amastigotes in culture. We suspected that neutrophil interactions with *L. amazonensis* and *L. braziliensis* amastigotes would differ based on 2 pieces of prior evidence. Firstly, these 2 species induce clinically distinct forms of leishmaniasis in

human patients, as discussed in Chapter 1. Secondly, we have previously demonstrated that *L. amazonensis* weakly triggers activation and cytokine production in murine dendritic cells, while *L. braziliensis* potently induces dendritic cell activation and IL-12 production [82]. Accordingly, we tested the hypothesis that *L. braziliensis* amastigotes would be potent inducers of neutrophil activation and would therefore be more susceptible to neutrophil microbicidal mechanisms. **Prior to this study, a side-by-side comparison of neutrophil interactions with *L. amazonensis* and *L. braziliensis* had not been performed.** Importantly, we observed *L. braziliensis* amastigotes induced significantly more neutrophil activation, oxidative burst, degranulation, and cytokine release than *L. amazonensis* amastigotes. These findings highly correlated with the ability of neutrophils to clear amastigotes, as *L. braziliensis* was highly susceptible to neutrophil-mediated killing while *L. amazonensis* efficiently resisted neutrophil microbicidal mechanisms (even when co-cultured with PMA-activated cells). We believe that these novel findings help to further explain the dramatic immunological differences observed in infections with *L. amazonensis* and *L. braziliensis*.

Because this study critically relied on the use of axenic amastigotes, we were fortunate to have access to *L. amazonensis* and *L. braziliensis* strains (LV78 and LC1418, respectively) that grow as amastigotes *in vitro*. However, these strains have been maintained under laboratory conditions for decades [133, 134], and it is unclear whether they continue to accurately represent the characteristics of parasite populations that can be obtained from current clinical cases. Therefore, an important future direction for this research is the assessment of neutrophil functions against fresh field isolates. In addition, it will be exciting to determine whether the dramatic differences in neutrophil responses

to *L. amazonensis* and *L. braziliensis* are also observed when using human cells and in the context of mouse models of infection.

The mechanisms responsible for heightened neutrophil activation in response to *L. braziliensis* amastigotes remain unclear. Importantly, we have previously reported that *L. braziliensis*-induced activation of dendritic cells is MyD88-dependent, implicating Toll-like receptors (**TLRs**) in parasite recognition [135]. Neutrophils are also highly sensitive to pathogen-associated molecular patterns that are identified by TLRs [136], but the specific parasite ligands responsible for TLR-dependent activation remain uncharacterized at this time. In the future, it will be interesting to determine how differences in surface molecule expression between parasite species drive differential cell activation outcomes.

FUTURE DIRECTIONS: ANTI-NEUTROPHIL TREATMENT DURING THE CHRONIC PHASE OF EXPERIMENTAL CUTANEOUS LEISHMANIASIS

As discussed in Chapter 2, anti-neutrophil antibody treatment has become a popular method for assessing the role of neutrophils in various infectious and inflammatory diseases *in vivo* [69-71, 137]. A handful of studies have utilized this approach to determine the role of neutrophils after *Leishmania* infection, but considerable differences in experimental design between studies complicate our ability to make concrete conclusions (Table 4.1). For example, Chen et al. and Tacchini-Cottier et al. both examined the effects of neutrophil depletion on *L. major* burden and lesion progression in BALB/c mice, but reported contradictory findings [72, 73].

Parasite Species/Strain	Treatment	Host Strain	Effects of Antibody Treatment
L. major LV39	NIMP-R14 1mg -6 h	BALB/c	Delayed lesion progression, reduced parasite burden
		C57BL/6	Accelerated early lesion progression, no differences in parasite burden [73]
L. major 5ASKH	RB6-8C5 250µg Day -1	BALB/c	Accelerated lesion progression, enhanced parasite burden
		C3H/HeJ	No change in lesion progression [72]
L. major Bokkara	RB6-8C5 2 mg Days -3, 0, 3	BALB/c	Accelerated lesion progression between weeks 1 and 6, chronic progression unaltered
		C57BL/6	Accelerated lesion progression at weeks 3 and 4, higher dLN burden at days 16 and 29, chronic progression unaltered [138]
L. major Friedlin	RB6-8C5 500 µg -16h	C57BL/6	Depletion prior to sandfly-mediated infection reduced parasite burden at the site of infection at 1 and 4 weeks [43]
L. donovani LV82	NIMP-R14 250 µg Days 0, 3, 6, 9, 12	BALB/c	Increased burden in the spleen and bone marrow, hepatosplenomegaly [139]
L. donovani LV9	RB6-8C5 200 µg Days -1 , 2, 5, 8, 11, 14	BALB/c C57BL/6	Enhanced parasite burden in the liver and spleen in both mouse strains [140]
L. infantum MON1	RB6-8C5 200 µg -48h, -5h, 72h, 168h	BALB/c	Reduction in spleen parasite burden
	RB6-8C5 200 µg starting at 3 months, 2 doses/week for 4 weeks		No effect [141]
L. braziliensis BA788	RB6-8C5 500 µg Days -1, 3, 6, 9, 12	BALB/c	Increased parasite burden at 2 weeks, increased IL-2, IL-4, IL-5, IFN-γ, TNF-α, faster early lesion progression [124]
L. amazonensis PH8	RB6-8C5 500 µg -16h	BALB/c	Increased lesion size and parasite burden at 1 week
		C57BL/6	No effect
	RB6-8C5 500 µg Days -1, 1, 3, 5, 7	BALB/c	Increased lesion size at weeks 4 and 8
		C57BL/6	No effect [142]

Table 4.1: Summary of previous anti-neutrophil studies in mouse models of leishmaniasis

One confounding factor in past studies is likely the anti-neutrophil antibody clone utilized. The majority of previous studies utilize RB6-8C5, but in recent years the specificity of this clone for neutrophils has been questioned [99]. Importantly, RB6-8C5 also appears to target non-neutrophil cell populations, including some plasmacytoid dendritic cells [143], monocytes [144], eosinophils [145], and T lymphocytes [146]. Additionally, reports of a compensatory increase in neutrophil numbers above control in the footpad [142] and blood [138, 141] of *Leishmania*-infected mice after completing RB6-8C5 treatments make it difficult to determine whether the observed phenotypes were due to initial neutrophil depletion or subsequent neutrophil influx. With the advent of newer anti-neutrophil antibody clones (such as 1A8), it has become possible to target these cells with greater precision. Interestingly, Wang et al. demonstrated that very low 1A8 doses can block neutrophil recruitment to sites of inflammation without inducing protracted periods of systemic neutropenia [137], which may help preserve unrelated neutrophil functions at distal sites, such as immunoglobulin production in the spleen [147].

Importantly, there is a paucity of anti-neutrophil studies assessing the role of neutrophils during the later stages of leishmaniasis. To accompany the work discussed in Chapters 2 and 3 and to increase the overall impact of my research, I conducted a pilot study utilizing a simple mouse model to assess neutrophil contributions to anti-parasite immunity during the chronic phase of *L. amazonensis* infection. Briefly, I infected mice with $2x10^5$ metacyclic promastigotes and allowed infections to proceed until the onset of visible footpad swelling at 4 weeks. Subsequently, mice received 300 μg of anti-Ly6G (1A8) or isotype control (2A3) i.p. every 6 days to assess whether anti-neutrophil

treatment impacted disease progression. Repeated 1A8 treatment efficiently blocked Ly6G on circulating neutrophils, causing a reduction in binding of APC-conjugated anti-Ly6G upon flow cytometric analysis (Fig. 4.1A). However, because our treatment antibody and APC-conjugated antibody are homoclonal (1A8), I developed a Ly6G-independent flow cytometry panel to determine whether anti-neutrophil treatment efficiently cleared neutrophils from the circulation. I found that MPO and 7/4 could be used in lieu of Ly6G to identify the neutrophil population in circulating $CD11b^{+}$ cells, and that $MPO^{hi}7/4^{int}$ cells were equivalent to $Ly6G^{+}$ cells (Fig 4.1B). Analysis of circulating leukocyte populations utilizing this panel revealed that anti-neutrophil treatment did not substantially alter the frequency of neutrophils in the blood (Fig 4.1C), consistent with the aforementioned study by Wang et al. [137]. Nevertheless, prolonged anti-neutrophil treatment significantly exacerbated lesion progression in infected mice (Fig. 4.1D). We were surprised to observe, however, that anti-neutrophil treatment did not significantly alter parasite burden in infected footpads (Fig. 4.1E). The results of this pilot study suggest that prolonged neutrophil recruitment during the chronic phase of *L. amazonensis* infection may be important for limiting disease pathogenesis, although neutrophils may exert these functions without drastically altering parasite clearance at the site of infection.

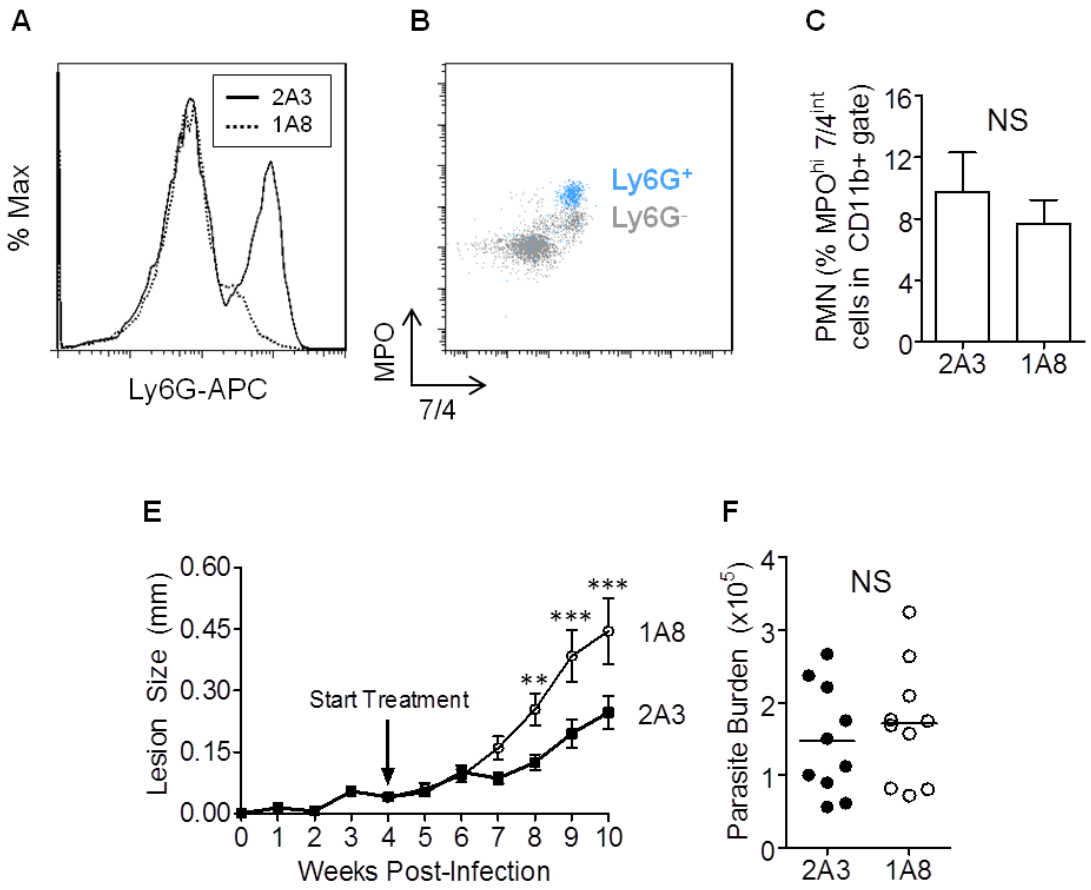

Figure 4.1: Effect of repeated 1A8 treatment on the progression of chronic cutaneous leishmaniasis in *L. amazonensis*-infected mice

(A) Histogram gated on $CD11b^+$ cells demonstrating that repeated 1A8 treatment reduces binding of APC-conjugated anti-Ly6G on circulating neutrophils. Solid line: isotype (2A3)-treated animals. Dotted line: anti-neutrophil (1A8)-treated animals. (B) Utilization of a Ly6G-independent panel to identify neutrophils in 1A8-treated animals. (C) Graphic representation of the circulating neutrophil population in 1A8-treated and 2A3-treated animals. (D) Lesion progression in *L. amazonensis*-infected C57BL/6 mice receiving antibody treatments starting at 4 weeks post-infection. 2-way ANOVA indicates the progressions are statistically different. ** ($p < 0.01$), and *** ($p < 0.001$) indicate statistically significant differences between groups at designated time points using the Bonferroni method. (E) Parasite burden in footpads at 10 weeks post-infection. NS, not significant.

CONCLUDING REMARKS

The goal of this dissertation was to characterize the interaction between neutrophils and *Leishmania* amastigotes. The findings presented herein suggest the following:

- Murine neutrophils interact with *L. amazonensis* promastigotes and amastigotes differently. Specifically, neutrophil interactions with promastigotes result in partial parasite clearance and TNF-α release. In contrast, *L. amazonensis* amastigotes are highly resistant to neutrophil microbicidal mechanisms and induce neutrophil IL-10 release. These observations indicate that neutrophils may play different roles during the acute (promastigote-mediated) and chronic (amastigote-mediated) phases of leishmaniasis.
- Amastigotes of *L. braziliensis* efficiently trigger neutrophil activation, oxidative burst, degranulation, and the release of pro-inflammatory and anti-inflammatory cytokines. Potent neutrophil activation corresponded with improved *L. braziliensis* clearance when compared to neutrophil killing of *L. amazonensis* amastigotes. These results are consistent with clinical findings that *L. braziliensis* and *L. amazonensis* induce distinct disease manifestations in infected patients. These findings are also in agreement with a previous report from our laboratory that *L. braziliensis* potently induces DC activation and cytokine production, while *L. amazonensis* interacts with DCs in a more silent manner.
- Initiating anti-neutrophil treatment in mice with established *L. amazonensis* infection exacerbates lesion progression without significantly impacting pathogen burden. Although the mechanism for this phenomenon is unclear at this time, it

appears that neutrophils play an important role in limiting disease severity during the chronic phase of leishmaniasis in an experimental animal model.

Overall, data presented in this dissertation help bridge a gap in our current understanding of the role of neutrophils in chronic leishmaniasis, and establish a foundation for future studies to determine whether modifying neutrophil functions can improve anti-parasite immunity and clinical leishmaniasis outcomes.

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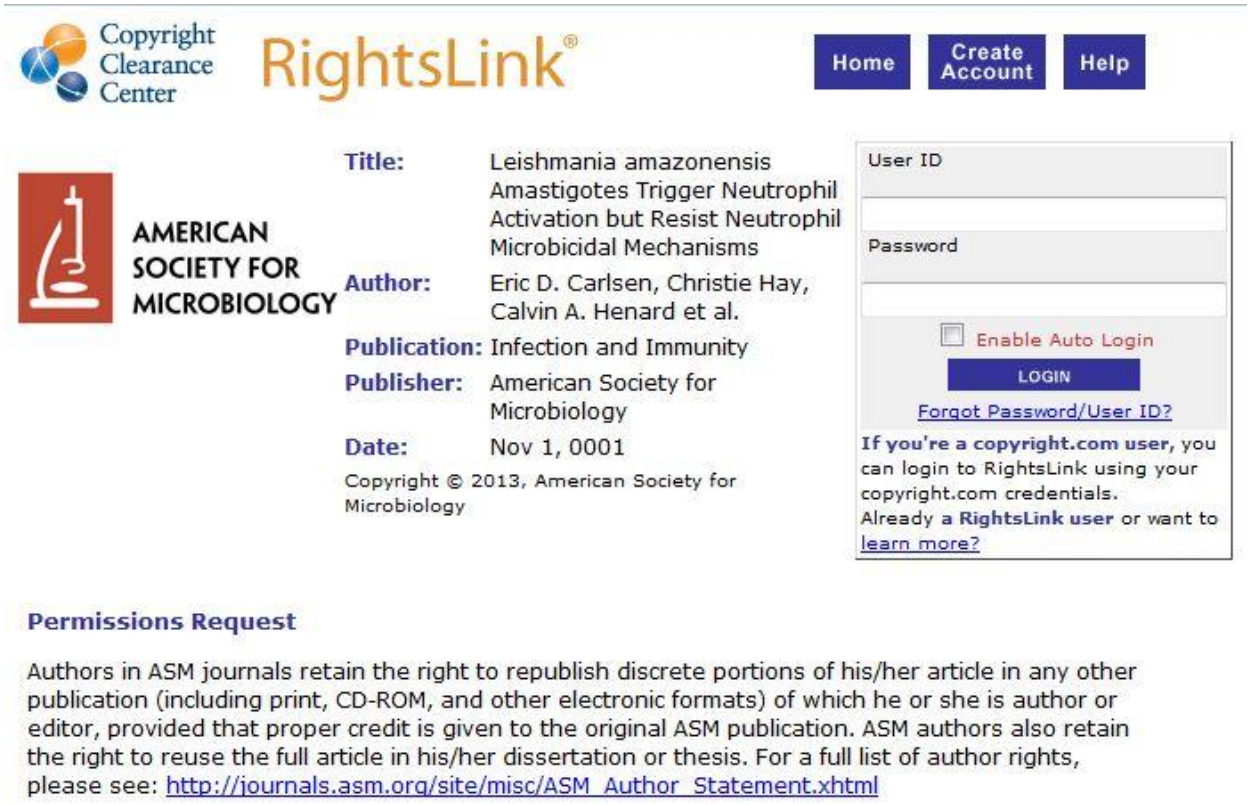

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Vita

Eric Donald Carlsen was born in Turnersville, NJ on January 12, 1986 to Wayne and Holly Carlsen. He completed his undergraduate work in Cellular and Molecular Biology at Ohio University in 2008. Eric became interested in tropical infectious diseases after working with the Ohio University Tropical Disease Institute Chagas Disease Control Program, which makes yearly visits to rural Ecuador to promote community awareness, improve epidemiological data through patient screening, and establish socioeconomic and ecological risk factors for people living in endemic areas. After finishing his Bachelor's degree, Eric joined the Combined MD-PhD Program at the University of Texas Medical Branch in 2008. In 2009, Eric received a Fogarty Travel Fellowship to assess chemokine receptor expression on circulating lymphocyte populations in leishmaniasis patients in Lima, Perú. While working in Lima, Eric worked closely with clinical faculty to learn more about leishmaniasis diagnosis and treatment options. This experience cemented his interest in neglected tropical disease as a topic for his dissertation.

After completing the first 2 years of medical school, Eric joined Dr. Lynn Soong's laboratory in 2010. Portions of his dissertation work were supported by a T32 Training Grant in Emerging and Infectious Tropical Disease (2010-2012) and by the McLaughlin Endowment Fund Pre-Doctoral Fellowship (2012-2013). The remainder of Eric's dissertation work was supported by the Blocker Scholar Fellowship in Biomedical Research (2013-2014). During his PhD studies, the Graduate School of Biomedical Sciences recognized Eric with the following awards: the David and Janet Niesel Scholarship Award (2010), the Robert Bennet Tuition Scholarship Award (2011), the John Stanton Scholarship (2013), the Arthur V. Simmang Academic Scholarship (2013), and the James W. McLaughlin Travel Award (2014). After

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EDUCATION

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PUBLICATIONS

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ABSTRACTS

Vargas-Inchaustegui DA, Espinosa D, **Carlsen E**, Tulliano G, Pacheco R, Llanos-Cuentas A, Arevalo J, and Soong L. The expression of inflammatory mediators and its association with the susceptibility to mucosal leishmaniasis in Peru. The IHII/McLaughlin Colloquium and at the National Student Research Forum (UTMB), Galveston, TX, 2009.

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