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The impact of vector mediated neutrophil recruitment on cutaneous leishmaniasis

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Summary

The dynamic process of pathogen transmission by the bite of an insect vector combines several biological processes that have undergone extensive co-evolution. Whereas the host response to an insect bite is only occasionally confronted with the parasitic pathogens that competent vectors might transmit, the transmitted parasites will always be confronted with the acute, wound healing response that is initiated by the bite itself. Invariably, this response involves neutrophils. In the case of *Leishmania*, infection is initiated in the skin following the bite of an infected sand fly, suggesting that *Leishmania* must possess some means to survive their early encounter with recruited neutrophils at the bite site. Here, we review the literature regarding the impact of neutrophils on the outcome of infection with *Leishmania,* with special attention to the role of the sand fly bite.

Introduction

Neutrophils are a key component of innate immunity and are essential for protection from bacterial infections due to their ability to recognize, phagocytose, and ultimately destroy these pathogenic organisms (Segal, 2005, Nauseef, 2007, Xu *et al.*, 2007, Weinrauch *et al.*, 2002). The importance of neutrophils is evidenced by uncontrolled dermal infections caused by group A *Streptocuccus* or *Staphylococcus aureus* in humans when neutrophil functions are impaired (Hidalgo-Grass *et al.*, 2006, Hidalgo-Grass *et al.*, 2004, Gilad *et al.*, 2006). The protective role of neutrophils is associated with rapid recruitment to sites of tissue damage and pathogen entry, and the subsequent clearance of these recruited neutrophils by macrophage/monocyte populations. Despite the neutrophil's potent anti-microbial arsenal, *Helicobacter pylori, Francisella tularensis,* and *Anaplasma phagocytophilum* have all evolved mechanisms to resist direct killing following phagocytosis (Allen *et al.*, 2007, Carlyon *et al.*, 2006). In addition, persistent neutrophil recruitment is associated with immuno-pathology and adverse outcomes, as observed during chronic conditions such as arthritis and gout (Jakus *et al.*, 2009, Eyles *et al.*, 2006, Cronstein *et al.*, 2006).

Recently, we reported on the dynamics of neutrophil recruitment to a site of *Leishmania* inoculation into the skin by the bite of an infected sand fly, the natural insect vector for this infectious, protozoan, parasite (Peters *et al.*, 2008). Numerous species of *Leishmania* exist, all of which share a digenetic lifecycle, alternating between the promastigote form in the sand fly and the obligate intracellular amastigote form in the mammalian host. The clinical outcomes of infection with different *Leishmania spp.* is highly diverse, ranging from self-limiting, cutaneous lesions following infection with *L. major*, to fatal, systemic involvement of the liver spleen, and bone marrow following infection with *L. donovani*. Regardless of the *Leishmania spp.*, parasite deposition in the skin coincides with tissue damage caused by the

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sand fly bite. The highly conserved, wound healing response involving neutrophils is therefore likely to influence the early establishment of infection, and both host protective and disease promoting roles for neutrophils have been reported (Ribeiro-Gomes *et al.*, 2004, Ribeiro-Gomes *et al.*, 2006, Tacchini-Cottier *et al.*, 2000, Chen *et al.*, 2005, van Zandbergen *et al.*, 2004, McFarlane *et al.*, 2008, Laskay *et al.*, 2008, Peters *et al.*, 2008). Here, we incorporate the influence of sand fly bite on current ideas regarding the role of neutrophils in Leishmaniasis.

Protective and Non-protective Roles for Neutrophils during in-vivo infection with *Leishmania*

Early experimental observations of *Leishmania*-neutrophil interactions during acute disease *in vivo* employed histological analysis of infected skin lesions and indicated both the early presence of neutrophils at sites of needle inoculation, and the presence of parasites inside neutrophil phagosomes. This was observed for *L mexicana, L. donovani,* and *L. chagasi* (Andrade *et al.*, 1984, Grimaldi *et al.*, 1984, Pimenta *et al.*, 1987, Wilson *et al.*, 1987, Laurenti *et al.*, 1996). Although the presence of degraded parasites within neutrophils was interpreted to indicate neutrophil-mediated killing, this analysis was not able to discriminate between phagocytosis of damaged or dead parasites and direct killing of viable organisms, an interpretation further complicated by the use of large doses of heterogeneous parasites. Early *in-vitro* observations employing human neutrophils indicated that *L. donovani* promastigotes or amastigotes were killed within hours of phagocytosis in an oxygen free-radical dependent manner (Chang, 1981, Pearson *et al.*, 1981). In contrast, acid phosphatases from *L. donovani* and *L. major* were shown to confer resistance to lysosome-derived toxic oxygen metabolites and to inhibit their production by neutrophils, suggesting these *Leishmania* strains have evolved mechanisms to avoid neutrophil killing (Remaley *et al.*, 1984, el-On *et al.*, 1990, al Tuwaijri *et al.*, 1990).

Subsequent observations on the impact of *Leishmania*-neutrophil interactions have been interpreted within the context of strains of mice that are resistant or susceptible to infection with *L. major*. In BALB/c mice, which do not resolve parasitic lesions and eventually succumb to disseminated disease, neutrophils were found to dominate the early cellular infiltrate following s.c. needle inoculation of 2×10^7 stationary phase promastigotes, and importantly were maintained over the first 12 days of infection (Beil *et al.*, 1992, Sunderkotter *et al.*, 1993). Furthermore, depletion of neutrophils at the time of *L. major* challenge in BALB/c mice inhibited the IL-4 response and promoted partial resistance (Tacchini-Cottier *et al.*, 2000). In contrast, neutrophil recruitment in resistant C57Bl/6 mice was shown to be short-lived, dropping to baseline levels by day 3 (Beil *et al.*, 1992). Furthermore, the transient depletion of neutrophils at the time of s.c. footpad challenge with *L. major* in resistant mice enhanced early parasite growth and lesion pathology, though in each case these mice eventually healed (Lima *et al.*, 1998, Tacchini-Cottier *et al.*, 2000, Ribeiro-Gomes *et al.*, 2004, Chen *et al.*, 2005). Interestingly, the mouse strain differences in the kinetics of neutrophil recruitment and maintenance in the *L. major* injection site was also shown to occur within non-immune granulomas initiated using inert particles (Belkaid *et al.*, 2000), suggesting that inherent differences in acute inflammation influence the subsequent Th1/Th2 balance that controls susceptibility and resistance to *L. major*. This notion has been extended to include intrinsic differences in the neutrophils themselves. Specifically, whereas co-injection of dead, syngeneic neutrophils exacerbated *L. major* growth in BALB/c mice, a similar injection of dead, syngeneic neutrophils into C57Bl/6 mice enhanced parasite killing (Ribeiro-Gomes *et al.*, 2004). And whereas BALB/c neutrophils elicted the production of PGE_2 and TGF-β by macrophages, C57Bl/6 macrophages were activated by syngeneic neutrophils to produce TNF-α. More recently, Charmoy et al. (Charmoy *et al.*, 2007) reported distinct neutrophil phenotypes responding to i.p. injection of *L. major* in resistant

and susceptible mice that differed in expression of cell surface integrins, TLRs, and secreted cytokines. The production of IL-12p70 by *L. major* activated neutrophils from C57Bl/6 mice, as compared to the secretion of inhibitory IL-12-p40 homodimers by *L. major* activated BALB/c neutrophils, seems an especially relevant finding that could account, at least in part, for the subsequent Th1 vs Th2 polarization that occurs in these mice.

As strong as these various associations between the innate neutrophilic response to *L. major* and infection outcome appear to be, these observations must contend with a number of reports in which quite opposite conclusions were drawn. Both Lima et al. (Lima *et al.*, 1998) and Chen et al., (Chen *et al.*, 2005) reported exacerbation of footpad lesion development in BALB/c mice treated with a neutrophil depleting antibody, and more recently, our own studies have documented a role for neutrophils in promoting sand fly transmitted infections in C57BL/6 mice (Peters *et al.*, 2008). Differences in the specificity and regimen of the depleting antibody, the strain, dose, and inoculation site of the *L. major* parasites, might all account for these discrepant results, and addressing some of these differences in a series of parallel experiments will be needed to reconcile these prior findings.

We would, in the meantime, argue that the role of neutrophils in the pathogenesis of cutaneous leishmaniasis and in modulation of host immunity is most appropriately studied in the context of infection in the skin initiated by the bite of an infected sand fly. In this setting, parasites deposited into the skin of neutrophil-depleted, C57BL/6 mice were only half as successful at establishing infection as the parasites transmitted to neutrophil sufficient mice. The enhanced host resistance was associated with an increase in pro-inflammatory cytokines (Peters *et al.*, 2008). While both needle and sand fly inoculation of parasites induced acute neutrophil recruitment in C57BL/6 mice, an intriguing aspect of sand fly inoculation was the persistence of neutrophils at the bite site, and this occurred following exposure to both infected and uninfected flies (Peters, et. al. 2009 PLoS Pathogens *accepted for publication*, (Peters *et al.*, 2008). Thus, the acute neutrophilic response following sand fly bite or needle inoculation is likely part of the host defense mechanism responsible for wound repair and sterilization. For example, a dynamic infiltration of neutrophils to localized sites of laser-induced brain injury has been observed (Kim *et al.*, 2006), suggesting endogenous factors released during tissue damage can drive this response. In contrast, the mechanism(s) of neutrophil maintenance at bite sites is likely mediated by sand fly derived factors that either mimic a tissue damage signal or activate an additional chemokine/chemokine receptor pathway (Teixeira *et al.*, 2005). Thus, the vigorous and neutrophilic host response to sand fly bite has likely influenced the evolution of *Leishmania* towards counter acting and even exploiting the presence of these cells.

The Influence of Apoptotic Neutrophils on the Initiation of Infection with Leishmania major

In protozoan infections, macrophage killing of *Trypanosoma cruzi* was shown to be inhibited by exposure to apoptotic, but not necrotic, T cells, and was one of the first studies to suggest that apoptotic cells might play an important role in promoting infection (Freire-de-Lima *et al.*, 2000). The suppressive influence of apoptotic cells on macrophage function was extended to *L. major* following the observation that human neutrophils efficiently phagocytosed *L. majorin vitro*, and that the parasite was capable of surviving within these cells (Laufs *et al.*, 2002, Aga *et al.*, 2002). Moreover, the infected neutrophils acquired an apoptotic phenotype but remained intact (van Zandbergen *et al.*, 2004). Additional *in vitro* observations gave rise to the “Trojan Horse” model of infection, whereby macrophages acquire *L. major* by phagocytosing infected, apoptotic, neutrophils (van Zandbergen *et al.*, 2004, Laskay *et al.*, 2003). Our own observations employing flow-cytometry and 2 photon-intra vital microscopy (2P-IVM) following needle or sand fly inoculation of RFP expressing

L. major showed that the majority of parasites were phagocytosed by neutrophils during acute infection without being killed, followed by a transition of the RFP signal into macrophage/monocytes (Peters *et al.*, 2008). While ourin vivo imaging failed to definitively capture this transition in real time, and in fact revealed release of parasites from stationary neutrophils that appeared to be undergoing apoptosis in the site, the clearance of these apoptotic bodies along with the large number of apoptotic, non-infected neutrophils in the localized bite site, would still be expected to modulate the function of infected macrophages in the site.

The cell contact-dependent induction of the non-inflammatory cytokines TGF-β and PGE_2 by macrophages following interactions with apoptotic neutrophils potentially facilitates both the 'silent entry' of parasites following uptake of infected neutrophils, and the silencing of infected macrophages that co-ingest non-infected by-stander neutrophils (Lucas *et al.*, 2006, van Zandbergen *et al.*, 2007). Observations by Ribiero-Gomes et. al., involving cell-contact dependent interactions of macrophages with uninfected, thioglycolate elicited neutrophils containing a proportion of apoptotic cells, support a model in which Fas ligand-dependent neutrophil apoptosis drives acute infection in BALB/c mice (Ribeiro-Gomes *et al.*, 2004, Ribeiro-Gomes *et al.*, 2005, van Zandbergen *etal.*, 2007). In contrast, the authors reported a cell-contact independent, protective, role for apoptotic neutrophils in C57BL/6 mice under the same conditions, which was later shown to be dependent upon TLR4 recognition of secreted neutrophil elastase (NE) (Ribeiro-Gomes *et al.*, 2007). Recently, Afonso et. al. (Afonso *et al.*, 2008)reported that induction of NE dependent killing of *L. amazonensis* by human macrophages occurred following exposure to necrotic, but not apoptotic, neutrophils. Therefore, one potential explanation for contact dependent *enhancement* of infection by neutrophils in BALB/c mice versus the contact independent *reduction* of infection in B6 mice is the propensity of thioglycolate neutrophils from these two strains to become necrotic under similar conditions. We would again argue that the most relevant population of neutrophils are those present in the dermis following transmission by sand fly bite, since these neutrophils will have undergone the natural exposure to the inflammatory mediators involved in their recruitment, extravasation and interstitial migration in the dermis, and may therefore be unique in their apoptotic vs necrotic cell death programs. Furthermore, a proportion of these neutrophils will have undergone a degree of modulation following parasite phagocytosis (Aga *et al.*, 2002, van Zandbergen *et al.*, 2004). The opposing outcomes of high-dose, sub-cutaneous infection of the footpad versus sand fly transmitted infection of the dermis in neutrophil depleted C57BL/6 mice may be the degree to which these two routes induce populations of either necrotic versus apoptotic neutrophils, or neutrophils secreting elastase.

The modulation of macrophage functions by apoptotic neutrophils likely contributes to the establishment of the early, 'silent,' phase of disease (Belkaid *et al.*, 2000), characterized by a remarkable avoidance of a host protective Th1 response during a prolonged phase (4–5 wks) of *L. major* amplification in the skin. As immature dendritic cells (DC) might also be expected to participate in the clearance ofapoptotic cells, their encounter with infected and/or uninfected neutrophils in the skin might have an especially important bearing on the delayed onset of T cell priming that is observed during *L. major* infection. There is ample evidence that the clearance of apoptotic cells by DC is critical for the maintenance of peripheral tolerance (Steinman *et al.*, 2000). Immature DCs are capable of extensive phagocytosis, and DC maturation can be inhibited by the engulfment of apoptotic cells, resulting in reducede xpression of costimulatory molecules CD40, CD80, andCD86, impaired allogeneic T cell responses, and down-regulation of LPS-driven interleukin-12 production(Stuart *et al.*, 2002, Clayton *et al.*, 2003) Neutrophil elastase has been shown to convert human immature DCs into TGF-beta secreting cells that modulate their immunostimulatory capacity (Maffia *et al.*, 2007). With respect to *Leishmania*, 2P-IVM

confirmed that within minutes after injection, at least some parasites were directly incorporated into the phagocytic vacuoles of dermal DCs (Ng *et al.*, 2008). Our own studies have indicated, nonetheless, that the majority of the infected DCs recovered from the skin after 24 hr contain granulocyte markers, suggesting that their early encounter with *L. major* occurs in the context of parasitized neutrophils (Ribeiro-Gomes, F.L., et al., unpublished). Furthermore, we have observed that the DCs recovered following their uptake of infected neutrophils *in vivo* or *in vitro* are incapable of cross-priming, or of activating *Leishmania*-specific CD4+ and CD8+ memory cells. The compromised function of DC with respect to the expression of secondary responses at the site of infected sand fly bite may be especially relevant to the failure to date of *Leishmania* vaccines to confer significant protection in humans against natural challenge. We have recently found that depletion of persistent neutrophils during an ongoing secondary response in vaccinated mice promoted the efficacy of a killed vaccine against *L. major* transmitted by sand fly bite (Peters, et. al. 2009 PLoS Pathogens *accepted for publication*). In this setting, neutrophil depletion was associated with an increase in the frequency of IFN-γ and TNF-α producing CD4+ T cells specific for *L. major* at the site of infection in the skin. These observations suggest that sand fly bite mediated neutrophil persistence at a site of parasite deposition inhibits effector function beyond the down-modulatory influence of neutrophils at the initial stage of infection.

It is important to note that neutrophils elicited in response to other infections, e.g. *Toxoplasma gondii*, BCG, and *Candida albicans*, have been reported to deliver activation signals to DC and to promote Th1 cell-mediated responses (Bennouna *et al.*, 2003, Morel *et al.*, 2008, Megiovanni *et al.*, 2006). These organisms appear to possess stimuli, absent in *Leishmania*, that activate neutrophils to produce type I inflammatory cytokines and chemokines that instruct recruitment and activation of DC during infection. The cross talk between neutrophils and DC might be further influenced by differences in the apoptotic programs that are induced by these infections in distinct inflammatory settings. Live, non-apoptotic neutrophils appeared to be involved in the activation of DC capable of priming T cell responses to BCGand *C. albicans*. By contrast, the suppression of macrophage (van Zandbergen *et al.*, 2004)and DC functions (Ribeiro-Gomes, F.L., et. al. unpublished) following *L. major* infection was clearly associated with apopotic neutrophils, which at least in the case of the infected neturophils recovered from the *L. major* loaded dermis, were accelerated in their apoptotic program.

Conclusion

Examination of the earliest events following transmission of *Leishmania major* by infected sand flies reveals that neutrophils are recruited to the site of sand fly bite and phagocytose deposited parasites. This process appears to be critical for disease progression since parasites transmitted in the absence of neutrophils are less likely to establish infection. (Peters *et al.*, 2008). In addition, the chronic maintenance of neutrophils appears to compromise the expression of adaptive immunity (Peters, et. al. 2009 PLoS Pathogens *accepted for publication*). These findings and others (van Zandbergen *et al.*, 2007, Laskay *et al.*, 2008), suggest that neutrophils play a prominent role in promoting the pathogenesis of cutaneous leishmaniasis, see Figure 1. While other studies suggest that in different settings, and under different experimental conditions, neutrophils are able to promote resistance, we would emphasize that those models which best replicate the natural route of infection are more likely to reveal the true nature, friend or foe, of the neutrophil in the clinical outcome of infection with *Leishmania.*

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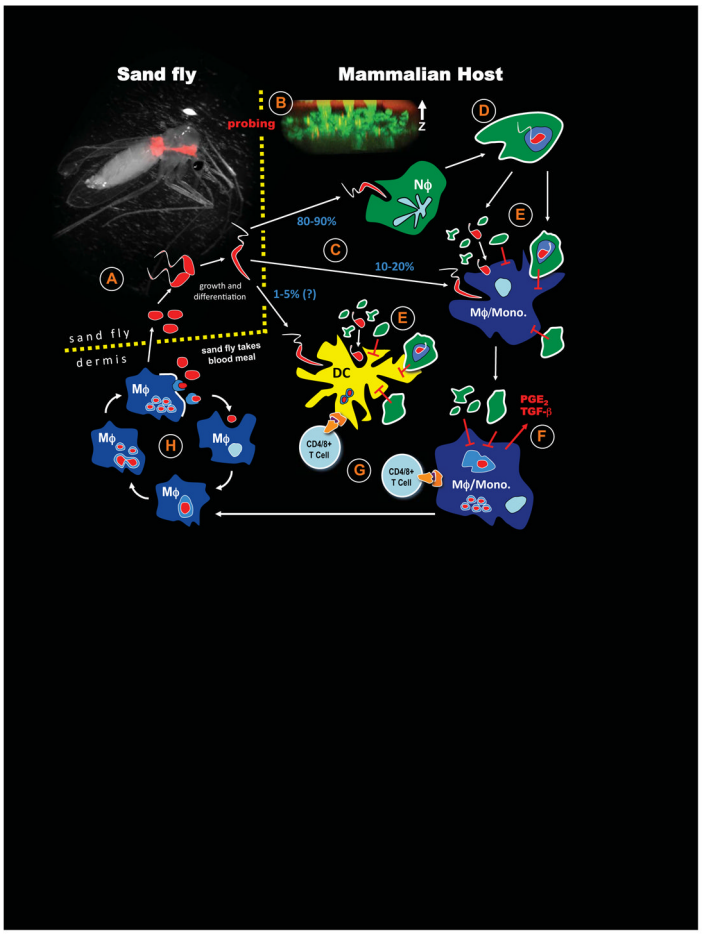

Figure 1.

Life cycle of *L. major* incorporating the influence of sand fly bite and the role of neutrophils in the pathogenesis of disease. (A) *Phlebotomine spp.* of sand flies take a blood meal from an infected individual, acquiring parasites in the process. Intracellular amastigotes undergo transformation, growth and differentiation in the sand fly midgut, eventually becoming metacyclic promastogites positioned in the anterior gut. The picture is of *P. dubosqi* infected with RFP-expressing *L. major.* (B) In search of a second blood meal, sand flies again probe the skin of a mammalian host, creating a hemorrhagic pool from which to feed while simultaneously depositing parasites into the dermis and epidermis. Tissue damage caused by sand fly probing induces highly localized neutrophil recruitment and the formation of neutrophil 'plugs' in the regions of skin punctured by the sand fly proboscis. The picture is of *L.m.*-RFP metacyclic promastigotes deposited among the Lys-GFP^{hi} neutrophils, and neutrophil plugs extending up through the epidermis and stratum corneum. (C) Neutrophils dominate in sites of tissue damage following sand fly probing and phagocytose the majority (80–90%) of parasites. Smaller numbers of parasites also gain direct access to macrophages/monocytes (10–20%). A few parasites may gain direct access to resident dermal dendritic cells (1–5%). (D) Infected neutrophils in the skin fail to kill *L.m.*, and the parasitized cells acquire apoptotic markers such as phosphatidylserine (indicated by a white cell surface). (E) At later time points (1–2 days), parasites begin to transition out of neutrophils into macrophage/monocytes and DCs. Parasite acquisition by these phagocytes can occur via uptake of released, viable organisms from infected neutrophils, or via uptake of parasitized neutrophils, i.e. the 'Trojan Horse' route. Infected macrophage/monocyte and DC

populations are also exposed to apoptotic bodies and large numbers of uninfected, apoptotic neutrophils that will down-modulate both innate mechanisms of parasite killing and APC functions. (F) During the ‘silent’ phase of parasite growth, neutrophils are persistently recruited to and maintained in the bite site by sand fly derived factors, and parasite killing and APC functions will continue to be suppressed, associated with production of non-inflammatory cytokines PGE_2 and TGF-β. (G) Parasites propagate in inflammatory, monocyte –derived cells in the skin, and increase to sufficient numbers so that they are eventually taken up by immunocompetent DCs in the skin or draining lymph node, initiating T cell priming and the healing phase of disease.