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Review Article

Innate Immunity Plays a Key Role in *Leishmania* Infection: Implications for Vaccine Design

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Neutrophils are part of the first line of immune response and are essential for resistance against a variety of pathogens. They professionally mediate direct killing of pathogens, recruit other phagocytes by specific chemokines, produce cytokines and interact with different immune cells to shape the adaptive response. *Leishmania* as an obligatory intracellular parasite has evolved to benefit this early innate response to find its way into macrophages, the final host cells. Therefore it is important to reconsider the role of neutrophils for further improvement of the current vaccine status. *J Med Microbiol Infec Dis, 2016, 4 (3-4): 39-44*.

Keywords: Innate immunity, Neutrophil, *Leishmania*, Vaccine.

INTRODUCTION

Leishmaniasis is a vector-borne disease mainly affecting Asia, Africa, South America, and the Mediterranean basin. The unicellular parasite from *Leishmania* genus inhabits some natural reservoirs and is transmitted to human by infected sandfly vectors. The result of infection in human as host is multifactorial and ranges from self-healing local cutaneous to disfiguring mucocutaneous or lethal visceral leishmaniasis. Sandfly saliva-derived proteins and microbiota [1], parasite species and associated viruses and most importantly competency of host immune system together with host microbiome [2], are factors that determine the infection outcome. The resolution, in general, is Th1 cellular immune response mediated, and Th2 response promotes disease establishment. There are several pieces of evidence indicating that innate immune response and in particular neutrophils as sentinels play a critical role in Th1/Th2 polarization early after infection. Therefore it is evident that for efficient vaccine development, the innate immune response must be considered together with adaptive immunity.

Neutrophils are sentinels of the innate immune response against invaders

Polymorphonuclear leukocytes also known as neutrophils, are the most abundant circulating human blood leukocytes (50-70%) that accumulate in the inflammation site very rapidly before any other cell type. They originate from the same precursors as mononuclear phagocytes in bone marrow in a constitutive manner. Neutrophils are short-lived cells that are programmed to die by apoptosis within a few hours. This is necessary to regulate their functions tightly. These cells are well equipped to fight against bacterial and fungal infections and are responsible for wound healing after sterilization of the site. They harbor diverse types of granules with exclusive roles within the cytoplasm [3]. Azurophilic or primary granules contain Myeloperoxidase and Serin proteases, and specific or secondary granules contain mostly antimicrobial peptides as LL-37, defencins, lactoferrin and pro-cathelicidin. These granules are especially involved in the direct killing of invaders. The rest of granules including tertiary granules which are filled with matrix metalloproteinase 9 and secretory vesicles containing receptors required for cell adhesion (like integrins) are involved in digestion of extracellular matrix and extravasation of neutrophils at the inflamed site respectively [4]. Evidently, inappropriate activation of neutrophils may lead to tremendous tissue damage during an autoimmune or uncontrolled inflammatory response [5].

Early after infection and/or tissue damage, pattern recognition receptors (PRRs) on tissue resident macrophages, fibroblasts, keratinocytes and endothelial cells effectively sense the pathogen-associated molecular patterns (PAMPs) and/or the danger-associated molecular patterns (DAMPs) and produce neutrophil-attracting chemokines which are IL-8 in human and CXCL1 and CXCL2 in mice [6-9]. These chemokines signal via CXCR2 to activate neutrophils and promote their adhesion to the endothelial cells. Activated neutrophils firmly adhere to the endothelium and extravasate at inflamed site. Neutrophils express CD11b (complement receptor for C3b/iC3b components) also immunoglobulin Fc-γ receptors for phagocytosis of opsonized microbes.

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After phagocytosis, microbes are killed by direct killing mechanisms. Reactive oxygen species (ROS) produced by membrane-associated NADPH oxidase [10], the fusion of primary and secondary granules including proteases and antimicrobials with phagosomes [11] and even degranulation into the periphery, directly kills the pathogen. Furthermore different signals such as ROS mediate nuclear chromatin release from these cells together with some granule contents as LL-37, defencins and neutrophil elastase during NETosis (Neutrophil Extracellular Traps) [12, 13]. Primarily, NETs entrap and block further dissemination of the pathogens in addition to killing some pathogens and facilitating phagocytosis by other phagocytes [4, 14]. Neutrophils can play an additional role which is orchestrating the adaptive immunity [15]. Neutrophil-derived/induced chemoattractants recruit immature dendritic cells (iDCs) to the inflammation site [16]. Human and mouse studies suggest that neutrophils directly interact with dendritic cells by Mac-1/DC-SIGN engagement to activate iDCs [17]. Mouse neutrophils can transport peripheral antigens to draining lymph nodes to prime antigen-specific Th1, Th17 and $CD8^+$ T cells [18-20]. IL-18 produced by neutrophils jointly with IL-12 produced by dendritic cells also activates natural killer cells. Human neutrophils release IFN-γ for macrophage activation and T cell differentiation [21].

The programmed cell death in neutrophils could be delayed by different signals such as pro-inflammatory cytokines and pathogens [22]. Macrophage recruitment by neutrophil-secreted chemokine including antimicrobial peptides and MIP-1 and reciprocal interaction between these two cells induce apoptosis even after days of survival [23, 24]. Apoptotic neutrophils are removed by macrophages and dendritic cells (efferocytosis) to avoid further tissue damage by hazardous components of neutrophils [25]. Eventually, neutrophil levels return to their baselines.

Leishmania parasites have evolved to advantage early innate response of neutrophils

Leishmania parasites are obligatory intracellular pathogens transmitted via sandfly bite and do not survive out of the host cells after deposition in the skin. Therefore upon transmission, the parasite benefits a massive recruitment of neutrophils to sandfly bite site instead of stopping neutrophil accumulation. Tissue injury caused by sandfly bite is sufficient to recruit neutrophils. However, the sandfly saliva [26] together with parasite factors including promastigote secretory gel (PSG) [27] and *Leishmania* chemotactic factor (LCF) [28] in the inoculum, augment neutrophil recruitment within first 30-45 min post infection. Although many others had previously demonstrated the early accumulation of neutrophils, Peters *et al.*, were the first group to image the massive and sustained recruitment of neutrophils *in vivo* (detectable up to 8 days) after sandfly deposition of the parasite in the ear dermis by using 2Photon Intravital Microscopy [29]. According to their findings, the neutrophils uptake 80-90% of inoculated parasites compared to 10-20% engulfed by macrophages [30]. LPG and GP63 surface molecules are well-known targets for C3b-component of alternative complement pathway. Surface receptors for complement components enable neutrophils to efficiently uptake opsonized parasites [31]. Meanwhile, LPG [32] and GP63 [33] molecules block further deposition of membrane attack complex (MAC) components of complement pathways on C3b and resist direct complement killing this way.

Leishmania parasites have evolved to survive in the hostile environment inside neutrophils by different evading mechanisms [34]. Some parasites like *Leishmania major* and *Leishmania donovani* can inhibit toxic oxygen metabolite production by acid phosphatases [35, 36]. Laskey *et al.* have recently proposed that *L. major* parasites can survive within human neutrophils because ingestion of uninfected apoptotic neutrophils by infected neutrophils inhibits ROS production [37]. This is entirely reasonable since high numbers of neutrophils are present at the infection site. Even uptake of apoptotic promastigotes in the inoculum can suppress further ROS production [38]. *Leishmania* parasites are also able to block fusion of the neutrophil granules with phagosome which is partly explained by surface LPG [39]. Many different species of *Leishmania* also induce NETosis such as *L. major* and *Leishmania amazonensis* [40], *L. donovani*, *Leishmania infantum* and *Leishmania mexicana*. However, several species resist NET killing by structural hindrance of surface LPG like *L. donovani* [41], by parasite-derived 3'-nucleotidase/nuclease activity like *L. infantum* [42] and by an unknown mechanism like *L. mexicana* [43]. Furthermore, a recent work by Chagas *et al.* has identified a novel NET-destroying endonuclease (Lundep) in *Lutzomyia longipalpis* (*L. longipalpis*) saliva which promotes survival against the leishmanicidal activity of NET [44].

Leishmania parasites delay the apoptosis of neutrophils *in vitro* [45, 46]; however, controversial results have been obtained by *ex vivo* experiments: once engulfed, surviving parasites accelerate neutrophil apoptosis [47]. Neutrophil apoptosis is further facilitated by sandfly saliva components [48]. Sandfly components also augment macrophage chemoattractant production by neutrophils [48]. Recruited macrophages, first encounter apoptotic neutrophils [22]. *Leishmania* parasites then translocate into macrophages by different mechanisms. In "Trojan Horse" model macrophages directly engulf apoptotic bodies of infected neutrophils [49]. In "Trojan Rabbit" model free parasites are ingested along with neutrophil apoptotic bodies [50]. Parasites can even be released from neutrophils to enter macrophages this time even more infectious than other pathways [22] and evade macrophage killing mechanisms. Evidently, apoptotic body ingestion by both macrophages and dendritic cells [51], known as "silent entry," has an adverse effect on activation of these cells and compromises appropriate adaptive immune response polarization by decreasing IL-12 and increasing TGF-β production [52]. Eventually, the parasite propagates in macrophages as host, however depending on some host factors including Neutrophil Elastase (NE), Toll-Like Receptor-4 (TLR-4) on macrophages and Tumor Necrosis Factor (TNF), the fate of the disease could be determined. Thus massive neutrophil infiltration induced by sandfly saliva is exploited by

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Leishmania to impair the early reaction of macrophages and dendritic cells and to delay cellular immune responses [22]. This might be the reason why vaccines against *Leishmania* fail to protect against natural infection [30].

A robust effector response is crucial to control early inflammatory response after parasite deposition by sandfly

Employing 2Photon Intravital Microscopy to record the early *in vivo* events, massive recruitment of neutrophils to mouse ear epidermis within a few minutes after an intradermal challenge by *L. major* infected sand fly was confirmed [29, 53]. Interestingly neutrophil depletion before needle and sandfly challenge dramatically reduces the parasite number per ear [54]. These observations were then used to explain why leishmanization remains the most efficient vaccine formulation so far. After leishmanization, healed mice are effectively protected against secondary sandfly challenge. Instead, neither ALM-CpG [53] nor polyprotein-GLA-SE vaccines (KSAC-GLA-SE or LeishF110-GLA-SE) [55] match the potency of leishmanization in protecting against sandfly challenge while protective against needle challenge. As investigated by Peters *et al.*, the key differences between healed and ALM or polyprotein vaccinated mice are attributable first to a robust immune response early after sandfly challenge mediated by $CD3^+CD4^+$ T cells and then to a higher IFN-γ/IL-17 ratio leading to low numbers of neutrophils 4 weeks post infection. Of particular note, neutrophil depletion after sandfly challenge in ALM-CpG vaccinated mice enhances the protective effect, comparable to healed mice, by increasing IFN-γ producing $CD4^+$ T cells [53]. This means that only a rapid and robust immune response soon after a sandfly infective bite can control the local immunomodulatory conditions at early time points post-infection when neutrophils have accumulated at the site.

Further investigation revealed that parasite persistence after healing is the key to leishmanization success. Persistent parasites after healing, maintain an effector and not memory population of $CD4^+$ T cells that produce IFN-γ and are characterized by high levels of Ly6C and t-bet molecules. These cells are introduced as the primary correlates of the immunity conferred by leishmanization since they infiltrate the bite site very rapidly after sandfly probing (opposite of a memory profile). They are short-lived in the absence of antigen and disappear as soon as the antigen level drops [56] (a common characteristic of non-living vaccines like ALM or polyproteins). They potentially modulate the inflammatory milieu in favor of Th1 response early after infection.

Immunity to sandfly saliva plays a major role in disease control

Together with the parasite, sand flies co-inject salivary components that facilitate getting a blood meal [57]. Further investigation demonstrated that co-inoculation of sand fly saliva and *Leishmania* exacerbates the disease and increases parasite load [58-61]. However pre-exposure to salivary gland homogenates (SGH) [59], bites of uninfected sand flies [62] or even vaccination with specific salivary molecules, either alone [26, 63-67] or in combination with parasite immunogenic proteins [68, 69], potentially protects against infectious challenge. Also, in endemic areas, anti-saliva immunity coincides with delayed-type hypersensitivity (DTH) response against *Leishmania* antigens [70-72]. Altogether these data raised the concept that immune response against sandfly saliva confers a long term protection against *Leishmania*. In 2007, a group of healthy volunteers was experimentally exposed to uninfected (*L. longipalpis*) bites and high levels of IgG1, IgG4 and IFN-γ were detected. One year after exposure, recall responses efficiently produced IFN-γ [73]. Furthermore, Valenzuela *et al.* demonstrated that immunization with DNA encoding *Phlebotomus papatasi* (*P. papatasi*) SP15 (PpSP15) protein protects against infectious challenge three-month post-immunization [26].

There are two alternatives to explain this fact. First, immunization with saliva neutralizes exacerbating factors. These could be mainly chemoattractants that recruit neutrophils to the sandfly probing site. Previously, components of *L. longipalpis* [74] and *L. intermedia* [75] saliva were demonstrated to induce a rapid recruitment of neutrophils in Balb/c model. Recently, C57BL/6 neutrophil migration towards salivary glands of the *P. duboscqi* and *L. longipalpis in vitro* was illustrated which was compromised by Proteinase K treatment indicating the protein nature of recruiting factors [76]. In this respect, pre-treatment with SGH from *L. longipalpis* [77] or *P. papatasi* [78] reduced neutrophil recruitment by different mechanisms. Alternatively, cellular anti-saliva immunity provides a DTH environment that controls early inflammatory events at infection site [79]. Oliveira *et al*. have correlated this with early recruitment of lymphocytes and IFN-γ/IL-12 production within 2 hours after the bite [80]. Biopsies taken at the site of a DTH response 48 hours after experimental bites were dominated by lymphocytes, macrophages and high levels of IFN-γ indicative of a Th1 response [81]. In another word, Th1 response against sandfly saliva promotes a Th1 response against *Leishmania*.

Concluding remarks: implications for vaccine development

As explained so far, *Leishmania* parasites are able to benefit neutrophils and bypass their hostile environment to establish a progressive disease. In this respect, sandfly saliva plays a crucial role in the massive recruitment of neutrophils, apoptosis induction, and NET disruption to support parasites' moving to their final destination in macrophages. The silent entry shuts down all leishmanicidal activities of macrophages and dendritic cells and eventually suppresses Th1 deviation. This could be one possible explanation why vaccine formulations with promising results in experimental needle challenge models fail in the field to protect against sandfly challenge. This point has drawn full attraction to re-think about the role of neutrophils in vaccine design against leishmaniasis. On one side, the humoral immune response could be raised against defined neutrophil chemoattractants in saliva to restrict massive recruitment of neutrophils. On the other side, multiple proteins like PpSP15 in *P. papatasi* or its counterparts in other sandfly species, used alone or in

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combination with immunogenic proteins of parasite, could be advantaged in DNA or dendritic cell context (vaccine modalities with more sustained antigen production and presentation) to promote long-lasting cellular immune response that rapidly colonize at the sandfly bite site. This reaction could confer protection by modulating the environment for Th1 deviation and parasite killing following sandfly challenge. Then there is an urgent need to further characterize immunogenic proteins in different sandfly and different *Leishmania* species in each endemic country and also define the saliva chemotactic factors of each sandfly species. These might promisingly lead us one step forward in improving vaccines against leishmaniasis although we still need to fully understand the role of the innate immune system in *Leishmania* infection and the function of other innate cells in the skin including γδ-T cells and innate lymphoid Cells (ILCs) and even the skin related microbiota. We should keep in mind that the parasite species and the experimental models used are important factors to be considered in every vaccine concept.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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