Review Article

Subversion of Immunity by *Leishmania amazonensis* Parasites: Possible Role of Phosphatidylserine as a Main Regulator

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*Leishmania amazonensis* parasites cause progressive disease in most inbred mouse strains and are associated with the development of diffuse cutaneous leishmaniasis in humans. The poor activation of an effective cellular response is correlated with the ability of these parasites to infect mononuclear phagocytic cells without triggering their activation or actively suppressing innate responses of these cells. Here we discuss the possible role of phosphatidylserine exposure by these parasites as a main regulator of the mechanism underlying subversion of the immune system at different steps during the infection.

1. *Leishmania* Parasites

*Leishmania* parasites are heteroxenous kinetoplastid protozoan organisms, which undergo complete differentiation upon a cycle of proliferation/differentiation in the midgut of phlebotomine sand flies followed by the transmission of infective metacyclic promastigotes [1, 2] to mammalian hosts during the insect blood meal. Once infecting mammalian hosts, these organisms, from free-living protozoa, become obligate intracellular parasites, residing and proliferating inside phagolysosomes of mononuclear phagocytic cells as amastigote forms.

In humans, *Leishmania* parasites can cause a broad spectrum of clinical manifestations from mild, self-resolving skin diseases to potentially fatal, disseminated visceral diseases. The outcome of the infection is dependent on multiple, interdependent factors, such as vector species, parasite species and strain, genetic background, and immunological status of the host. There are two main groups of parasites, stratified upon the clinical outcome of the infection: the ones capable of causing tegumentar and the ones capable of causing visceral diseases. In both cases, disease is initiated by the bite of an infected sand fly, followed by the generation of a skin lesion, mainly caused by the inflammatory response induced on that site. In some cases the disease is confined to the skin or mucosal tissues, and is termed cutaneous (CL) or mucocutaneous (MCL) leishmaniasis, respectively. In addition, diffuse cutaneous leishmaniasis (DCL) occurs when the parasite disseminates causing the appearance of multiple skin lesions, in distal sites relative to the transmission site [3]. In a similar way, in visceral leishmaniasis, there is parasite dissemination through blood and lymphatic vessels from the initial lesion site. However, these parasites establish in organs that comprise important populations of mononuclear phagocytes, such as bone marrow, spleen, and liver [4]. Among the clinical manifestations observed in humans with the tegumentary disease, diffuse and mucocutaneous leishmaniasis are the most severe forms. In both cases, most patients were found in the South and Central America, associated with *L. amazonensis* infection for DCL and *L. braziliensis* infection for MCL.
1.1. Diffuse Cutaneous Leishmaniasis. Diffuse cutaneous leishmaniasis (DCL) is a rare clinical manifestation and is characterized by the appearance of several nonulcerated nodular skin lesions, uncontrolled parasite proliferation, and resistance to most therapeutic strategies [5, 6]. The lesions are characterized by a dense dermal infiltrate of vacuolated macrophages heavily parasitized. The intense parasitism in the DCL lesions reflects the functional state of macrophages, which are considered permissive. The deficient macrophage activation in DCL hinders the elimination of Leishmania resulting in a disorganized inflammatory process, unable to control the infection. The determinants of DCL are multifactorial and may be associated with both immunologic and genetic events of the patient and the pathogenic factors related to the parasite and vector. The participation of factors associated with the parasite has been shown by some authors although it is a point that still remains to be further explored. In this context, the exhibition of markers of apoptosis by the parasite could be a contributing factor during host-parasite interactions as a possible immunosuppressive mechanism of DCL [7].

2. Immune Response

2.1. Classical L. major Infection. Experimental infection models with Leishmania parasites have been extensively used as a tool to study immune responses, especially regarding T-cell differentiation [8, 9]. This is due to the fact that inbred mice strains demonstrate specific patterns of susceptibility and resistance to the disease [9, 10] which correlate with the immune response built by these animals. The classical experimental model that generated this knowledge was infection with L. major parasites. C57BL/6 mice infected with this parasite develop a Th1 CD4+ T-cell response, which is highly effective to activate leishmanicidal and inflammatory mechanisms in macrophages, leading to intracellular parasite destruction. In this case, a skin lesion is formed, which regresses, becoming undetected around 6–8 postinfection [9]. Nevertheless, latent parasites remain in the infected tissue, providing antigens to maintain a protective immune response that prevent reinfections [11]. On the other hand, BALB/c mice infected with the same parasite species and strain developed a Th2 CD4+ T-cell response, which is not efficient to promote macrophage classical activation, leading to progressive disease. At the cellular level, this difference is mainly due to the activation of a population of cells that express a highly restricted T-cell receptor, Vβ4 Va8, which recognizes the LACK (Leishmania homologue of receptors for activated kinase) antigen and rapidly produces IL-4, necessary to deviate the immune response towards Th2 [12]. Currently, it is clear that the proposed model of susceptibility and resistance to Leishmania infection is quite reproducible when working with some specific strains of L. major though, for other strains and/or species, the picture is relatively more complex. Indeed, effective macrophage activation is the key to control the infection; however, the phenotype displayed by T cells in different situations is not as polarized as observed in the classical model. Actually, there are several papers that suggest that most correlations between CD4+ T-cell response and disease development are not straightforward. BALB/c IL-4 receptor knock-out (KO) mice remained susceptible to L. major infection when infected with LV39 strain, which seems to be due to an increased production of IL-10 by T cells [13]. C57BL/6 mice infected with a L. major strain, isolated from a patient with nonhealing lesions, still developed a Th1 response but displayed a progressive disease [14]. In addition, when infected with the IR173 strain of L. major, CD4+ T cells from both BALB/c and the resistant mice strain B10.D2 produce IL-4 very rapidly [15]. Other factors such as infection route, number of parasites inoculated, and type of infection (needle versus sand fly inoculation) are crucial to determine the type of response elicited (reviewed in [9]). The complexity of the interactions that determines the clinical and immunological outcome of the disease is much less known, and apparently much more multifactorial in other infection systems, such as the ones that involve L. amazonensis infection.

2.2. L. amazonensis Infection: Beyond the Paradigm. Experimental infection with L. amazonensis parasites leads to progressive disease and uncontrolled lesion development in all inbred mouse strains, including those ones that are highly resistant to L. major infection. However, there is a gradient of disease severity, ranging from BALB/c mice, which develop a very fast lesion, that ulcerate, generating extensive areas of necrotic tissue, to C3H. HeN mice that still develop nonhealing lesions, however, displaying slow progression rates [16, 17]. Nonetheless, the phenotype displayed by different mouse strains does not correlate with dichotomous Th1/Th2 responses. Actually, in the analyzed mouse strains such as BALB/c, C57BL/6, and C3H. HeN, it was possible to observe CD4+ T cells capable of producing different types of cytokines such as Th2 cytokines (IL-4, IL-5, and IL-13), Th1 cytokines (IFNγ, and TNFα), and regulatory cytokines (TGFβ and IL-10), which characterizes an unpolarized cellular response [18–20]. Targeted deletion of the Il4 or Il10 gene [21, 22] causes minimal effects on lesion development and parasite tissue loads as well as treatment of infected mice with IFNy [23] or IL-12 [22]. Interestingly, L. amazonensis promastigotes and, especially amastigotes, are able to get through the innate immune response almost unnoticed. As mentioned before, the main host cell for Leishmania proliferation in the mammalian host is the macrophage, which is, together with dendritic cells (DCs), the main antigen presenting cells of the innate immune response. When compared to L. braziliensis parasites, for example, L. amazonensis parasites are much less capable of triggering the expression of CD40 and CD80 [24], both costimulatory molecules for T-cell activation, and the production of IL-12p40 [24]. Actually, amastigote infection is able to downregulate the expression of MHC class II molecules [25], which, during macrophage infection, is depending on sequestering these molecules inside the parasitophorous vacuole for degradation [26, 27]. During the first week of infection in C57BL/6 mice, chemokines such as CCL5, CCL3, CCL2, CCL4, and CCL11 as well as their receptors, are not upregulated when compared to
L. major infection, both at the lesion site and draining lymph node [19]. Additionally, amastigote infection downregulates several intracellular pathways that lead to DC activation such as STAT 1, STAT 3 and Erk 1/2 phosphorylation and the expression of the interferon-responsive elements IRF8 and 1, suggesting a global inhibition of inflammatory responses of these cells [25]. The most well-characterized ligand for amastigote recognition and internalization in macrophages is the opsonizing antibodies produced throughout infection. Triggering of Fc receptors on the host cells lead to IL-10 production and has a pathogenic role [28]. These events are necessary to evade the early immune response culminating with the ineffective T-cell response observed in most cases. In parallel to L. major infection in BALB/c mice, where the oligolcional Vβ4 Vα8 CD4+ T-cell population is necessary to the development of susceptibility in the host and is considered as pathogenic T cells, in L. amazonensis infection, T cells, in a general way, seem to be highly pathogenic. First of all, there is no clonal or oligolcional T-cell population involved, since there is no predominance of a single or a group of Vβ chains expressed on T cells that respond to L. amazonensis infection [29]. However, the disruption of CD4+ T cell effector functions, as observed in recombinae-activating gene KO mice (RAG KO), MHC class II transactivator KO mice (CIITA KO) and nude mice leads to transient resistance to L. amazonensis infection, measured by lesion development [30]. In addition, the adoptive transfer of regulatory T cells also restrains pathogenic effector T cells, diminishing lesion size and parasite tissue loads [31]. The mechanisms underlying pathogenic role of T cells for the disease need to be determined.

3. Phosphatidylerine Exposure

3.1. Homeostasis and Efferocytosis. Phosphatidylerine (PS) is a structural phospholipid present in virtually all membranes and cell types. In normal cells these molecules face the cytoplasmic leaflet of the plasma membrane, whereas during apoptotic cell death these molecules translocate to the outer surface. Once outside the cell, PS becomes one of the ligands recognized by surrounding phagocytes to clear dying cells [32]. However, PS in this model is not just one eat-me signal [33]. PS is the most characterized tickling [34] ligand of apoptotic cells, which means that PS provides the signals for the phagocyte to activate immunosuppressive and anti-inflammatory mechanisms. PS recognition is mandatory to prevent the establishment of a response to the self-antigens engulfed by these cells during apoptotic cell clearance and to avoid triggering inflammatory responses, especially during the embryogenesis, when massive amounts of apoptotic cells are generated and therefore, cleared [35–37], but also in adults to prevent inflammatory immunopathologies [32]. The intracellular events, receptors, and soluble factors involved in this mechanism are still being deciphered and are not the focus of this discussion. However, the effects of PS recognition in macrophages and DCs have a direct impact in immune responses. Apoptotic cells actively induce the production of the anti-inflammatory cytokines TGFβ, PGE2, and PAF [35] and actively inhibit the production of TNFα and IL-1β, even upon LPS challenge [35, 38]. Recognition of apoptotic cells also decreases the expression of several activation markers and costimulatory molecules by both human and murine DCs [39, 40] and regulates the expression of cytokines involved with T-cell differentiation at the transcriptional level [41, 42]. At the single cell level, DCs that ingested an apoptotic cell and bacteria at the same time are able to discern between them and only present bacterial antigens. This is possible because the generation of peptide-MHC class II complexes is controlled by toll-like receptors (TLRs) in a strictly phagosome autonomous manner. Since apoptotic cells do not trigger TLR activation, the generation of stable complexes is inhibited or abrogated [43]. All these effects are fundamental to maintain homeostasis and comprehend the last step of the efferocytosis [44] or apoptotic cell clearance. However, it seems that intracellular parasites elegantly make use of these mechanisms to establish in the host [45–47]. Furthermore, some parasites mimic the features of apoptotic cells to avoid host immune response, as discussed in the next section.

3.2. Conserved Immune-Evasion Mechanism? One of the most common PCD phenotypes is phosphatidylerine (PS) exposure, which can be observed upon chemotherapy, starvation, and heat shock conditions in several unicellular organisms [48–51] or is actively displayed in normal conditions [52]. Our group observed that lesion-derived amastigotes of L. amazonensis actively expose high levels of PS, and by blocking this molecule there is a drastic decrease in the ability of these parasites to infect and establish in the macrophages [52]. These parasites are viable and capable of differentiating into promastigote forms in vitro (unpublished data) and inside the sand fly vector [53] and to infect macrophages and mice [52, 54] and did not display other markers of PCD. Therefore we denominated this mechanism as apoptotic mimicry. PS exposure on amastigotes of L. amazonensis occurs in virtually 100% of the parasites; however, the amount of PS molecules depends on the infected host. Parasites obtained from BALB/c mice expose higher amounts of PS than the ones obtained from C57BL/6 mice [54]. This observation demonstrates that the amount of PS at the surface of the amastigotes has a positive correlation with the severity of the disease and suggests that the host is able to modulate this phenotype of the parasite. Following our description several other groups demonstrated the role of PS exposure and recognition in different infection models. Blood and cell-derived trypomastigotes of Trypanosoma cruzi are able to expose PS, in contrast with epimastigotes, which are not. In addition, infection with PS-exposing trypomastigote forms induces Smad nuclear translocation and inducible nitric oxide synthase inhibition (iNOS), suggesting an autocrine modulation of the host cell dependent on TGFβ [55]. It is interesting to note that, among all T. cruzi parasite stages, only the ones that are infective for mammalian cells evolve the ability to expose PS, suggesting the presence of an evolutionary link between PS exposure and the ability to infect host cells. Similarly, Toxoplasma gondii peritoneal tachyzoites expose PS at their surface, and the recognition of this molecule seems to be necessary to downmodulate
iNOS expression and activity upon macrophage infection [56]. More recently, several papers have demonstrated the role of exposed PS molecules for the infection by enveloped viral particles. For human immunodeficiency Virus-1 (HIV-1), PS at the viral envelope is a cofactor for monocyte infection [57]; in vaccinia virus infection, PS recognition modulates the activity of proteins involved in cytoskeleton reorganization such as p21-activated kinase (PAK) and the small Rho GTPase Rac, leading to increased macropinocytic activity and uptake of viral particles [58]. In addition, PS exposure by tumor cell, microvesicles shed by transformed cells, or endothelial cells in the intratumor environment seems to be involved in different events in tumor development, maintenance, and metastasis [59, 60]. This knowledge stimulated some researchers to evaluate the efficacy of anti-PS antibodies to treat viral and tumoral diseases. Actually the results so far are promising. In murine models of Lassa fever (Pichinde virus) or murine cytomegaloviruses the treatment efficacy was very high, reaching complete cure (total absence of detectable viral loads) in combination with available antiaviral drugs [61]. For experimental tumoral disease, lung cancer, pancreatic tumors, and glioblastomas were efficiently treated, decreasing tumor growth and metastasis in some cases or potentiating the effect of chemo- and radio-therapies [62–64].

3.3. Leishmania amazonensis Infection: New Insights. Our group has been committed to study the role of PS exposure on the surface of different isolates of L. amazonensis. We worked with the hypothesis that L. amazonensis isolates from DCL patients would have higher PS exposure compared with localized cutaneous leishmaniasis (LCL), and this would contribute to macrophage deactivation, favoring parasite replication. For this, we compared PS exposure in L. amazonensis isolates from DCL clinical cases in the active phase of the disease, reported in Maranhão state in Brazil, to those isolated from LCL patients of clinical cases from Bahia. The results indicate that the isolates obtained from DCL patients indeed displayed more PS than isolates from LCL patients at early times postinfection. In addition, isolates from DCL patients were more infective than the ones obtained from LCL patients (Franca-Costa et al., unpublished results). On the other hand, independent of parasite strain analyzed, the parameters of infectivity correlated positively with the exposure of PS in the parasites. These data suggest that in human infections the pattern observed in mice when comparing BALB/c versus C57BL/6 mice is maintained. However, it is necessary to investigate the mechanisms by which the recognition of PS on the surface of the isolates of L. amazonensis deactivate the macrophage response. Particularly, it would be necessary to evaluate whether freshly isolated parasites display this phenotype to validate our analysis made on amastigotes derived from macrophages infected in vitro with cultured promastigote parasites isolated from human lesions. We believe that understanding the dynamics of PS expression, along with identification of the mechanisms involved in the immunosuppression of DCL patients, can result in therapeutic targets for intervention in the immunopathogenesis of this chronic and severe form of leishmaniasis.

In a similar way we are interested in the immunomodulatory mechanisms underlying PS exposure in different inbred mice strains. For that we are currently evaluating these mechanisms during BALB/c infection, which induces high levels of PS exposure on intracellular amastigotes. We observed that PS exposure is intrinsic to the intracellular parasite and cannot be observed in axenically cultured amastigotes but upregulates very fast after internalization. However, these levels are dramatically increased when infected macrophages are in the presence of previously primed T cells or their soluble products. We confirmed these results by infecting BALB/c nude mice where we observed that the amastigotes obtained from these mice display minimal levels of PS, which are completely restored if we adoptively transfer primed CD4+ T cells to nude mice (Wanderley et al. unpublished results). Interestingly, these data indicate that one possible role for the previously reported pathogenic T cells [31] would be to induce PS exposure on intracellular amastigotes and, therefore, contributing to the generation of highly infective parasites. The T-cell-dependent PS exposure on amastigotes seems to be dependent on the induction of iNOS expression on host macrophages, and parasite survival is dependent on the concomitant induction of arginase 1 expression (Wanderley et al. unpublished results). We propose that high levels of PS exposure are induced by parasite stress delivered by iNOS activity. In this case, it is still unknown whether PS exposure on amastigotes is indeed a phenotype triggered by PCD or a specific process involving modulation of PS translocation. Under PS-inducing conditions, macrophages express high levels of arginase 1 (Wanderley et al. unpublished results), that is the enzyme necessary to produce ornithine, the precursor of polyamines. In this situation, polyamines could protect the parasite from the iNOS-dependent stress, stimulating parasite growth [66, 67] and increasing DNA stabilization [68, 69]. We understand that the unique characteristics of the T-cell response to L. amazonensis infection contribute to the generation of a perfect environment to stimulate and maintain increased levels of PS on the surface of intracellular parasites. Probably the balance observed in infected BALB/c mice, when disrupted, leads to the differences observed among different mouse strains. In Figure 1 we summarized our hypothesis regarding the T-cell-dependent modulation of PS exposure on intracellular amastigotes of L. amazonensis.

4. Final Remarks

The observation of PS exposure as a strategy to evade the immune system and persist in the mammalian host, made initially in the experimental model of L. amazonensis infection, was a breakthrough since it stimulated different groups around the world to look for the possibilities for basic and applied research on the field. Our group is still studying the immunological, cellular, and molecular mechanisms underlying control of PS exposure in parasites and the effects of its recognition by parasitized cells and organisms. We believe that this could be a major strategy in different systems
where avoidance from immune surveillance is necessary to establish a disease.

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