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Apoptotic mimicry: an altruistic behavior in host/*Leishmania* interplay

J.L.M. Wanderley[1,2], A. Benjamin[1,2], F. Real[1,3], A. Bonomo[1,4], M.E.C. Moreira[1] and M.A. Barcinski[1,5]

[1]Instituto Nacional de Câncer, Rio de Janeiro, RJ, Brasil
[2]Programa de Pós-Graduação em Ciências Morfológicas, ICB, UFRJ, Rio de Janeiro, RJ, Brasil
[3]Instituto de Ciências Biomédicas, and
[4]Instituto de Microbiologia Professor Paulo de Góes, UFRJ, Rio de Janeiro, RJ, Brasil
[5]Departamento de Parasitologia, ICB, USP, São Paulo, SP, Brasil

Abstract

Apoptosis is the most common phenotype observed when cells die through programmed cell death. The morphologic and biochemical changes that characterize apoptotic cells depend on the activation of a diverse set of genes. Apoptosis is essential for multicellular organisms since their development and homeostasis are dependent on extensive cell renewal. In fact, there is strong evidence for the correlation between the emergence of multicellular organisms and apoptosis during evolution. On the other hand, no obvious advantages can be envisaged for unicellular organisms to carry the complex machinery required for programmed cell death. However, accumulating evidence shows that free-living and parasitic protozoa as well as yeasts display apoptotic markers. This phenomenon has been related to altruistic behavior, when a subpopulation of protozoa or yeasts dies by apoptosis, with clear benefits for the entire population. Recently, phosphatidylserine (PS) exposure and its recognition by a specific receptor (PSR) were implicated in the infectivity of amastigote forms of *Leishmania*, an obligatory vertebrate intramacrophagic parasite, showing for the first time that unicellular organisms use apoptotic features for the establishment and/or maintenance of infection. Here we focus on PS exposure in the outer leaflet of the plasma membrane - an early hallmark of apoptosis - and how it modulates the inflammatory activity of phagocytic cells. We also discuss the possible mechanisms by which PS exposure can define *Leishmania* survival inside host cells and the evolutionary implications of apoptosis at the unicellular level.

Correspondence
M.A. Barcinski
Coordenação de Pesquisa
Instituto Nacional de Câncer
Rua André Cavalcanti, 37
20231-050 Rio de Janeiro, RJ
Brasil
E-mail: barcinsk@inca.gov.br

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Leishmania parasites

Parasites of the genus *Leishmania* are the causative agents of human leishmaniasis. The disease ranges from a healing skin lesion to a potentially fatal disseminating disease. *Leishmania* affects over 80 countries and a half million people, being a global health problem. The parasites are transmitted by sandflies of the subgenera *Phlebotomus* (Old World) and *Lutzomyia* (New World) by inoculating infective metacyclic promastigote forms. These forms are internalized by host macrophages and differentiate into non-mo-

tile amastigote forms which are able to proliferate inside phagolysosomes, thus being responsible for the maintenance and propagation of the infection (1).

Leishmania parasites have developed different strategies to invade host macrophages. Promastigotes display two major surface molecules - lipophosphoglycan (LPG) and glycoprotein 63 (gp63) - that play a fundamental role in *Leishmania* virulence. LPG is the major component of the promastigote's glycocalix, which blocks complement-mediated lysis in metacyclic forms due to a specific molecular alteration (2). gp63 is a metalloprotease that cleaves the C3b form of complement into the inactive iC3b form and also blocks complement-mediated lysis (3). Therefore, iC3b can opsonize the parasite for phagocytosis through complement receptors 3 and 1 (CR3 and CR1), targeting the parasite for uptake by macrophages (4). In amastigote forms the antibody-mediated phagocytosis by Fcγ receptors plays an important role in parasite internalization (5). However, there is little evidence to date of intrinsic virulence factors in amastigote forms. Recently, we observed that amastigote forms expose phosphatidylserine (PS) on their surface - an early hallmark of apoptosis. This phospholipid plays an important role in amastigote internalization and survival inside host macrophages (6). The possible mechanisms underlying PS participation in the pathogenesis of leishmaniasis and the implication of apoptotic features exhibited by unicellular parasites will be discussed here.

Programmed cell death: unicellularity x multicellularity

Programmed cell death (PCD) is a mechanism involving differential expression of specific target genes. Apoptosis, described by Kerr et al. (7), is the most common phenotype of PCD. Apoptotic cells are characterized by morphological markers such as cell shrinkage, PS exposure, membrane blebbing, DNA fragmentation, and packaging of cell contents into apoptotic bodies (8-11). This kind of cell death is non-inflammatory, especially because it prevents the release of cytoplasmic contents since apoptotic bodies are quickly cleared by phagocytes.

Multicellular organisms depend on extensive renewal of senescent, damaged or harmful cells and on an efficient mechanism for death and removal of these cells. Cell death is also required for the maintenance of homeostasis and participates in organogenesis during development. Undoubtedly, apoptosis is advantageous for multicellular organisms but not necessarily for unicellular organisms. However, nowadays much evidence of apoptosis is available also for unicellular organisms. The slime mold *Dictyostelium discoideum* exposes PS on the surface when submitted to nutritional deprivation (12). Chloroquine-treated strains of *Plasmodium falciparum* present DNA fragmentation in an oligonucleosomal pattern (13). Some evolutionary forms of *Trypanosoma brucei rhodesiense* die inside the vector, displaying DNA fragmentation and membrane blebbing (14). A subpopulation of *Saccharomyces cerevisiae* exposes PS and presents DNA fragmentation in aged cultures (15).

What are the benefits of apoptosis for unicellular organisms? Two main hypotheses have been proposed to answer this question. First, cell death can be important for population size control when there are insufficient nutrients (free-living organisms) or to avoid host death (parasitic organisms). In this case unicellular apoptotic cells show an altruistic behavior, dying for the benefit of others. The second explanation is that apoptotic cells, which will not necessarily die, could provide signals that enhance the survival of the entire population.

Phosphatidylserine and infectivity

PS is a structural phospholipid normally

localized on the inner surface of the plasma membrane. Apoptotic cells lose membrane asymmetry, and PS is exposed on the outer leaflet of the plasma membrane. Recognition of PS on the surface of apoptotic cells drives phagocytes to internalize these cells (16,17). In mammals there are redundant receptors for apoptotic cell clearance. Most of them use PS as their ligand but the only one able to recognize PS specifically and directly is the recently described PS receptor (PSR) (18). PS/PSR interaction leads to a macropinocytic activity of macrophages generating the endocytic pathway for apoptotic cell clearance. PS recognition by this receptor also leads to transforming growth factor $ß_1$ (TGF-$ß_1$) production by phagocytic cells (19). This cytokine inhibits the inflammatory response in an autocrine and paracrine manner. It has been recently reported that $\alpha_v ß_3$ integrin can also mediate TGF-$ß_1$ production through PS recognition (20). We have previously shown that amastigote forms of *Leishmania* (*L*) *amazonensis* expose PS on their surface and use this ligand to penetrate host macrophages. PS recognition leads to enhanced TGF-$ß_1$ secretion and interleukin-10 (IL-10) mRNA production. Pretreatment of amastigotes with annexin V - which binds to PS at high calcium concentrations - inhibited amastigote infectivity by at least 50% and significantly reduced TGF-$ß_1$ production by infected macrophages (6). Thus, PS signaling in amastigote forms functions like apoptotic cells according to a mechanism denoted by us as "apoptotic mimicry".

Some anti-leishmanial pharmacological agents induce apoptotic death of the parasite. For example, 12 h after antimony addition to macrophages infected with *L. donovani*, intracellular parasites expose PS and display DNA fragmentation (21). The high levels of drug-induced parasite death very probably preclude any eventual advantage of PS signaling. PS exposure is a natural phenotype displayed by a subpopulation of purified amastigote forms and this characteristic enhances the survival of the entire population. The exact mechanism by which PS exposure increases amastigote infectivity is not fully understood.

Effects of phosphatidylserine signaling: a working hypothesis

Arginase activity

Macrophages are the effector cells in the control of *Leishmania* infection although they are the preferential host cells for these parasites. Macrophage biology defines leishmanial survival. Activated murine macrophages metabolize L-arginine via two main pathways that are catalyzed by the inducible enzymes nitric oxide synthase (iNOS) and arginase. Nitric oxide (NO) is generated from the oxidation of L-arginine by iNOS. L-arginine can be converted by arginase to L-ornithine, the substrate for ornithine decarboxylase (ODC), which is the main enzyme in polyamine synthesis (22). Arginase and iNOS can be differentially induced in murine macrophages by two antagonistic cytokines. The Th1-type pro-inflammatory cytokine IFN-γ up-regulates iNOS, whereas Th2-type IL-4 and IL-10, which are anti-inflammatory cytokines, induce arginase activity (23). TGF-$ß_1$ also enhances arginase activity in macrophages and hence increases polyamine synthesis (24). This dual capacity can be part of distinct metabolic programs since M-1 macrophages - derived from Th1-prone strains (C57Bl/6) - are easily activated to produce NO, and M-2 macrophages - derived from Th2-prone strains (BALB/c) - are easily activated to produce polyamines. Since NO is an inhibitor of cell replication while polyamines can stimulate replication, it seems that macrophages from Th1 and Th2 mice can influence immune reactions in opposite ways. In fact, it has already been shown that N-hydroxyl-L-arginine (a physiological inhibitor of arginase) controls cellular infection of *L. infantum* and *L. major*

amastigotes, since it was able to inhibit arginase and parasite growth (25). Recently, it was shown that treatment of *L. major*-infected BALB/c macrophages with IL-4, IL-10 or TGF-$ß_1$ led to a substantial increase in arginase activity and in the number of intracellular parasites. Furthermore, infected macrophages from C57Bl/6 mice displayed significantly less arginase activity when compared to infected BALB/c macrophages in response to these cytokines (26). These results can help to explain the course of infection observed in susceptible (BALB/c) and resistant (C57Bl/6) mice.

Freire-de-Lima et al. (20) reported convincing data correlating apoptotic cell recognition with polyamine synthesis and survival of intracellular *Trypanosoma cruzi*. Peritoneal macrophages treated with apoptotic cells showed a 5-fold enhancement in putrescine production and a 20-fold increase in ODC activity. Moreover, putrescine addition increased parasite growth in infected macrophages. ODC activity induced by apoptotic cell recognition was inhibited by an anti-TGF-$ß_1$ monoclonal antibody.

Based on these published data, we can postulate a model for the mechanisms by which PS enhances macrophage permissiveness to *Leishmania*. Recognition of PS on the surface of amastigotes leads to TGF-$ß_1$ release by infected macrophages. This cytokine then stimulates the arginase activity of infected and surrounding cells, consequently reducing NO production and increasing polyamine synthesis (Figure 1). Furthermore, we cannot exclude the fact that direct activation of PSR can lead to increased arginase activity, since PSR activation by an agonist antibody induces arginase protein expression (27).

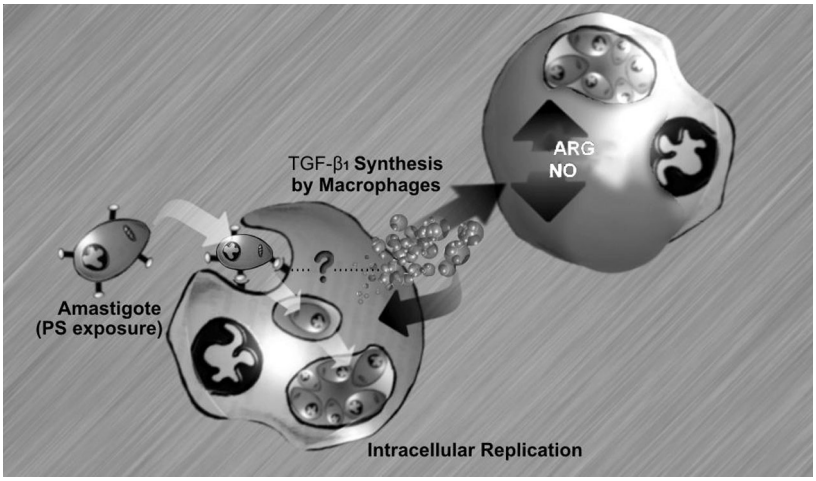

Figure 1. Macrophage modulation by amastigotes exposing phosphatidylserine. ARG = arginase activity; NO = nitric oxide; PS = phosphatidylserine; TGF-$ß_1$ = transforming growth factor $ß_1$.

Immune system modulation

Apoptotic cells seem to influence immune system physiology through interactions with dendritic cells (DC). Sauter et al. (28) showed that DC exposed to tumoral apoptotic cells do not mature. These cells, in contact with T lymphocytes, are unable to induce T cell proliferation because they lack proper expression of CD86 and CD80 molecules, contrasting with those that have engulfed fresh or necrotic tumor cells (28). These findings suggest that apoptotic cells can induce toleration of T lymphocytes, acting as an antigen carrier to maintain peripheral tolerance. This hypothesis was corroborated by Albert et al. (29) who demonstrated the necessity of DC maturation for cross-toleration of $CD8^+$ T cells. They used a system to test the role of $CD4^+$ T cell/DC interplay in the activation of cross-presentation by DC. When antigen delivery to DC was through an apoptotic cell infected with influenza virus, these cells were unable to activate $CD8^+$ T cells. The same occurred when DC did not interact with $CD4^+$ T cells or lacked a CD40 stimulus (29). Many questions are still open concerning the mechanism by which apoptotic cells modulate antigen processing and presentation. In a recent review, Albert (30) postulated that many immunosuppressor signals given by apoptotic cells can participate in this mechanism such as TGF-$ß_1$, IL-10 and PS recognition by CD36 and PSR.

The role of DC in immune responses against *Leishmania* is well characterized, but only recently these cells were characterized as host cells for amastigote and promastigote proliferation, at least *in vitro*. Interestingly, only antibody-opsonized amastigotes or metacyclic promastigotes can induce DC maturation while amastigotes derived from nude mice - which produce only IgM antibodies - internalize and proliferate inside DC without inducing their maturation (31).

We propose that PS on the surface of amastigote forms of *Leishmania* may play a role in DC maturation and antigen presentation. In fact, DC matured in the presence of a modified PS molecule of *Schistosoma mansoni* induce a subpopulation of T cells that produce high levels of IL-10 (32). This suggests a potential role of PS signaling in the regulation of immune responses against pathogens.

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