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Mimicry of Apoptotic Cells by Exposing Phosphatidylserine Participates in the Establishment of Amastigotes of *Leishmania (L) amazonensis* in Mammalian Hosts

João L. M. Wanderley,*† Maria E. C. Moreira,* Aline Benjamin,*† Adriana C. Bonomo,*† and Marcello A. Barcinski²§

Signaling through exposed phosphatidylserine (PS) is fundamental for the TGFβ₁-dependent, noninflammatory phagocytosis of apoptotic cells. This same mechanism operates in the internalization of amastigotes of *Leishmania (L) amazonensis (L(L)a)* in a process quoted as apoptotic mimicry. Now we show that the host modulates PS exposure by the amastigotes and, as a consequence, BALB/c mice-derived amastigotes expose significantly more PS than those derived from C57BL/6 mice. Due to this difference in the density of surface PS molecules, the former are significantly more infective than the latter, both in vivo, in F₁ (BALB/c × C57BL/6) mice, and in vitro, in thioglycollate-derived macrophages from this same mouse strain. PS exposure increases with progression of the lesion and reaches its maximum value in amastigotes obtained at the time point when the lesion in C57BL/6 mice begins to decrease in size and the lesions in BALB/c mice are still growing in size. Synthesis of active TGFβ₁, induction of IL-10 message, and inhibition of NO synthesis correlate with the amount of surface PS displayed by viable (propidium iodide-negative) infective amastigotes. Furthermore, we also show that, similar to what happens with apoptotic cells, amastigotes of *L(L)a* are internalized by macrophagocytosis. This mechanism of internalization is consistent with the large phagolysosomes characteristic of *L(L)a* infection. The intensity of macrophage macrophagocytic activity is dependent on the amount of surface PS displayed by the infecting amastigote. *The Journal of Immunology, 2006, 176: 1834–1839.*

Mononuclear phagocytes play a fundamental role in host defense mechanisms. Paradoxically, diverse microorganisms, by means of different strategies, have evolved the capacity to evade the microbicidal activity of these cells and to establish as obligate intracellular parasites (1). Such is the case with protozoa of some species of the genus *Leishmania* spp., responsible for debilitating diseases in men, with a wide spectrum of clinical forms. Several studies, mainly in promastigotes, have focused on the ability of different surface molecules to modulate parasite entry and allow for intracellular survival by interfering with macrophage function (2–5). On the contrary, in amastigotes, this type of information is still very scarce. We have shown recently that, in amastigotes of *Leishmania amazonensis (L(L)a)*, signaling via exposed phosphatidylserine (PS)³ is an important mechanism for leishmanial establishment in the vertebrate host. PS on the surface of amastigotes inhibits macrophage inflammatory activity in a way similar to what has been described to occur with apoptotic cells, and this mechanism has thus been quoted as apoptotic mimicry (6). This lipid moiety, by interacting with macrophages, participates in parasite internalization and induces an anti-inflammatory response by inhibiting macrophage NO activity, increasing IL-10 message and TGFβ₁ secretion (6). In this study, we show that PS exposure by amastigotes is modulated by the host, and consequently, parasites derived from susceptible BALB/c mice display significantly more exposed PS than those derived from resistant C57BL/6 mice, and that the amount of surface PS correlates with parasite infectivity. We demonstrate that PS-dependent macrophagocytosis (7) plays an important role in the internalization of *L(L)a* amastigotes by macrophages. Furthermore, we show that macrophage leishmanicidal activity is down-regulated when infected by BALB/c-derived amastigotes, because these cells produce more TGFβ₁ and less NO than macrophages infected with C57BL/6-derived amastigotes.

**Materials and Methods**

**Animals, infection, and parasite purification**

Six- to 8-wk-old BALB/c and C57BL/6 mice maintained at our own facilities were infected in one of the hind footpads with 2 × 10⁶ amastigotes of *L(L)a* strain LV79 (MPRO/BR/72/M 1841-LV-79). When the lesion reached a workable size, the animals were sacrificed, and the lesion was removed under sterile conditions. The tissue was finely minced and homogenized with a tissue grinder (Thomas Scientific). The cell suspension was resuspended and centrifuged at 50 × g for 10 min at 4°C. The supernatant was carefully removed, further centrifuged, and washed three more times at 1450 × g for 17 min at 4°C. After 2-h incubation under rotation at 34°C to liberate endocytic membranes (8), the amastigotes were further centrifuged and incubated for 16 h at 34°C, at the end of which they were centrifuged and washed three times before use.
Flow cytometric analysis

For surface PS detection, amastigotes were washed and resuspended in binding buffer (10 mM HEPES, 150 mM NaCl, 2.5 mM CaCl₂) at pH 7.3. Cells were incubated at room temperature for 15 min with Annexin V-FITC (1/20 dilution; Molecular Probes). At the time of acquisition, propidium iodide (PI) was added to each sample to the final concentration of 0.7 mg/mL. For surface IgG detection, amastigotes in PBS were incubated with a goat anti-mouse Fc-FITC (Sigma-Aldrich). To confirm the absence of adherent macrophage in the amastigote preparation, the parasites were analyzed with anti-mouse CD14-FITC and CD11b-FITC in the first series of experiments. Data were collected in a FACScan (BD Biosciences) and analyzed by CellQuest software (BD Biosciences). Ten thousand gated events were harvested from each sample.

Macrophage infection

Thioglycollate (Sigma-Aldrich)-induced macrophages from (BALB/c × C57BL/6) F₁ mice were pooled and washed in medium containing 10% FCS. After differential cell count, 10⁴ macrophages were plated in 24-well chambers (TPP) at 37°C. After 2 h of incubation for adherence, cells were washed and incubated with 10⁵ amastigotes for an additional 2 h at 34°C. After incubation, the cells were fixed and stained, and the infectivity index (percentage of infected macrophages × average number of amastigotes in infected macrophages) was defined by microscopic analysis. The infectivity index was assessed after different times of culture. To discriminate the parasites’ PS-dependent from Fc-dependent binding, the incubation was performed in the presence of respectively Annexin V (AX) or a purified rat anti-mouse Fcγ III/II receptor (BD Pharmingen) or, alternatively, normal mouse serum (NMS).

Quantification of TGFβ₁ and NO synthesis

Active TGFβ₁ production was assayed by ELISA (Duoset kit; R&D Systems) after 72 h of culture in serum-free medium containing 100 ng/ml LPS from Escherichia coli serotype 026:B6 (Sigma-Aldrich). The concentration of TGFβ₁ in each experiment was determined by comparison with a curve generated from TGFβ₁ standards. For NO synthesis determination, thioglycollate-induced peritoneal macrophage cultures were infected with amastigotes for 2 h and stimulated with 0.1 ng/ml murine IFN-γ (PeproTech) and 100 ng/ml LPS at the same time. Cultures were then washed and maintained with LPS and IFN-γ for an additional 90 h. Supernatants were collected and assessed for NO concentration by Griess reaction (Sigma-Aldrich) and comparison with a standard NaNO₂ curve.

Detection of macrophage macropinocytic activity

Macropinocytic activity was quantified by means of the Lucifer Yellow uptake assay adapted from Hoffmann et al. (7). Briefly, thioglycollate-induced peritoneal macrophages were stimulated for 2 h at 37°C with parasites or 50 ng/ml PMA. Cells were washed three times at 300 g for 10 min to remove parasites and PMA and were then resuspended in DMEM containing 10% FCS and 250 mg/ml Lucifer Yellow (Molecular Probes) for 20 min at 37°C. After this time, cells were washed five times with buffered saline to remove free Lucifer Yellow and analyzed in a F4500 fluorescence spectrophotometer (Hitachi). Macrophage macropinocytic activity was inhibited by incubating cells with amiloride (Aml) hydrochloride (Sigma-Aldrich).

Statistical analysis

One-way ANOVA or unpaired t test with Welch correction were performed using the InStat software (GraphPad).

Results

Differential PS exposure by amastigotes modulates infectivity

As shown in Fig. 1A, at 10 wk postinfection, a higher frequency of amastigotes derived from BALB/c mice exposes PS (47.9% of PIP⁺/AX⁻ cells), compared with the frequency of PS-exposing amastigotes derived from C57BL/6 mice (11.2% of PIP⁺/AX⁻ cells). The density of PS molecules on the cell surface is significantly higher in the former than in the latter, as measured by the mean fluorescence intensity (MFI) of AX-labeled cells (Fig. 1B). The difference in exposure increases with progression of the lesion (Fig. 1, B and C). Despite some variation on the time course of lesion size in different experiments, the difference of PS exposure between amastigotes from different origins becomes significant at the moment when lesion size begins to discriminate one mouse strain from the other and continues to increase with lesion size progression. After 10–12 wk postinfection, the experiment is usually interrupted due to difficulties in obtaining an adequate number of viable amastigotes from C57BL6 mice for analysis and to the appearance of areas of necrosis in BALB/c lesions. Analysis of PS exposure was always conducted before the appearance of necrosis in BALB/c mice and when amastigotes derived from both origins displayed similar viability after purification, as assessed by measuring PI incorporation by FACScan and dehydrogenase activity by the MTT assay (data not shown). It also is important to point out that, because the frequency of PIP⁺/AX⁻ cells also changes with the course of infection (Fig. 1A), all the functional assays (see below) were performed with a population of amastigotes containing a maximum of 20% of such cells. In addition, amastigotes were negative for macrophage markers as a control for the possibility of adhered host membranes contributing for the presence of surface PS (data not shown). When infecting (BALB/c × C57BL/6) F₁ mice (Fig. 2A) or thioglycollate-induced peritoneal macrophages (Fig. 2B), amastigotes derived from BALB/c mouse are significantly more infective than those of C57BL/6 origin. In the in vitro experiment, it becomes clear that amastigotes from BALB/c origin not only multiply more efficiently inside the macrophages but also display a more efficient rate of internalization (Fig. 2B). Because
amastigote opsonization and consequent internalization into macrophages via Fc receptors is a known mechanism of infectivity (9), we assessed the amount of IgG on the surface of parasites by flow cytometry using an anti-mouse γ-chain Ab. As shown in Fig. 3A, no difference between amastigotes from both origins could be detected, suggesting that the observed differential infectivity cannot be attributed to internalization via Fcγ receptors. To discriminate the contribution of PS and Fcγ recognition for amastigote internalization, we inhibited the former with AX and the latter with NMS or an anti-Fcγ receptor Ab. As shown in Fig. 3B, each treatment by itself, inhibits ~40% of BALB/c-derived amastigote internalization, and together (AX and NMS or anti-Fc receptor) they decrease 90–95% of the infection. In C57BL/6-derived amastigote infection only both, AX plus NMS are capable of inducing a significant decrease in parasite internalization. Considering that, in the case of apoptotic cell clearance, PS is able to induce macropinocytosis (7), and that the phagolysosomes induced by IL-10 internalization are morphologically compatible with macropinocytic endocytosis (10–12), we next looked at whether this process operates in the macrophage amastigote uptake.

Macropinocytosis mediated by PS is a mechanism for amastigote internalization

To measure the competence of PS on the surface of amastigotes to stimulate macrophage macropinocytic activity, we used a Lucifer Yellow uptake assay (7, 10). As can be seen in Fig. 4A, amastigotes are able to increase the uptake of Lucifer Yellow by thioglycollate-induced peritoneal macrophages from (BALB/c × C57BL/6) F1 mice. Lucifer Yellow uptake correlates with the amount of surface PS exposed by the amastigotes and is completely abolished by previous incubation of amastigotes with AX, showing that the process is entirely surface-PS dependent. Furthermore, macrophages’ treatment with 1.5 mM Aml hydrochloride, a selective inhibitor of macropinocytosis, in a concentration that yields inhibition of the uptake of Lucifer Yellow induced by PMA (data not shown), significantly reduces parasite internalization and has no effect on the internalization of fixed RBC (Fig. 4B).

However, binding of amastigotes to the surface of phagocytes was not inhibited by treatment with Aml and was significantly higher with amastigotes from BALB/c, compared with those of C57BL/6 origin (Table I and Fig. 5). In addition, the number of free parasites in the coculture supernatant was higher in Aml-treated cultures than in control cultures (data not shown). Treatment of macrophages with Aml does not alter phagocytosis of fixed or opsonized RBC, indicating that the macrophage phagocytic capacity is undamaged (Fig. 5B). Furthermore, amastigotes viability and PS exposure are not affected by Aml (data not shown). As shown in Table I and Fig. 5A, anti-Fcγ receptor Ab as well as AX significantly abrogates binding of amastigotes to Aml-treated macrophages, indicating that both ligands (Fc and exposed PS) play a role in amastigote/macrophage interaction.

To show that the effect on parasite virulence of the exposed PS is due not only to the induction of a higher internalization rate but also to a differential production of macrophage inactivation cytokines and a consequent reduction on the leishmanicidal capacity of the phagocyte, we next looked for TGFβ1 and NO synthesis and expression of IL-10 message by F1 macrophages when exposed to parasites displaying different amounts of surface PS.
As shown in Fig. 6, the density of PS molecules exposed on the surface of amastigotes displayed by the infecting amastigotes. The density of PS molecules exposed by the parasites and is reversed by the addition of anti-TGFβ1. Uptake induced by PMA is used as a positive control. ΔMFI represents the MFI of AXpos/PIneg amastigotes. B, Endocytosis of amastigotes from BALB/c and C57BL/6 origin and glutaraldehyde-fixed mouse RBC in the absence and in the presence of Aml (+Aml) after 2 h of coculture. *, p < 0.03.

Expression of IL-10 message and TGFβ1 synthesis by macrophages correlates with the amount of PS molecules on the parasite surface: TGFβ1 secretion defines NO production

PS recognition triggers IL-10 message and TGFβ1 production by macrophages (6, 13) and dendritic cells (14), leading to an anti-inflammatory response. It also has been shown that both IL-10 and TGFβ1 prevent macrophage activation and exacerbate leishmanial infections. Abs to both cytokines reversed the increased susceptibility to parasite growth (15–17). The effect of anti-TGFβ1 has been attributed to an increased synthesis of NO by the macrophages (18). As can be seen in Fig. 6A, the amount of IL-10 and IL-15 message is higher in macrophages infected with amastigotes of BALB/c origin than in those infected with parasites of C57BL/6 origin, and both are higher than the amount produced by noninfected control macrophages. Also, the amount of TGFβ1 produced by (BALB/c × C57BL/6) F1 macrophages correlates with the density of surface PS moieties displayed by the infecting amastigotes. As shown in Fig. 6B, left panel, when the density of PS molecules on the surface of amastigotes derived from different hosts is clearly different, as revealed by the differences in MFI of AX binding (MFI of 5.33 in amastigotes derived from BALB/c mice and of 2.69 in amastigotes derived from C57BL/6 mice) the production of active TGFβ1 induced by both amastigotes is proportionally different. On the contrary (right panel), when the amastigotes display similar amounts of surface PS (MFI of 5.85 in amastigotes derived from BALB/c mice and of 5.79 when derived from C57BL/6 mice) the amounts of induced TGFβ1 secretion are not statistically different. In addition, the inhibition of NO synthesis by macrophages activated by LPS and IFN-γ also is proportional to the amount of surface PS exposed by the parasites and is reversed by the addition of anti-TGFβ1 (Fig. 6C).

Discussion

Engulfment of apoptotic cells and active suppression of the release of proinflammatory mediators are both dependent on PS recognition by neighboring phagocytes (13, 19, 20). Interestingly, amastigotes of L(L)α rely on this same event, PS exposure, for establishing in their mammalian host (6). In our previous study (6), and that holds for the data presented in this study, we raised the point that our amastigote preparation was negative for macrophage surface markers. This type of data, though not definitive, makes very unlikely the possibility of our parasite preparation being contaminated by host macrophages, host macrophage membranes or even host macrophage membranes adhered to the parasites. This last possibility is further minimized by a step of overnight incubation, a procedure specifically described to eliminate host cell membranes, during amastigote purification (8). However, the absence of host protein surface markers in the amastigotes, by no means precludes the very interesting possibility of lipid transfer from the host to the parasite. New data, which certainly do not prove that PS exposed by amastigotes is of parasite origin but reinforce the possibility that the parasite is able to expose its own PS, are the finding by us (J. L. M. Wanderley, L. H. Pinto-da-Silva, E. Saraiva, and M. A. Barcinski, manuscript in preparation) that in vitro grown promastigotes also are able to expose PS.

In this study, we show that the percentage of parasites exposing PS as well as the density of PS molecules exposed on the external layer of the amastigotes cell membrane is modulated by the host, being higher in parasites recovered from BALB/c mice, compared with parasites derived from C57BL/6 mice. The intensity of both parameters, assessed respectively by the frequency of AXpos/Pneg cells (Fig. 1A) and by the MFI of Annexin V-FITC binding (Fig.

Table I. Attachment of purified amastigotes from different hosts to amiloride-treated macrophages

<table>
<thead>
<tr>
<th>Amastigotes</th>
<th>Percentage of Macrophages with Attached Amastigotesa</th>
<th>Attached Amastigotes per Macrophagea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BALB/c-derived</td>
<td>47.0 ± 1.40</td>
<td>1.53 ± 0.007</td>
</tr>
<tr>
<td>C57BL/6-derived</td>
<td>32.0 ± 1.40</td>
<td>1.53 ± 0.050</td>
</tr>
<tr>
<td>AX-treated parasites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BALB/c-derived</td>
<td>19.0 ± 2.80</td>
<td>1.37 ± 0.007</td>
</tr>
<tr>
<td>C57BL/6-derived</td>
<td>13.7 ± 0.35</td>
<td>1.18 ± 0.020</td>
</tr>
<tr>
<td>Anti-FcγR-treated macrophages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BALB/c-derived</td>
<td>9.5 ± 0.70</td>
<td>1.19 ± 0.030</td>
</tr>
<tr>
<td>C57BL/6-derived</td>
<td>7.5 ± 0.70</td>
<td>1.27 ± 0.080</td>
</tr>
</tbody>
</table>

a A total of 200 cells were analyzed per experimental point.
1B) changes in the course of the lesion-size curve. As a consequence, BALB/c-derived amastigotes are more infective in vitro and in vivo than C57BL/6-derived ones. The mechanism by which the host modulates PS exposure by the intracellular amastigote is still under study. However, it is important to point out that ATP-dependent and independent phospholipid translocases, have been described (21) albeit in promastigotes, and their exact nature and control of expression are now under study. Cytokines such as IL-4, IL-10, and TGFβ have been shown to promote intramacrophagic multiplication of amastigotes (12, 15, 22). Of those, IL-10 expression and TGFβ secretion were up-regulated in macrophages in response to signaling by PS in the amastigote surface (6) and, as shown in this paper, the level of up-regulation correlates with the amount of surface PS (Fig. 6, A and B). Together with IL-4, these cytokines were shown to enhance arginase I activity leading to the synthesis of polyamines essential for leishmanial growth (23, 24). Intracellular polyamine levels were shown to modulate PS exposure in mammalian cells (25). As such, an alternative hypothesis to our findings is that the same mechanism operates in amastigote infection and that the concentration of polyamines varies in macrophasmes from different mouse strains due to their differential capacity to respond to arginase-activation cytokines (26).

It has been shown that PS exposed by apoptotic cells is capable of inducing macropinocytosis through the additive participation of tethering ligands, able to promote the adherence of the apoptotic cell to the phagocyte surface (7). Considering that typical phagocytosomes of amastigotes of L(L)a are compatible with internalization by macropinocytosis, we next looked at whether PS on the parasite surface also is able to modulate this process and whether the intensity of the macropinocytic process correlates with the surface density of PS moieties. Indeed, our results show that differential induction of macropinocytosis by amastigotes can explain the higher rates of internalization displayed by amastigotes from BALB/c origin, compared with those derived from C57BL/6 hosts (Fig. 4). Trying to reconcile our findings with the known property of opsonizing Abs to induce amastigote internalization (9), we showed no difference in the density of Abs on the surface of amastigotes from different origins. Interestingly, however, when macrophages were treated with Aml, a known inhibitor of macropinocytosis (7, 11), parasite internalization was inhibited but amastigotes attached to the phagocyte surface could be found (Figs. 4B and 5). An anti-Fc RI/II Ab inhibited this surface attachment with more efficiency than AX, indicating that parasite’s attachment is dependent, in addition to exposed PS, also on the presence of parasite opsonizing Abs (Fig. 5A and Table I). The observed higher binding rate of parasites from BALB/c origin than from C57BL/6 origin can be due to the higher affinity for Fc receptors of the Abs produced during a Th2 response than those generated during the course of a Th1 response (27, 28), even though it is clear that the immune response to L(L)a infection is not so evidently dichotomized as it is with the infection with Leishmania major (29). Differential affinity for Fc receptors also can reconcile the finding of equal amounts of opsonizing Abs in amastigotes of different origins with the fact that NMS blocked more efficiently the infectivity index of BALB/c-derived amastigotes than of C57BL/6-derived ones (Fig. 3B). At this point, we can only speculate that the internalization of C57BL/6-derived amastigotes is preferentially due to exposed PS and less-dependent on opsonizing Abs. We still have no explanation for the fact that the attached opsonized amastigotes are not internalized by Fc mediated phagocytosis, because phagocytosis of opsonized RBC is maintained after Aml treatment of macrophages. Finally, we show that
PS exposure by amastigotes functions as an escape mechanism from the leishmanicidal capacity of host macrophages by inducing cytokines well known for their capacity to promote leishmanial growth such as IL-10 (16, 17) and TGFβ2 (15), via inhibition of NO production by the infected cell (Fig. 6). Interestingly, IL-15, which is a cytokine associated with T cell proliferation, was enhanced in the same proportion of IL-10 (Fig. 6A). This can be due to the autocrine role of IL-10 in promoting IL-15 production by macrophages (30). An interesting possibility, which has not been ruled out by our results, is that IL-10 production is induced by the binding of opsonizing Abs to Fc receptors present on the macrophage surface (31). Adding to the list of possible escape mechanisms it also has been very recently described that signaling by PS is sufficient to induce inhibition of IL-12 (32) production by activated macrophages, an essential step for the differentiation of a curative Th-1 CD4+ response (33, 34). It must be pointed out that mimicking apoptotic cells by obligate intracellular pathogens besides inducing host anti-inflammatory responses and inhibition of the Th-1 differentiation pathway also may be a mechanism for inducing PS-dependent immunosuppression, as recently described by Fadok and colleagues (14). This can be very important in the case of L. (L)a infection, because this pathogen is the most important agent of cutaneous disseminated leishmaniasis, a disease that causes with severe and usually noncurable immunosuppression. In addition, PS from liposomes or parasites can alter dendritic cell case of by Fadok and colleagues (14). This can be very important in the inducing PS-dependent immunosupression, as recently described in Th-1 differentiation pathway also may be a mechanism for mimicking apoptotic cells by obligate intracellular pathogens be-

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Disclosures
The authors have no financial conflict of interest.