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DISTINCT CELLULAR MIGRATION INDUCED BY *Leishmania infantum chagasi* AND SALIVA FROM *Lutzomyia longipalpis* IN A HEMORRHAGIC POOL MODEL

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SUMMARY

Recruitment of a specific cell population after *Leishmania* infection can influence the outcome of the disease. Cellular migration in response to *Leishmania* or vector saliva has been reported in air pouch model, however, cellular migration induced by *Leishmania* associated with host's blood and vector saliva in this model has not been described. Herein we investigated cellular migration into air pouch of hamster after stimulation with combination of *L. chagasi* and host's blood and *Lutzomyia longipalpis* saliva. Migration induced by saliva was 3-fold more than those induced by *L. chagasi* alone. Additionally, *L. chagasi* associated with blood and saliva induced significantly even more leukocytes into air pouch than *Leishmania* alone. *L. chagasi* recruited a diverse cell population; however, most of these cells seem to have not migrated to the inflammatory exudate, remaining in the pouch lining tissue. These results indicate that *L. chagasi* can reduce leukocyte accumulation to the initial site of infection, and when associated with vector saliva in the presence of blood components, increase the influx of more neutrophils than macrophages, suggesting that the parasite has developed a strategy to minimize the initial inflammatory response, allowing an unlimited progression within the host. This work reinforces the importance of studies on the salivary components of sand fly vectors of leishmaniasis in the transmission process and the establishment of the infection.

KEYWORDS: *L. infantum chagasi;* Sand fly saliva; Leukocytes; Inflammation.

INTRODUCTION

In the New World, visceral leishmaniasis is caused by protozoan parasites of the genus *Leishmania* (*L. chagasi*, synonymous *L. infantum*) and transmitted by the female phlebotomine sand fly, *Lutzomyia longipalpis,* during blood repast. To obtain a blood meal, sand flies locate blood by introducing their mouthparts into the skin, tearing tissues, lacerating capillaries and creating hemorrhagic pools upon which they feed[26]. During this process, salivary gland contents are injected together with *Leishmania* promastigotes into the host's skin[32]. In an attempt to probe and feed sand flies must first circumvent the host's homeostatic system, and the innate and acquired immune responses[2]. It has been well documented that to overcome these obstacles, sand flies evolved within their salivary secretion an array of potent pharmacological components, such as anticoagulants, anti-platelet, vasodilators and, importantly, immunomodulator and anti-inflammatory molecules[2].

Analysis of early promastigote-host cell interactions indicates that after inoculation of *Leishmania* into the dermis, the promastigotes interact rapidly with serum components. Metacyclic promastigotes of *Leishmania* have been shown to activate complement in both classical and alternative pathways[7]. After C3 opsonization, promastigotes undergo an immune adherence reaction and bind to CR1 erythrocyte receptors[9]. Opsonization of *Leishmania* metacyclic promastigotes with complement is rapid and lysis, via the membrane, attack complex (C5b-C9 complex) that begins 60 s after serum contact[7]. This results in efficient killing of approximately 90% of all inoculated parasites within a few minutes. Upon encounter of macrophages, parasites bind to CR3 on their surface that facilitates the uptake of parasites into their main host cell[11].

It has also known that cell number and composition of the cellular infiltrate in the initial stages after infection greatly influences innate immune response and the development of acquired immune response. Along gradients of C3a and C5a and several chemokines, such as MIP-1β and MIP-2, inflammatory cells, monocytes/macrophages and polymorphonuclear leukocytes (PMN) migrate to the inoculation site in the skin[31]. Once in the dermis, *Leishmania* can penetrate granulocytes, and macrophages or dendritic cells[13,24,25]. Macrophages are the reservoir cells for parasite replication and regulate the infection by their ability to potentially phagocytose and kill the parasite if it can avoid *Leishmania*-mediated functional inhibition[21]. Neutrophils and eosinophils possess leishmanicidal activity that restrains parasite progression at the initial

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step of the infection[13,24]. However, it was demonstrated that *Leishmania* can survive transiently within neutrophils[5], and following infection with *Leishmania*, the lifespan of neutrophils can be increased to two days, through the inhibition of procaspases processing in the infected cells[1].

Here, we investigated the cell number and composition in the initial response to infection induced by *L. chagasi* combined or not with the host's blood or saliva from *Lu. longipalpis* in hamster employing an air pouch model of inflammation. There are same reports using this model, but only in mice, where the authors evaluated the cellular recruitment induced by *L. major* and *L. donovani*[15], or by *L. braziliensis*[30], as well as reports of *Lu. longipalpis* saliva in macrophage recruitment[29], and the chemotactic effects of *Lu. intermedia* combined or not with *L. braziliensis*[18]. However, migration of cells induced by *L. infantum chagasi* associated with host's blood and *Lu. longipalpis* saliva in the hamster model has not been described. The hamster air pouch model was chosen here because it is the animal model for visceral leishmaniasis that best mimics the various aspects of human disease[17].

The complex microenvironment where the parasite, vector saliva, and the host's blood components encounter for the first time can determine the outcome of infection. The description of the initial inflammatory response is important especially when the three major components, parasite, saliva and blood, are analyzed together.

MATERIALS AND METHODS

Animals and study design: Two-month-old Syrian hamsters (*Mesocricetus auratus*) of both sexes were obtained from the central animal facility of Departamento de Patologia e Medicina Legal of Universidade Federal do Ceará (DPML/UFC), and held under specific pathogen-free conditions. For the experiments, the animals were divided in the following groups (15 to 30 animals per group): Group 1, inoculated with saline sterile; Group 2, inoculated with *L. chagasi*; Group 3, inoculated with the heparinized blood collected from each animal; Group 4, inoculated with vector saliva; Group 5, inoculated with *L. chagasi* associated with heparinized blood; Group 6, inoculated with *L. chagasi* associated with vector saliva; and Group 7, inoculated with *L. chagasi* associated with heparinized blood and vector saliva. The Animal Care and Utilization Committee from UFC approved all experimental procedures (process No. 029/2008).

Parasite culture: *L. infantum chagasi* (MHOM/BR/BA-262) promastigotes were grown in Schneider's *Drosophila* medium (Sigma-Aldrich, St. Louis, MO) at 25 °C supplemented with 20% heat-inactivated fetal calf serum, 2 mM L-glutamine, 200 U/mL of penicillin and 200 g/mL streptomycin (all from Sigma-Aldrich), and 2% sterile human urine. The infectivity of parasites was maintained with regular passage through hamsters, and for use in the experiments, parasites were expanded for several days to reach stationary growth phase.

Sand fly salivary glands: Salivary glands of females of *Lu. longipalpis* (Jacobina strain) were obtained from Jesus G. Valenzuela, Laboratory of Malaria and Vector Research (LMVR), Vector Molecular Biology Section (NIAID-NIH),USA. Twenty females of *Lu. longipalpis* reared at LMVR (NIAID-NIH) were used for dissection of salivary glands 5-8 days post-eclosion; salivary glands were dissected and stored in sterile PBS (pH 7.4) at -70 °C. To obtain the homogenate, salivary glands were disrupted by ultra-sonication and the supernatant collected after centrifugation at 15,000 g for two minutes. Salivary glands were lyophilized in groups of 10 pairs. Each 10 pairs of glands were resuspended with sterile distilled water and before the use were diluted in sterile saline in the concentration of one pair of gland per injection.

Air pouch and *in vivo* cell migration: Air pouches were induced on the back of anesthetized hamsters (5-6 animals per group for each time) by injection of 5 mL of sterile air, as described elsewhere[19], and immediately inoculated with 100 µL either one of the following stimuli (all prepared in endotoxin-free saline): endotoxin-free saline alone; stationary-phase *L. chagasi* promastigotes (10^7 parasites); salivary gland of *Lu. longipalpis* (equivalent to one pair of salivary glands/animal); heparinized blood collected from the animal itself; *L. chagasi* plus heparinized blood; *L. chagasi* plus salivary gland; or *L. chagasi* plus heparinized blood and salivary gland. One hundred to 150 µL of blood of each animal was obtained via orbital plexus after light anesthesia with ketamine and xylazine at the moment of the experiment, and used to inoculate the animal itself. The number of leukocytes present in the blood was determined in a Neubauer hemocytometer before inoculation (each 100 µL of blood injected contained 40-50 x 10^4 cells). After 6, 12 and 24 h, animals were lethally anesthetized, and the pouches washed with a total of 10 mL of endotoxin-free saline to collect leukocytes from the exudates. Lavage fluids (approximately 6 to 8 mL) were washed, and cell pellets were resuspended in saline plus 10% BSA, stained in Turk's solution, and counted in a Neubauer hemocytometer. Cells were cytoadhered to glass slides using Shandon cytospin2 and stained with hematoxylin and eosin to determine proportions of monocytes/macrophages, neutrophils, eosinophils, basophils, and lymphocytes. In all stimuli with blood, the cell values that were determined at 12 h were subtracted from the values of the cells found in the blood that was injected initially (average of 45 x 10^4 cells). The values of monocytes/macrophages, neutrophils, eosinophils, basophils, and lymphocytes found in the experiments were also subtracted from normal blood values found in hamsters. The viability of the cells obtained from the exudates was verified by trypan blue exclusion (0.1%).

Histological analysis: The pouches lining tissues were dissected and fixed in 10% neutral buffered formalin. Tissues were mounted in paraffin blocks, sectioned at 5-µm intervals, and stained with hematoxylin and eosin for histological analysis. The alterations were observed by analyzing 50 microscopic fields per section in the pouch lining tissue, on two sections from each animal (four to five hamsters per group), using a 40 x objective, taking into account the following parameters: hemorrhage, edema, hyperemia and fibrin. Each parameter was evaluated according to the intensity of the event through scores: 0 (absence), 1 (rare), 2 (moderate) and 3 (accentuated). Cellular analysis was evaluated in five fields, using the increase of 1000 x. The population of cells from each animal corresponds to the sum of the fields analyzed. The cellular population observed was: macrophages, neutrophils, eosinophils, and lymphocytes.

Statistical analysis: Statistically significant differences of the results were determined using nonparametric statistical tests: t-Student for comparisons between two groups; one-way ANOVA for comparisons between three or more groups. Statistical analysis and graphs were performed using GraphPad prism version 5.0 (GraphPad Software, San Diego, USA). Values of $p < 0.05$ were considered significant.

RESULTS

Leukocyte recruitment in air pouch exudates: Leukocyte migration reached a maximal peak at 12 h after injection and then declining over a 24-h period into the air pouch (Fig. 1A). *L. chagasi* and saliva induced significantly more cells into air pouches at 12 h after stimulation when compared with saline (Fig. 1A). Of interest, migration induced by saliva was 3-fold more than that induced by *Leishmania* alone (Fig. 1A). In relation to stimuli containing blood, is noteworthy that the number of cells in the blood injected containing an average of 45 x 10^4 cells, while the number of cells collected after 12 h of blood stimulation was 100 x 10^4 cells, which means that the number of cells that migrated induced by blood was in fact 55 x 10^4 cells (Fig. 1A). This number of cells was greater than those induced by *L. chagasi* or saline. Also, in Fig. 1B, the number of cells collected after 12 h of stimulation with *Leishmania* plus blood and *Leishmania* associated with blood and saliva was 144 x 10^4 cells and 250 x 10^4 cells, respectively, meaning a real number of migration of 99 x 10^4 cells for *Leishmania* plus blood, and 205 x 10^4 cells for *Leishmania* plus saliva plus blood. These data were significant in relation to stimulation with *Leishmania* alone. Interestingly, *L. chagasi* associated with saliva resulted in a reduced cellular migration, 55 x 10^4 cells (Fig. 1B), when compared with stimulation with saliva alone (76 x 10^4 cells) (Fig. 1A).

Types of cells that migrated in air pouch exudates: All stimuli induced migration of a mixed population of leukocytes with a predominance of neutrophils at 12 h (Fig. 2A and 2B). Neutrophils migration induced by saliva was more expressive than that induced in response to blood, *L. chagasi* or saline at 12 h after stimulation (Fig. 2A). Saliva also induced a considerable number of eosinophils after 12 h, as compared to all stimuli (Fig. 2A). *L. chagasi* associated to saliva was a great inducer of cell migration into the exudate; however the combination of *L. chagasi* with blood and saliva was even better inducer of all the types of cell to the inflammatory site (Fig. 2B). This combination induced migration of a large number of neutrophils and macrophages as compared to *L. chagasi* alone, and *L. chagasi* plus saliva (Fig. 2B).

Types of cells that migrated in the pouch lining tissue: Macrophages were the predominant cell type at 12 h and 24 h post-stimulation in the pouch lining tissue, followed by neutrophils. It was observed significantly almost 4-fold more macrophages than neutrophils at 12 h after stimulation by *L. chagasi* (Fig. 3A), however after 24h macrophages number decreased while neutrophils almost doubled (Fig. 3B). *L. chagasi* associated with blood or *L. chagasi* plus blood plus saliva induced similarly cell migration at 12 h and 24 h post-stimulation (Fig. 3A and 3B). The combination of *L. chagasi* with blood and saliva showed that the number of neutrophils dropped considerably after 24h, while the number of macrophages did not change (Fig. 3B). After 12 h of stimulation with *L. chagasi* and saliva was possible to observe a smaller number of cells in the pouch lining tissue, as compared with other stimuli, also showing a virtual absence of neutrophils and presence of a small number of eosinophils, however, 24 h after stimulation with *L. chagasi* associated with saliva, there was a slight increase in macrophages (Fig. 4B).

Histopathological findings in the pouch lining tissue: All stimuli alone presented a slight edema and hyperemia (Fig. 4A). Edema and hyperemia also appeared in all groups with association of stimuli, ranging from rare to moderate (Fig. 4B). The major inflammatory changes were observed when *Leishmania* was associated with blood and saliva (Fig. 4B). Hemorrhage was observed in a more pronounced manner following stimulation with *L. chagasi* associated with blood and saliva, when compared with other groups (Fig. 3B).

DISCUSSION

In this study we found that *L. infantum chagasi* proved to be a poor inducer of cellular migration into air pouch exudate, although there was migration of a diverse population of cells. The data observed

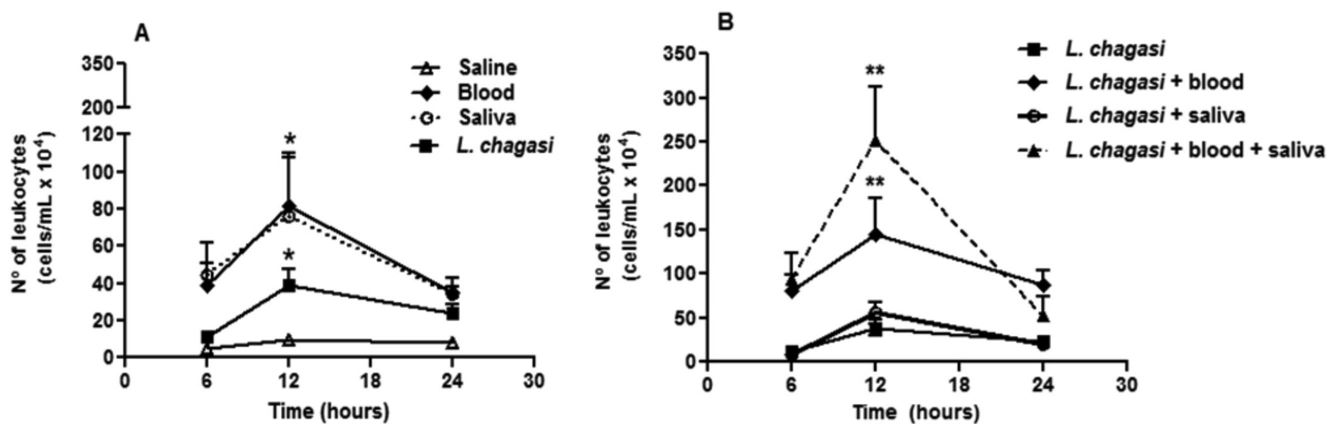

Fig. 1 - Number of leukocytes accumulating in air pouch exudate in response to **(A)** *L. chagasi,* blood, or *Lu. longipalpis* saliva; **(B)** *L. chagasi* plus blood, *L. chagasi* plus *Lu. longipalpis* saliva, or *L. chagasi* plus blood and plus *Lu. longipalpis* saliva. Air pouches were raised on the backs of male hamsters. One milliliter of endotoxin-free saline, *L. chagasi* (10^7 promastigotes), salivary gland of *Lu. longipalpis* (one pair of salivary glands/animal); blood; *L. chagasi* plus blood; *L. chagasi* plus salivary gland; or *L. chagasi* plus blood and salivary gland were injected into pouches, and exudate was collected at 6, 12, and 24 h after inoculation. Leukocytes were enumerated microscopically. Data are mean ± SE of 5 hamsters. (A) *$p < 0.05$, *Leishmania*- or blood- or saliva-stimulated hamster vs. saline-stimulated hamster. (B) **$p < 0.05$, *Leishmania* plus blood- or *Leishmania* plus blood plus saliva- stimulated hamster vs. *Leishmania*-stimulated hamster. The data are representative of three independent experiments.

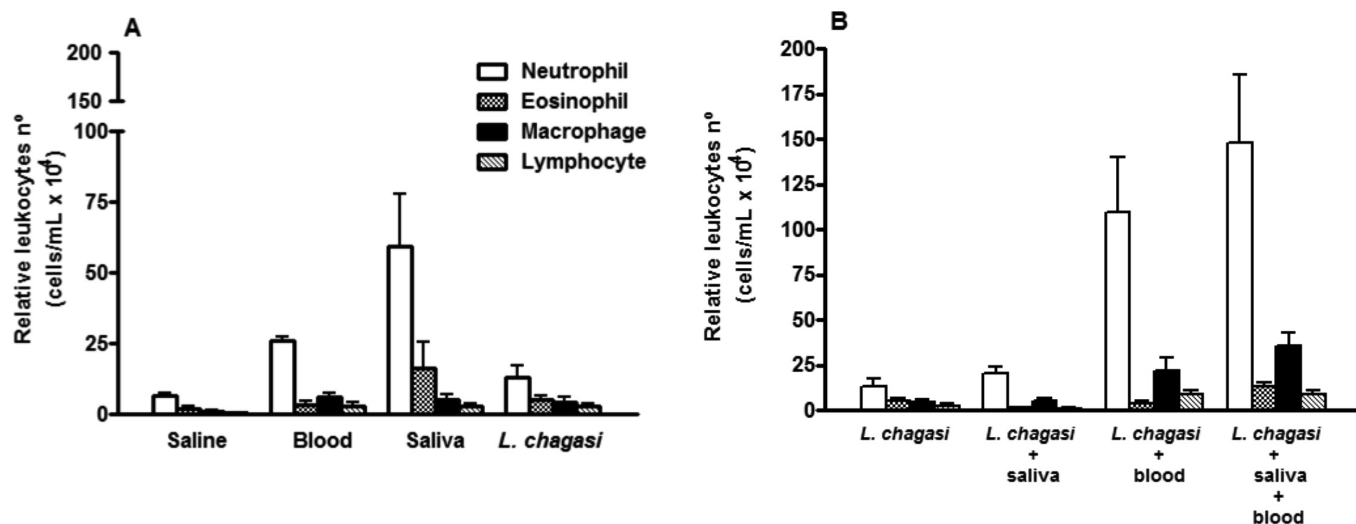

Fig. 2 - Total number of neutrophils, macrophages, eosinophils, and lymphocytes accumulated in air pouches in response to **(A)** *L. chagasi*, *Lu. longipalpis* saliva, or blood; **(B)** *L. chagasi*, *L. chagasi* plus blood, *L chagasi* plus *Lu. longipalpis* saliva, or *L.chagasi* plus blood plus saliva, at 12 h after stimulation. Stimulations were done as described in the legend to Figure 1. Exudate were placed onto microscope slides by use of cytospin and stained with Diff-Quik solution; proportion of neutrophils, macrophages, eosinophils, and lymphocytes/200 cells were enumerated and relative cell numbers were calculated from total exudate leukocytes. Data are mean ± SE of five hamsters. Differences observed for neutrophils, and macrophages in *Leishmania*-, saliva-, and blood-inoculated hamsters were significant ($p < 0.05$; n = 5), compared with saline control. Differences observed for eosinophils in *Leishmania*-, and saliva-inoculated hamsters were significant ($p < 0.05$; n = 5), compared with saline control. The data are representative of three independent experiments.

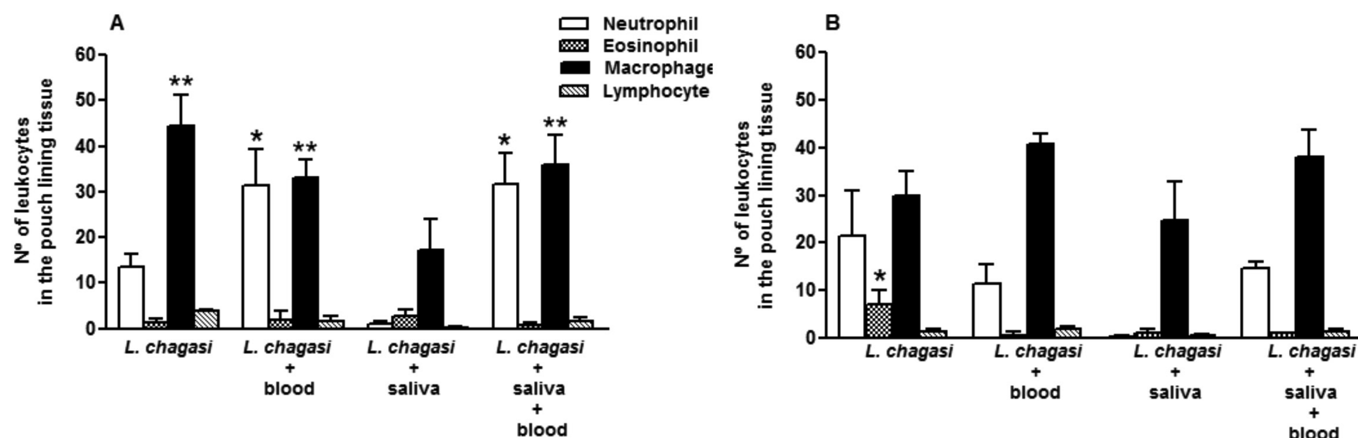

Fig. 3 - Number of leukocytes accumulating in the pouch lining tissue in response to *L. chagasi*, *L. chagasi* plus blood, *L chagasi* plus *Lu. longipalpis* saliva, or *L.chagasi* plus blood plus saliva at 12 h **(A)** or 24 h **(B)** after inoculation. Stimulations were done as described in the legend to Figure 1, and the pouches lining tissues were dissected at 12 and 24 h after inoculation. The data were obtained by analyzing 50 microscopic fields per section in the pouch lining tissue, on two sections from each animal and from five hamsters per group, using a 100 x objective. The cellular population observed was: macrophages, neutrophils, eosinophils, and lymphocytes. (A) *$p < 0.05$ (neutrophils: *L. chagasi* plus blood- or *L. chagasi* plus saliva plus blood-stimulated hamster vs. *L. chagasi*-stimulated hamster); **$p < 0.05$ (macrophages: *L. chagasi*- or *L. chagasi* plus blood- or *L. chagasi* plus saliva plus blood-stimulated hamster vs. *L. chagasi* plus saliva-stimulated hamster). (B) *$p < 0.05$ (eosinophil: *L. chagasi*-stimulated hamster vs. *L. chagasi* plus blood- or *L. chagasi* plus saliva or *L. chagasi* plus saliva plus blood-stimulated hamster. The data are representative of three independent experiments.

in pouch lining tissue suggest that most of these cells do not seem to have migrated to the inflammatory exudate, remaining in lining tissue. Corroborating these findings, a study demonstrated that infection with other viscerotropic species of *Leishmania,* such as *L. donovani*, did not induce a strong recruitment of leukocytes in the exudate[15]. Maybe one possible explanation to these findings is the fact that promastigotes of *Leishmania* have a large quantity of lipophosphoglycan (LPG) on their surface, which has potent inhibitory cell activity[33]. Studies have showed that LPG of *L. donovani* blocks expression of E-selectin, ICAM-1, and VCAM-1 on endothelial cells, suggesting the ability of *L. donovani* to prevent transendothelial migration of monocytes[14]. Thus, it is possible that infection with viscerotropic *Leishmania* parasites did not induce a strong recruitment of leukocytes in the exudate because cells are recruited but can fail to migrate to air punch exudate, suggesting the blockade of transendothelial cell migration. However, this fact needs to be clarified with further studies.

On the other hand, *L. chagasi* associated with blood and saliva recruited

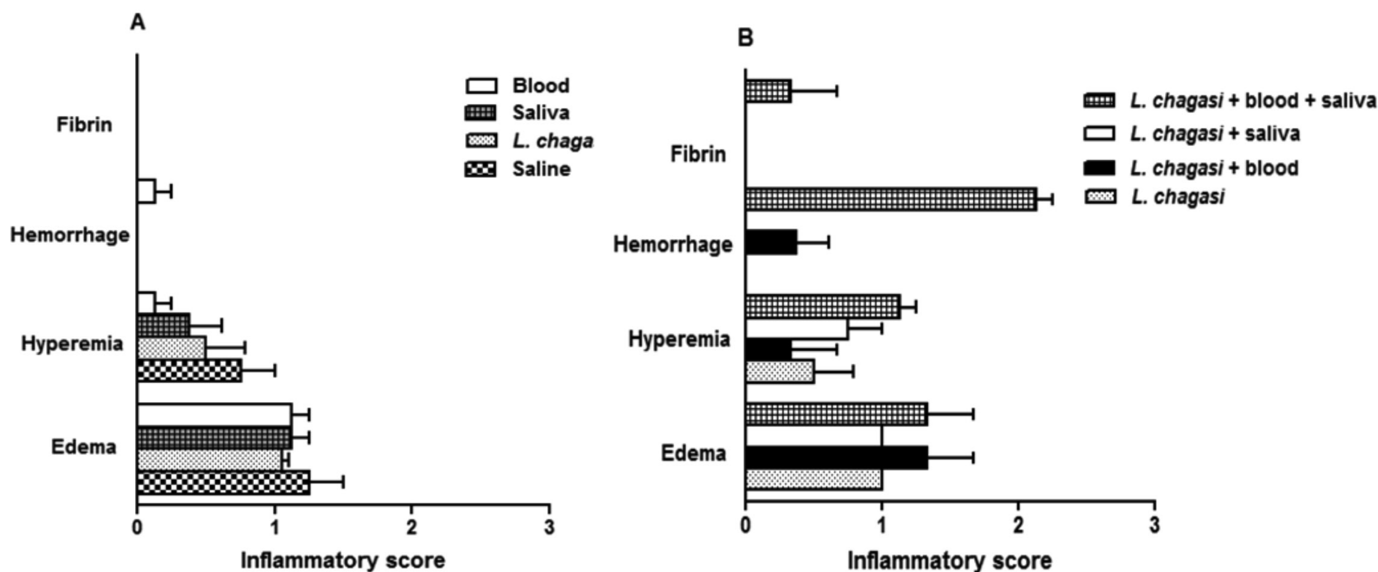

Fig. 4 - Inflammatory scores in the pouch lining tissue at 12 h of stimulation with **(A)** *L. chagasi*, saline, blood, or *Lu. longipalpis* saliva; **(B)** *L. chagasi*, *L. chagasi* plus blood, *L.chagasi* plus saliva, or *L. chagasi* plus blood plus saliva. Stimulations were done as described in the legend to Figure 1, and the pouches lining tissues were dissected at 12 and 24 h after inoculation. The data were obtained by analyzing 50 microscopic fields per section in the pouch lining tissue, on two sections from each animal and from five hamsters per group, using a 40 x objective. Inflammatory scores: 0 (absence), 1 (rare), 2 (moderate) and 3 (accentuated).

more leukocytes than *L. chagasi* did alone. *Leishmania* is an intracellular parasite that has adopted several strategies to survive and to replicate inside the host, and saliva may represent one such strategy, as has been suggested by several studies[2,10]. Herein we observed that sand fly saliva induces a significant influx of leukocytes into air punch exudate. The chemotactic effect for leukocytes, mainly macrophages, induced by sandy fly saliva was previously described in models of cell migration *in vitro* with *Lu. longipalpis* saliva, and other sand fly species as *P. duboscq* and *P. papatasi*[10]. Other study suggests that *Lu. longipalpis* saliva induces an important and diffuse inflammatory infiltrate characterized by neutrophils, eosinophils, and macrophages which are observed after 48 h in the dermis of the ear of BALB/c mice exposed to bites of uninfected sand flies[27]. Sand fly saliva has also been previously described as capable of exacerbating *L. major* infection resulting from an increased production of Th2 cytokines due by *P. papatasi* saliva, indicating that saliva can modulate the host immune response[16]. Other studies have shown that sand fly saliva modulates the response of macrophages to a phenotype more permissive to the survival of *Leishmania*[30]. Sand fly saliva is also able to suppress NO, H_2O_2, and antigen presentation by macrophages[20]. Maybe this anti-inflammatory activity of *Lu. longipalpis* saliva may partly explain the inhibition that occurred in cell recruitment when saliva was associated with *Leishmania*. Another explanation would be to consider some sort of modulation of the inflammatory response made by parasites as a strategy to increase your chance of survival in the animal model used in this study. It has been reported that hamsters are more susceptible to all species of viscerotropic *Leishmania* than mice[17], the model used in all studies cited above.

Besides the effect observed with sand fly saliva, *Leishmania* associated with blood also shown to have an important role in modulating the inflammatory response, suggesting synergism between these stimuli. Some studies have shown that inflammatory mediators present in blood may regulate the initial inflammatory response that develop after infection with *Leishmania*, particularly complement components. The complement system exerts a strong selective pressure on the survival of the parasite, and most of the parasites enter permissive monocytes rapidly to prevent their death[7]. Promastigotes opsonized by C3b bind to erythrocytes and are more readily phagocytized by PMN and macrophages[9]. In humans, the serum cytotoxic activity against promastigotes is the major effector mechanism during the initial invasion by *Leishmania*[8,9]. The immune adherence of promastigotes can facilitate the progression of infection by the transfer of parasites attached to red cells for blood phagocytes. Activation of the classical complement pathway by *Leishmania* in the deposition of C3 on the surface of parasites produces the C5 convertase that initiates the lytic cascade with the death of the parasite[8]. Phagocytosis of promastigotes by host cells during this period allows the *Leishmania* evade lysis by complement[7]. This is believed to be the most efficient mechanism of invasion of promastigotes in the host. This fact can explain the importance of blood to carry a greater induction of cells as a mechanism of escape and infection by *Leishmania* promastigotes.

Herein, *L. chagasi* was able to induce an inflammatory response characterized by the influx of neutrophils, macrophages and eosinophils. In addition, the association of *Leishmania* with blood and saliva induced the recruitment of more cells than did *L. chagasi* alone. Previous works demonstrated that *Leishmania* elicits the recruitment of a mixed population of inflammatory cells that can vary between species and strains[14,29]. Neutrophils were the main cells recruited after the stimuli in air pouch exudate, especially when *L. chagasi* was associated with saliva or blood and saliva. Studies showed that PMN are the first leukocytes to appear at the site of inflammation where they phagocytose the parasites, some of which are able to survive within these first host cells[6,23,35]. *Leishmania* is able to delay apoptosis of neutrophils, a mechanism that involves inhibition of caspase-3, which is known as an inducer of apoptosis in PMN[1]. Parasites that are ingested but not killed by PMN, at

this early stage, can benefit from this early accumulation of neutrophils to the site of infection, as has been shown for *L. major*[28]. The influx of neutrophils in the first 24 h modifies the T cells response, via IL-4 production, and the susceptibility to infection by *L. major*, by developing a Th2 response[28]. Furthermore, it was demonstrated that *Leishmania* promastigotes induce migration of PMN by releasing *Leishmania* chemotactic factor (LCF), and that the coincubation of *Leishmania* com PMN inhibits the chemokine CXCL10, suggesting that *Leishmania* inhibits the activity of Th1 or NK cells and, consequently, interferes with the development of the protective immune response[35], which may facilitate its progression within the host.

Also, the combination of *L. chagasi* with blood and saliva was capable of inducing an influx of many eosinophils to air pouch exudate. Other species of *Leishmania* such as *L. major*, *L. donovani* and *L. braziliensis* are also able to induce migration of eosinophils[15,30]. It is known that eosinophils, like neutrophils, are also able to phagocyte and kill *Leishmania*[13,24]. Eosinophils can also participate in the vasodilatation facilitating the blood meal and at the same time creating an inhospitable environment for pathogens transmitted by vector[4]. Sand fly saliva was also an important inducer of eosinophil migration to air pouch. In dogs, inoculation of *Lu. longipalpis* saliva resulted in an inflammatory response with the presence of an intense eosinophilia[22]. Eosinophils were also observed in the inflammatory process developed at the site of immunization with recombinant 15 kDa protein from saliva of *P. papatasi*[34]. In studies of sand fly saliva was observed that the exacerbation of infection has been linked to inhibition of production of Th1 cytokine and increased production of Th2 cytokines caused by saliva from *P. papatasi*, indicating that *Lu. longipalpis* saliva may have influenced the type of immune response, since the saliva promotes the recruitment of eosinophils, possibly by the production of eotaxin, a chemokine characteristic of the Th2 immune response[16]. The presence of eosinophils suggests a Th2 immune response induced by saliva of the vector, and this type of response would facilitate the survival of *Leishmania* in the early stage of infection.

Macrophage was the second predominant cell type in air pouch exudate, especially when the stimulus was with *L. chagasi* associated with blood and saliva. In the early phase of infection, the ability of macrophages to respond to activation signals of Th1 against intracellular pathogens is important in determining proliferation or elimination of the parasite. The recruitment of a small number of macrophages has been associated with smaller lesions in a model of immunodeficient mice[3]. The intracellular killing of *Leishmania* by macrophages depends on the production of ROS and NO, as already shown[11]. The proportion of neutrophils and macrophages changes during the first weeks of infection, and as expected, the number of neutrophils decreases soon with a concomitant increase of macrophages. Studies by[15] showed that the number of macrophages increased approximately 50% after 48 h of infection with *L. donovani*. In the present study, we observed that macrophages were more predominant cells in the punch lining tissue than neutrophils at 12 h after stimulation with *L. chagasi*. As expected, the number of neutrophils increased after 24 h of stimulation. However, in the pouch lining tissue stimulated by *L. chagasi* associated with saliva and blood the macrophages were the predominant cells with both 12 h and 24 h post-inoculation, suggesting that these cells were recruited, but may not have migrated into punch exudate, as discussed above, or actually migrated only later (after 24 h).

Histopathological analysis in punch lining tissue showed *Leishmania* associated with saliva and blood induced some important inflammatory changes, although mild to moderate, such as edema, hyperemia, hemorrhage and fibrin. Previous reports have showed that saliva from *Lu. intermedia* could induce inflammatory changes such as edema and hyperemia in the ear dermis of BALB/c[18]. The mild or moderate inflammation induced by *L. chagasi* in this study also suggests the parasite has developed a strategy to minimize the initial inflammatory response, allowing an unlimited progression within the host.

More detailed studies of leukocyte populations that migrate to the initial site of *Leishmania* inoculation, cytokine and chemokine production, as well as characterization of cellular phenotype, can clarify new aspects involved in the survival of *L. chagasi* in the host. This work reinforces the importance of studies on the salivary components of sand fly vectors of leishmaniasis in the transmission process and the establishment of the infection.

RESUMO

Migração celular distinta induzida por *Leishmania infantum chagasi* e saliva de *Lutzomyia longipalpis* em um modelo de pool hemorrágico

O recrutamento de uma população de células específicas após infecção por *Leishmania* pode influenciar o resultado da doença. A migração celular em resposta a *Leishmania* ou saliva do vetor tem sido reportada utilizando o modelo da bolsa de ar subcutânea, entretanto, a migração celular induzida por *Leishmania* associada com o sangue do hospedeiro e saliva do vetor neste modelo ainda não foi descrita. Neste trabalho foi investigada a migração celular no modelo da bolsa de ar subcutânea em hamster após a estimulação com a combinação de *L. chagasi*, sangue do hospedeiro e saliva de *Lutzomyia longipalpis*. A migração induzida por saliva foi três vezes maior do que a induzida por *L. chagasi* sozinha. Adicionalmente, *L. chagasi* associada com sangue e saliva induziu significativamente ainda mais leucócitos no exsudato inflamatório do que o estímulo com *Leishmania* sozinha. *L. chagasi* recrutou uma população de células distintas, no entanto, a maioria dessas células parece não ter migrado para o exsudato inflamatório, permanecendo no tecido da bolsa de ar. Estes resultados indicam que *L. chagasi* pode reduzir o acúmulo de leucócitos para o local inicial da infecção e que quando associada à saliva do vetor e na presença de componentes do sangue aumenta o influxo de mais neutrófilos do que macrófagos, sugerindo que o parasito desenvolveu uma estratégia para minimizar a resposta inflamatória inicial, permitindo uma progressão ilimitada dentro do hospedeiro. Este trabalho reforça a importância de mais estudos sobre os componentes da saliva dos vetores das leishmanioses no processo de transmissão e no estabelecimento da infecção.

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