ABSTRACT

EVALUATION OF THE EFFECTS OF *Leishmania braziliensis* EXOSOMES IN THE PRODUCTION OF INFLAMMATORY MEDIATORS BY MONONUCLEAR PHAGOCYTES

Exosomes are 30-100nm organelles secreted by various cell types. They occur when the endosomal membrane invaginates into multivesicular bodies (MVB) and are secreted through the melding of MVBs with the plasma membrane (PM). *Leishmania* exosomes are composed by several proteins such as heat shock proteins, annexins, GP63, proteins with signaling activity and also contain mRNAs and miRNA. Over the past 10 years it has been reported that these vesicles actively manipulate host signaling and immune cell functions, due to the enrichment by Leishmania exosomes of virulence factors such as GP63. Studies demonstrate that *Leishmania donovani* exosomes activate tyrosine-phosphatases, downregulating IFN-γ and inhibiting the expression of microbicidal molecules such as TNF and NO, which creates a microenvironment that favors parasite proliferation. Despite of not having immunological memory, studies suggest that after a first stimuli mononuclear phagocytes can get "trained", passing through epigenetic changes, and responding more effectively against a second stimuli. That way, the sensitization of macrophages with *L. braziliensis* exosomes, prior to its infection by this pathogen, may be associated with higher inflamatory cytokines production as well as inflammasome activation; once the pathways involved in this process would already be activated. **Methods and Results:** Healthy subjects' macrophages were sensitized for 24 hours with *L. braziliensis* exosomes. Afterwards, these cells were infected with *L. braziliensis* and cultured for 24 hours. We observed higher levels of IL-1β and IL-6 in cultures that were sensitized before infection than in cells only infected. Furthermore, stimulation with *L. braziliensis* exosomes induced production of IL-1β, IL-6, IL-10 & TNF by macrophages. We inhibited the secretion of exosomes by *L. braziliensis* prior to macrophage infection and observed that cells infected with those parasites induced less production of IL-1β, IL-6, IL-10 & TNF and cultures presented lower infection rate than cells that were infected with regular *Leishmania*. Exosome stimulation also induced the consumption of NLRP3 inflammasome in macrophages and the blocked of these receptors resulted in lower levels of IL-6, TNF and IL-1β. Our results suggest that *L.*

braziliensis exosomes stimulate macrophages leading to inflammation and those effects might be NLRP3 dependent.

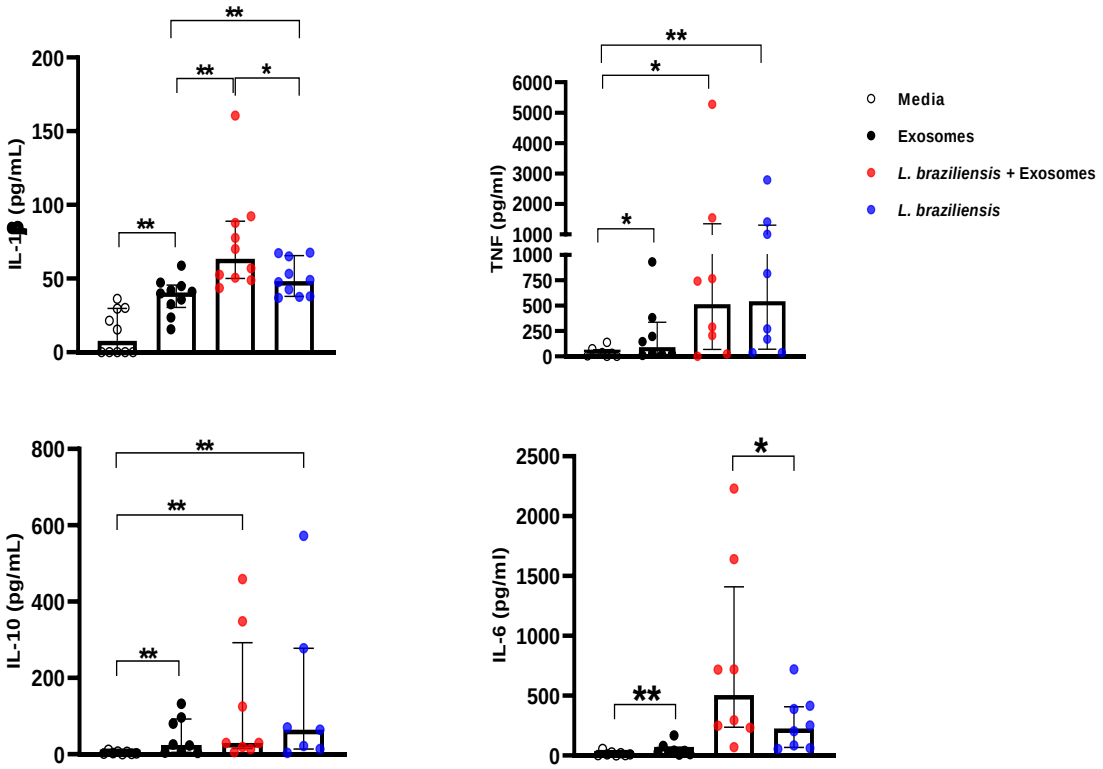
Key words: *Leishmania braziliensis*, macrophage, exosomes, inflammasome.

RESUMO

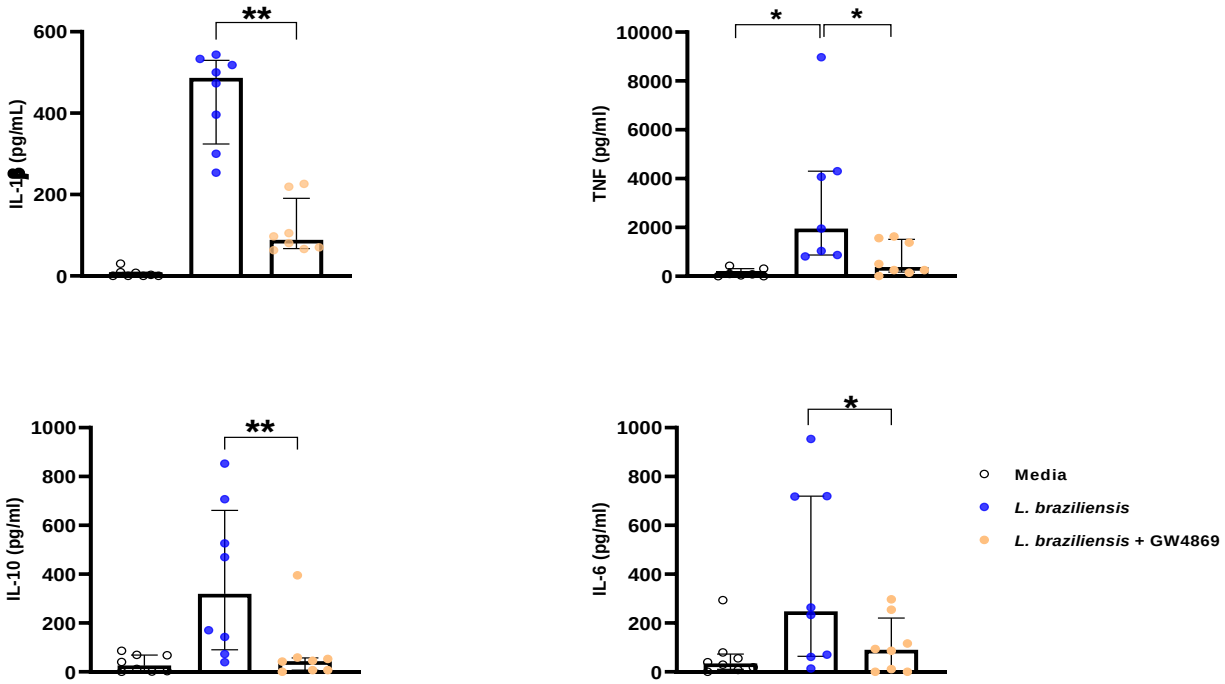
Exossomos são organelas de 30 a 100 nm secretadas por vários tipos de células. Elas ocorrem quando a membrana endossomal invagina em corpos multivesiculares (MVB) e são secretados através da fusão de MVBs com a membrana plasmática (PM). Os exossomos de Leishmania são compostos por vários fatores de virulência, como heat shock proteins, anexinas, GP63, proteínas de sinalização e também contêm mRNAs e miRNA. Nos últimos 10 anos, foi relatado que essas vesículas manipulam ativamente a sinalização do hospedeiro e as funções das células imunes, devido aos exossomos de *Leishmania* serem ricos em fatores de virulência como GP63. Estudos demonstram que os exossomos de *Leishmania donovani* ativam tirosina-fosfatases, regulando negativamente o IFN-γ e inibindo a expressão de moléculas microbicidas como TNF e NO, criando um microambiente que favorece a proliferação parasitária. Apesar de não possuir memória imunologica, estudos sugerem que após um primeiro estímuo os fagócitos momonucleares ´pdem ser “treinados”, passando por mudanças epigenéticas e respondendo de forma mais eficaza um segundo estímulo, dessa forma, a sensibilização de macrófagos com exossomos de L. braziliensis, antes de sua infecção por esse patógeno, pode estar associada à maior produção de citocinas inflamatórias, e ativação de inflamassoma, uma vez que as vias envolvidas neste processo já estariam ativadas. **Métodos e Resultados:** Macrófagos de indivíduos saudáveis foram sensibilizados por 24 horas com exossomos de *L. braziliensis*. Em seguida, essas células foram infectadas com *L. Braziliensis* e cultivadas por 24 horas. Observamos níveis mais elevados de IL-1β e IL-6 em culturas que foram
sensibilizadas antes da infecção do que em células apenas infectadas. Além disso, a estimulação com *L. braziliensis* induziu a produção de IL-1β, IL-6, IL-10

e TNF por macrófagos. Nós inibimos a secreção de exossomos por *L. braziliensis* antes da infecção de macrófagos e observamos que células infectadas com esses parasitas induziram menor produção de IL-1β, IL-6, IL-10 & TNF e as culturas apresentaram menor taxa de infecção do que células que foram infectadas com *Leishmania* normal. A estimulação com exossomos também induziu o consumo de inflamassoma NLRP3 em macrófagos e o bloqueio desses receptores resultou em níveis mais baixos de IL-6, TNF e IL-1β. Nossos resultados sugerem que os exossomos de L. braziliensis estimulam macrófagos levando a inflamação e esses efeitos podem ser dependentes de NLRP3.

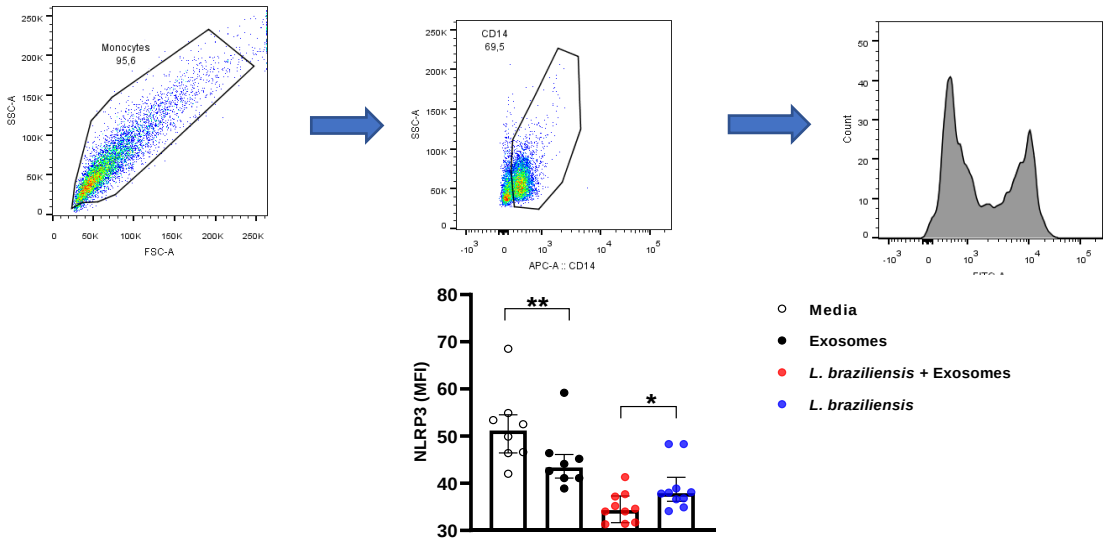
PRINCIPAIS GRÁFICOS



Cytokine production by macrophages from HS sensitized with *L. braziliensis* exosomes and infected with *L. braziliensis*. Macrophages from HS (n=8) were stimulated with exosomes isolated from *L. braziliensis* (300:1) for 24 hours and afterwards infected or not with *L. braziliensis* for another 24 hours. Levels of IL-1β (A), TNF (B), IL-10 (C) and IL-6 (D) were determined in culture supernatants by LUMNEX. Statistical analyses were performed using the Wilcoxon rank test *p<.05, **p<.01



Cytokine production by macrophages from HS infected with *L. braziliensis* with exosome production inhibited. Axenic culture of *L. braziliensis* promastigotes was treated with GW4869 (20ng/ml), exosome production inhibitor, for 2 hours. Macrophages from HS (n=8) were infected with GW4869 treated *L. braziliensis* (5:1) for 24 hours. Levels of IL-1β (A), TNF (B), IL-10 (C) and IL-6 (D) were determined in culture supernatants by LUMNEX. Statistical analyses were performed using the Wilcoxon rank test *p<.05, **p<.01.



Exosome stimulation enhance NLRP3 consumption by macrophages. Macrophages from healthy subjects (n=8) were sensitized with exosomes (300:1) for 24 hours and infected with *L. braziliensis* promastigotes for another 24 hours at a ratio of 5:1. Cells were stained with anti-CD14 and anti-NLRP3. Data were collected using flow cytometry and analyzed with FLOWJO® software. (A) Representative gating strategy on $CD14^{+}$ expression in macrophages from one healthy subject. NLRP3 MFI was taken from $CD14^{+}$ population. (B) The data represent the mean of fluorescence intensity (MFI) of NLRP3 expression by macrophages in the different stimulated groups. Statistical analyses were performed using the Mann-Whitney test for unpaired groups and Wilcoxon rank test for paired measurements *p<.05 **p<.01.