Original Article:
Comparision of the Effects of Leishmania Soluble Antigen (LSA) and Lipopolysaccharide (LPS) on C57BL/6 Mice Macrophage Function

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Citation Khosrowpour Z, Soudi S, Hashemi SM. Comparision of the Effects of Leishmania Soluble Antigen (LSA) and Lipopolysaccharide (LPS) on C57BL/6 Mice Macrophage Function. Immunoregulation. 2018; 1(1):39-44. http://dx.doi.org/10.32598/Immunoregulation.1.1.39

doi: http://dx.doi.org/10.32598/Immunoregulation.1.1.39

Article info:
Received: 03 December 2016
Accepted: 28 June 2017
Available Online: 01 April 2018

Keywords:
Lishmania,Macrophage, Lipopolysaccharide, Soluble antigen

ABSTRACT

Background: Macrophages activation is the important anti-leishmania immune response. Different signals could affect macrophages development and functional activation. In the present study, we compared the effect of Leishmania Soluble Antigen (LSA) and Lipopolysaccharide (LPS) on peritoneal macrophage responses. Appropriate activation of macrophages depends on the signals they receive from pathogens and their different functional differentiation is crucial for anti-leishmania effects of macrophages.

Materials and Methods: In order to assay C57BL/6 mice macrophage function after LPS or LSA treatment, we measured phagocytic activity, cytokine pattern, and Nitric Oxide (NO) production by macrophages.

Results: Phase contrast microscopy showed that LPS-treated macrophages became more granular and spindle-shaped and similar to untreated macrophages, LSA-treated cells displayed round and spindle-shaped morphology. In addition, Nitric oxide assay and cytokine analysis showed that IL-6, IL-10, and TNF-α production was significantly reduced in LSA-treated macrophages in comparison with LPS-stimulated cells. It was also found that LSA-treated macrophages represented an anti-inflammatory phenotype compared with LPS-treated macrophages.

Conclusion: This anti-inflammatory phenotype was related to increase in IL-10/TNF- α production of LSA-treated macrophages and there was no difference in the amount of TGF-β between LSA- and LPS-treated groups.

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Introduction

Leishmania promastigotes are taken up by neutrophils and macrophages. Macrophages are the final host cells for proliferation and differentiation of promastigotes. In addition, macrophages activation is the important anti-leishmania immune response. Different signals could affect macrophages' development and functional activation. There are two macrophage subsets: Classical and alternative activated macrophages. Interferon Gamma (IFN-γ) or microbial products such as Lipopolysaccharide (LPS) are important activators of macrophages. Pattern Recognition Receptors, such as Toll-like Receptors (TLRs) are involved in directing and initiating adaptive immunity and the outcome of parasite within macrophages. The phagocytosis and survival of parasites in the macrophages are determined by surface Lipophosphoglycan, GP63, and Proteophosphoglycans on L. major promastigotes [1, 2]. It is reported that alternative macrophage activation leads to survival of parasites [3-5].

Appropriate activation of macrophages depends on the signals they receive from pathogens and their different functional differentiation is crucial for anti-leishmania effects of macrophages. In the present study, attempts were made to study the effect of LSA and LPS on peritoneal macrophage responses. In order to assay C57BL/6 mice macrophage function after LPS or LSA treatment, we measured phagocytic activity, cytokine pattern, and NO production by macrophages.

Materials and Methods

Animals

Female C57BL/6 mice were used for macrophage. Female BALB/c mice were used for parasite maintenance. Mice were purchased from Pasture Institute of Iran. The animals were housed under standard laboratory conditions according to the guidelines of Shahid Beheshti University of Medical Sciences, Tehran, Iran, for animal care and handling.

Parasites and antigen preparation

In order to isolate Leishmania major (MRHO/IR/75/ER) promastigotes, spleens and lymph nodes of the infected BALB/c mice were minced and cultured in the liquid phase of Novy-MacNeal-Nicolle medium (NNN) and promastigotes were sub-cultured in RPMI medium supplemented with 5% FBS and expanded at 25°C until stationary growth. To prepare LSA, the parasites were centrifuged and washed three times using cold sterile Phosphate-Buffered Saline. Then, they were counted and diluted to 109 ml and a five-cycle rapid freeze and thaw process was used and the lysate was centrifuged at 8000 g for 15 min at 4 °C. The supernatant was collected and stored at -70 °C. Protein content of soluble antigens were determined via Bradford assay [6].

Peritoneal macrophage isolation and culture

Thioglycollate-elicited macrophages were extracted from C57BL/6 by peritoneal washing with ice cold RPMI and enriched via plastic adherence (1 h, 37°C, 5% CO_2). After one hour, non-adherent cells were removed. Macrophages were counted and cultured at 5×105 cells/well, and then cultured in RPMI, 2 mM glutamine, 50 U/mL of penicillin, and 50 µg/mL streptomycin supplemented with 10% FBS in 24-well culture plates (37°C, 5% CO_2). Next, macrophages were treated with 10µg/ml LPS or LSA for 72 hours. Non-treated macrophages were used as control.

Cytokine assay

After treatment of the macrophages, the supernatant of LPS- or LSA-treated and non-treated macrophages were collected and stored at -70 °C. IL-6, TGF-β, IL-10, and TNF-α production by macrophages were analyzed via enzyme-linked immunosorbent assay (ELISA) using Douset kits (R&D systems, USA), following the manufacturer's instructions.

Nitric oxide measurement

The supernatant of LPS- or LSA-treated and non-treated macrophages were analyzed for NO production 72 hours after the treatment period of macrophages. For this purpose, 100 µl of the Griess reagent were added to 100 µl of the supernatant. The absorbance of the developed color was read at 540 nm. NO concentrations were calculated using standard solution of sodium nitrite (Merck) prepared in culture media.

Statistical analyses

For in vitro measurements, One-way Analysis of Variance was run to determine the statistical difference between experimental groups. Data are presented as Mean±SD. Differences between mean values were considered significant when $P<0.05$. Data were analyzed using GraphPad Prism 5.0 software (Graph Prism Inc., San Diego, Ca).

Results

Phase contrast morphology of LSA and LPS treated macrophages

Phase contrast microscopy indicated that untreated macrophages showed a mixture of round and spindle-shaped cells. Upon treatment with LPS, macrophages became more granular and spindle-shaped. Similar to untreated macrophages, LSA-treated cells displayed round and spindle-shaped morphology (Figure 1).

Cytokine assay of LSA and LPS treated macrophages

To determine the LSA on C57BL/6 mice macrophage function, we measured the amount of pro- and anti-inflammatory cytokines in the macrophage-conditioned medium sampled 72 h after activation using commercial ELISA kits for TNF-α, TGF-β, IL-6, and IL-10 produced in the supernatant of stimulated macrophages. Cytokine analysis showed that IL-6, IL-10, and TNF-α production was significantly reduced in LSA-treated macrophages in comparison with that in LPS-stimulated cells (Figure 2). In addition, no significant differences were observed in

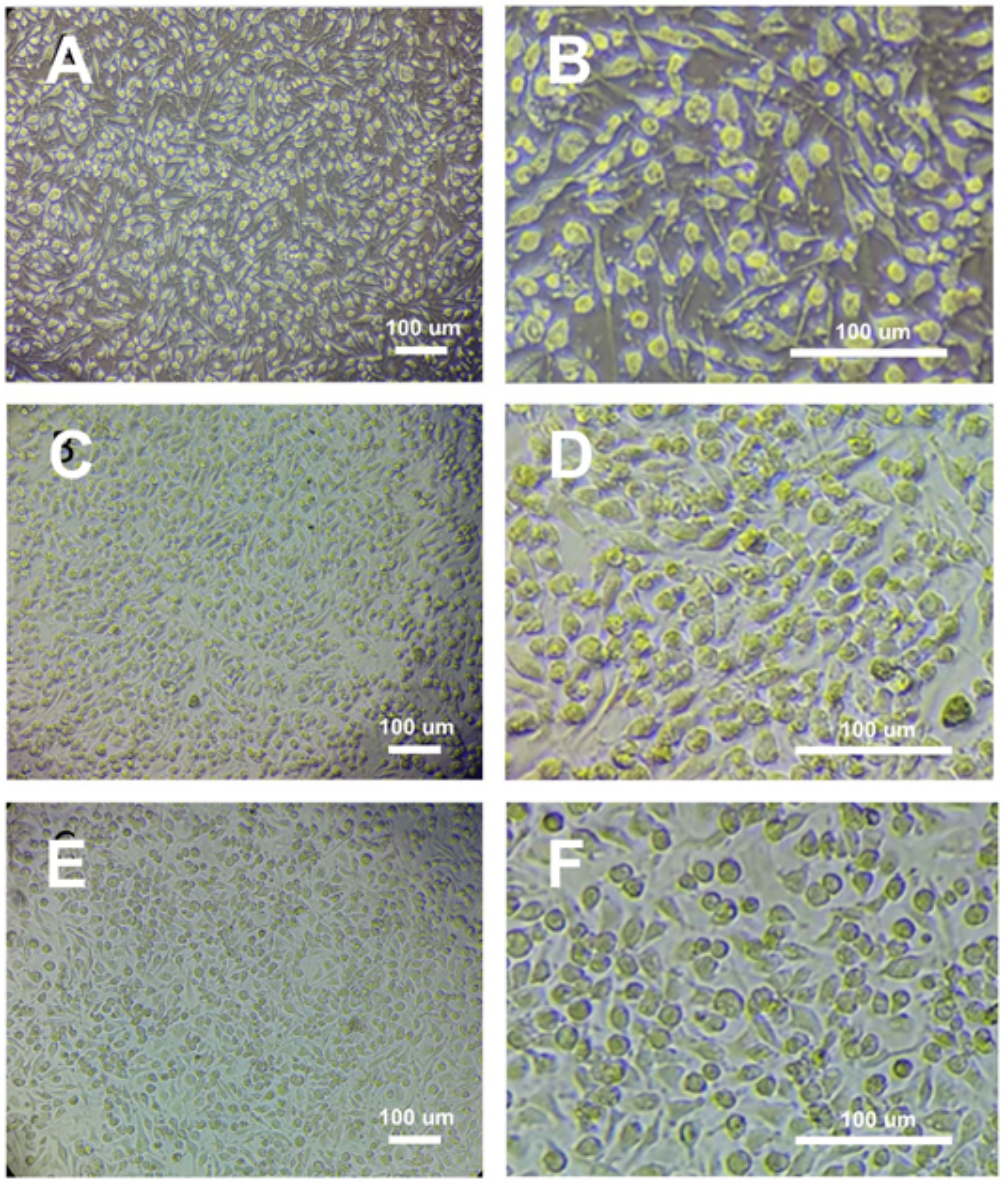

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Figure 1. Phase contrast morphology of LSA and LPS treated macrophages. Macrophages treated with LPS (A, B). Macrophages treated with LSA (C, D) untreated macrophages (E, F)

Cytokine assay of LSA and LPS treated macrophages

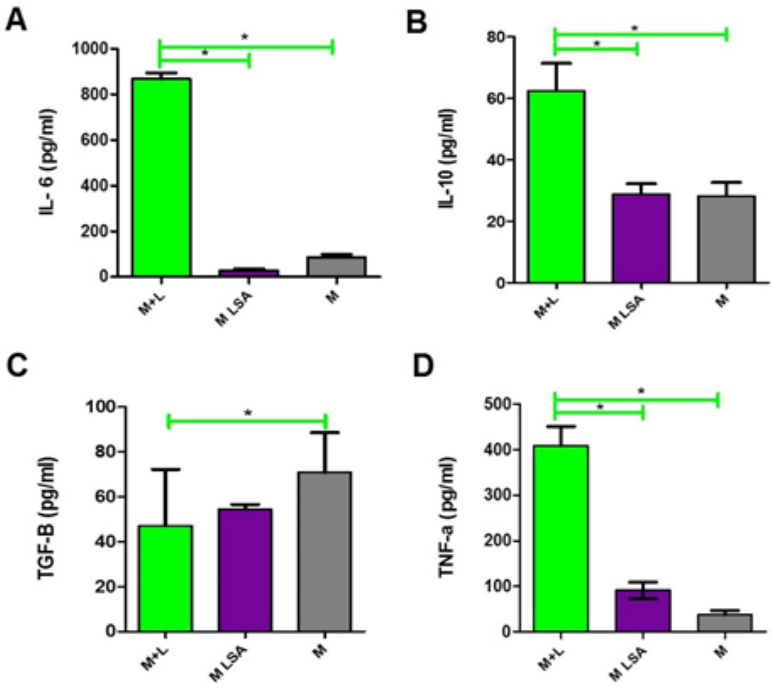

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Figure 2. Cytokine production in the supernatants of of LSA- and LPS-treated macrophages. Pro- and anti-inflammatory cytokines in the macrophage-conditioned medium sampled 72 h after activation using commercial ELISA kits for TNF-α, TGF-β, IL-6, and IL-10 produced in the supernatant of stimulated macrophages. Data are expressed as the Mean±SD of three independent experiments. * Indicated groups are significantly different from each other (P≤0.05).

NO assay of LSA- and LPS-treated macrophages

TGF-β, IL-6, and IL-10 production between LSA-stimulated macrophages and unstimulated cells (Figure 2).

NO assay of LSA- and LPS-treated macrophages

NO production was measured in the supernatant of C57BL/6 macrophages 72 h after activation by LPS or LSA treatment. NO production significantly increased after LPS stimulation in comparison to LSA-treated and untreated macrophages (Figure 3).

Discussion

In the present study, we compared the effect of LSA and LPS on peritoneal macrophage responses. Numerous studies have shown various stimulators, such as LPS, on the morphology of macrophages [7, 8], genetic reprogramming, and the expression of pro-inflammatory cytokine and phagocytic activity [9-12]. Although, activation of macrophages by LPS through TLR 4 and the mechanism of the TLR signaling pathway has been well documented [11, 13] in the current study we focus on LSA and its effects on macrophage activation and function.

The results indicated that LPS and LSA have different effects of macrophage responses even in their morphology. Phase contrast microscopy showed that LPS-treated macrophages became more granular and spindle-shaped and similar to untreated macrophages, LSA-treated cells displayed round and spindle-shaped morphology (Figure 1). Macrophages are divided into two main functional categories according to their inflammatory (M1) and

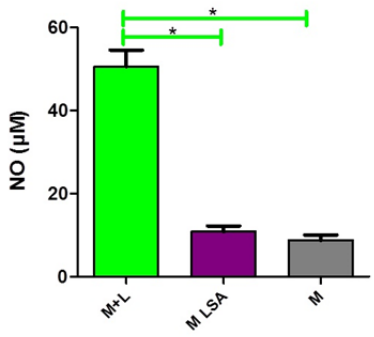

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Figure 3. NO production in the supernatant of LSA- and LPS-treated macrophages. Data are expressed as the mean± SD of three independent experiments. *Indicated groups are significantly different from each other (P≤0.05).

anti-inflammatory (M2) behavior [14-16]. Different activation methods induce macrophage polarization to M1 or M2 phenotype [17,18]. According to different studies, LPS can induce M1 macrophages with more pro-inflammatory cytokine production and upregulation of inducible NO Synthase (iNOS) that terminated to anti-microbial properties [19,20].

A similar macrophage phenotype was induced in the current experiment when C57BL/6 macrophages were treated for 72h with LPS. They showed higher IL-6, TNF-α, and NO production compared to non-treated macrophages. In addition LPS treatment induces anti-inflammatory IL-10 cytokine production to regulate the created inflammatory situation, while the TNF-α/IL-10 ratio is still to the benefit of TNF-α [21]. In contrast, LSA activation of macrophages demonstrated a significant lower IL-10, IL-6, and TNF-α cytokine production compared to LPS-treated group. However, the TNF-α/IL-10 ratio is to the benefit of IL-10 production that reflected the induction of M2 phenotype by LSA activation.

Because of the anti-inflammatory role of IL-10, it seems that LSA can induce immunoregulatory phenotype in peritoneal macrophages to ameliorate the immunopathological properties of inflammatory cytokines in infection [22]. However, although not significant, LSA-treated macrophages induced higher level of TNF-α cytokine compared to non-treated macrophages, which demonstrated the existence of TNF-α stimulatory PAMPs among the LSA. In accordance with low TNF-α production, LSA-treated macrophages produced lower NO level compared to LPS-treated group. So, the LSA-activated peritoneal macrophages cannot actively participate in the clearance of intracellular pathogens through detrimental role of nitric [23].

Our results indicated that NO assay and cytokine analysis showed that IL-6, IL-10, and TNF-α production was significantly reduced in LSA-treated macrophages in comparison with LPS-stimulated cells. In conclusion, we found that LSA-treated macrophages represented an anti-inflammatory phenotype compared to LPS-treated macrophages. This anti-inflammatory phenotype was related to increase in IL-10/TNF- α production of LSA-treated macrophages and there was no difference in the amount of TGF-β between LSA- and LPS-treated groups. According to the findings, LSA can be used for induction of anti-inflammatory responses through IL-10 function to control immune-pathological responses.

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