Linoleic Acid Inhibits the Release of *Leishmania donovani* Derived Microvesicles and Decreases Its Survival in Macrophages

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Visceral leishmaniasis is a neglected tropical disease caused by *Leishmania (L.) donovani* parasite in the Indian subcontinent. Macrophages (mφ) are the harboring cells for parasite and their interactions dictate the pathogenesis of this disease. Polyunsaturated fatty acids are an integral part of the mφ cell membrane and are derived from linoleic acid (LA), which is a principal essential fatty acid. Here, we have investigated the effect of the simultaneous presence of LA during *L. donovani* infection in mφ. Treatment with LA suppresses the parasitic load in mφ (kDNA expression) and promotes the Th-1 type immune response (IL-12, iNOS). However, no significant change in kDNA expressions was observed when *L. donovani* promastigotes were treated with LA. Intrigued by this observation, we explored mechanism(s) by which LA promoted the protective type immune response in infected mφ. Interestingly, LA decreased the release of *L. donovani* derived extracellular vesicle later characterized as microvesicles. Moreover, these microvesicles were suppressive concerning their bias toward the Th-2 type of immune responses (IL-10, Arginase) in mφ. We suggest that LA plays a protective role in the immune response against *L. donovani* infection by inhibiting the release to *Leishmania* derived microvesicles and thus promoting Th-1 type immune response in mφ.

**Keywords:** visceral leishmaniasis, *Leishmania donovani*, linoleic acid, *Leishmania* derived microvesicles, immune-modulation

**INTRODUCTION**

*Leishmania (L.) donovani* is the causative agent of disease visceral leishmaniasis (VL) in the Indian subcontinent. The disease affects primarily underprivileged people in Bihar, Jharkhand, West Bengal and eastern part of Uttar Pradesh. The global incidences of VL are between 50,000 and 90,000 (WHO, 2019). There is no human vaccine available against VL in the market and chemotherapy has its own drawbacks especially evolving drug resistance (Mishra et al., 2007). The emergence of post kala-azar dermal leishmaniasis (PKDL) cases in the past few years (NVBDCP, 2019), HIV-VL co-infection (Sinha et al., 2005), the emergence of drug resistance (Mishra et al., 2007) and unavailability of a human vaccine against VL suggest a need for more effective control measures. Advancement in controlling the disease requires an improved understanding of the host-parasite interaction in VL infection.
The pathogenesis of VL depends significantly upon macrophage (mϕ)-Leishmania interactions and further their encounter with T cells. As major components of the cellular membrane, polyunsaturated fatty acids (PUFA) play a pivotal role in maintaining the membrane fluidity and increase the innate immune threshold in mϕ, which is essential for appropriate antigen presentation to T cells (Sen et al., 2001). It is evident that mϕ enriched with PUFAs specially arachidonate showed ~50% enhancement of phagocytic and adhesion capacity (Calder et al., 1990). Linoleic acid (LA) is an essential polyunsaturated fatty acid (PUFA) and the precursor of long-chain PUFAs in the mammals. Various groups including ours have reported the role of prostaglandins and leukotrienes in Leishmania infection (Morato et al., 2014; Saha et al., 2014; Chaves et al., 2016; Saini et al., 2019). However, supplementation with their dietary precursor i.e., LA is a more viable and feasible approach in the treatment of human VL, but such studies are lacking.

In the present study, we have analyzed the effect of the simultaneous presence of LA during L. donovani infection in mϕ. LA suppressed parasitic load (kDNA gene expression) in infected mϕ and promoted Th-1 type protective immune response. However, it did not show any direct leishmanicidal activity in vitro on L. donovani promastigotes. Further exploration showed that LA reduces the release of extracellular vesicles from L. donovani parasite (promastigote form), which were later characterized as microvesicles (Muralidharan-Chari et al., 2010; Raposo and Stoorvogel, 2013; Marcilla et al., 2014). The immunomodulatory properties of these L. donovani derived microvesicles (LdMv) were also analyzed in vitro and were found to be immunsuppressive. Taken together, our data indicate that LA modulates the release of L. donovani derived microvesicles (LdMv) that restrain the parasitic load and promote pro-inflammatory type immune response.

MATERIALS AND METHODS

Reagents
RPMI-1640 media (Cat. No. 31800-022, Gibco Life Technologies, USA), FBS (Cat. No. 10270106, Gibco Life Technologies, USA), antibiotic cocktail (Penicillin + Amphotericin B + streptomycin; only for maintenance media; Cat. No. E485, Amresco Inc., USA), Kanamycin (only for infection media; Cat. No. Kanamycin-750, Macleod Pharmaceuticals Ltd., India), NaHCO3 (Cat. No. 27765, Fischer Scientific Pvt. Ltd., India), HEPES (Cat. No. MB016, Himedia Laboratories Pvt. Ltd., India), and L-Glutamine (Cat. No. G0063, TCI Chemicals Pvt. Ltd., India) were procured for cell culture purpose. Pure Linoleic Acid (LA; Cat. No. sc-200788A) was purchased from Santa Cruz Biotechnology, USA. Details of other reagents are given in their respective method sections.

Parasite
Promastigote form of L. donovani (dd8; MHOM/IN/80-DDd8; WHO reference strain of Indian origin) was maintained in M199 medium with 10% FBS at 25–27°C in anaerobic condition (Tiwari et al., 2016). The parasite strain was obtained from the Central Drug Research Institute, Lucknow, India. These parasites were being routinely maintained in our laboratory (MNNIT Allahabad) and were tested for expressions of L. donovani specific actin/tubulin gene regularly. For experiments, cells were fixed with formaldehyde (4%) and counted using a Neubauer chamber.

Culture of Macrophage Cell Line and in vitro Assays
The mouse mϕ cell line (J774A.1) was obtained from Central Drug Research Institute, Lucknow, India and maintained in RPMI-1640 media (with 10% FBS) at 37°C in 5% CO2 incubator (Model No. ESCO CellSafe, Esco Micro Pvt. Ltd., Singapore) at MNNIT Allahabad (Saini et al., 2019). For experiments, 2 x 106 mϕ cells were counted with the help of the Neubauer chamber and seeded in the six well plates. After 3–4 hours (h), non-adherent cells were removed and the assay was performed with adhered cells. To establish the infection of L. donovani to mϕ, adherent J774A.1 mϕ cells were incubated with the 20 x 106 L. donovani parasites (MOI; 1:10). After 12 h of incubation, the non-infecting parasites were removed by washing with sterile PBS/incomplete RPMi (thrice). As per the experimental plan, cells were treated with 500 nM LA in culture (on the basis of literature) along with the parasite infection. After completion of assays, cells were processed for RNA/DNA isolation.

Gene Expression Analysis Using Real-Time PCR (qPCR)
Total RNA was isolated from mϕ (uninfected as well as infected) and processed further as described by Saini et al. (2020a). All the primers used in the present study (Supplementary Table 1) were commercially synthesized from Eurofins genomics, India. Expression analysis of gene was performed using qPCR (Cat. No. TCR0096, PikoReal real-time PCR System). The housekeeping gene i.e., HGPRT (cell line)/α-tubulin (parasite) was used to normalized cT values in control and experimental tubes. The data is represented as fold change in gene expression (2−ΔΔcT value) considering untreated mϕ as reference/control (fold change = 1) (Schmittgen and Livak, 2008; Sindhu et al., 2018).

Measurement of Parasite Load Using kDNA
A modified Fan and Gulley method was used for the isolation of DNA from infected mϕ cells (Fan and Gulley, 2001). Briefly, the parasitic load in infected mϕ was quantified by measuring the copy number of L. donovani specific kDNA in isolated DNA using qPCR (Verma et al., 2019).

Fluorimetry Analysis
The culture supernatant of L. donovani parasite was incubated with 10 μM Diphenylhexatriene (DPH; Cat. No. 66525, SRL Pvt. Ltd., India) for 60 min at room temperature. After completion of incubation, the analysis was performed in a fluorescence spectrophotometer (LS-45, Perkin Elmer Inc., USA) in the Center for Interdisciplinary Research (CIR), MNNIT Allahabad. Scan between 385 and 650 nm was performed and maxima was observed at 430 nm. Hanks' balanced salt solution (HBSS) (Tiwari et al., 2016) alone was taken as background and was deducted to get normalized fluorescence intensity. Experiments
were performed three times independently and every time in triplicate.

**GC-HRMS Analysis**
To assess the fatty acid profile of culture supernatant of *Leishmania donovani*, GC-HRMS was used. Fatty acids from culture supernatant were extracted using Bligh and Dyer method (chloroform/methanol method) (Bligh and Dyer, 1959). Briefly, 3.75 ml chloroform and methanol (1:2) were added to 1 ml sample (supernatant). Heptadecanoic acid (5 mM/L) (Cat. No. H3500, Sigma Aldrich, USA) was added as the internal standard. Tubes were vortexed well and 1.25 ml Chloroform was added. After mixing, an equal amount (1.25 ml) of double distilled water was added. Then, the tubes were centrifuged at 1,000 rpm for 5 min at room temperature. The lower organic phase was recovered and the solvent was dried. The lipid extract obtained was dissolved in 2 ml BF₃-Methanol (14%). Sealed tubes were kept at 55°C for 1.5 h with vigorous shaking after every 20 min. After 1.5 h, 2 ml saturated NaHCO₃ solution and 2 ml Hexane was added. Tubes were centrifuged at 1,000 rpm for 5 min and the organic phase was collected for analysis. Final samples were transferred to screw cap glass tubes and were transported to SAIF- IIT Bombay for GC-HRMS analysis under appropriate conditions.

**Characterization of Extracellular Vesicles Released by *L. donovani* (Ld) Promastigotes**
5 × 10⁶/ml parasites were cultured for 6 h with and without LA (significant uptake of LA was observed by promastigotes form of parasite after 6 h). After 6 h, the supernatants were harvested after centrifugation at 1,200 rpm for 10 min to remove parasites. These stationary phase *L. donovani* promastigotes were washed with PBS and fixed in 2.5% glutaraldehyde overnight (25°C). Fixed parasites were washed, dissolved in sterile water, placed on glass coverslips and kept at 37°C for complete drying. The supernatant was further centrifuged twice at 10,000 rpm for 30 min to harvest secreted microvesicles (Mv) (Greening et al., 2015). These Mv and dried parasite samples were transported to All India Institute of Medical Sciences (AIIMS) New Delhi for further analysis. Samples were analyzed under the Scanning Electron Microscope (EVO18 Zeiss, Oberkochen, Germany) in the sophisticated analytical instrumentation facility of AIIMS New Delhi. Besides this, the isolated Mv was finally dissolved in PBS and analyzed in particle size analyzer (Nanotrac Wave, Microtrac, USA) in CIR, MNNIT Allahabad.

**Statistical Analysis**
Experiments were performed independently in three sets. Each reaction was carried out in duplicates. The data are represented as Mean ± S. D. To compare the differences between two groups, student’s *t*-test (paired and unpaired) was used. A *p* < 0.05 was regarded as significant and shown with the graph. The analysis was done using SPSS 15.0 and Graph Pad Prism-5.0. All the graphs and figures were made using Graph Pad Prism-5.0.

**RESULTS**

**LA Decreases the Parasitic Load in *mφ* and Promotes Pro-inflammatory Response**
Presence of LA at the time of *L. donovani* infection to *mφ* suppresses the parasite load in infected *mφ* ([Figure 1A](#fig1a){refig}); Mean ± S. D., 1.092 ± 0.3801) as compared to infected control (infected *mφ* without any treatment) (Mean ± S. D., 9.018 ± 1.243) ([Figure 1A](#fig1a){refig}). However, we didn’t observe any significant change in kDNA of *L. donovani* promastigotes upon treatment with LA (without *mφ*; [Figure 1B](#fig1b){refig}). Findings showed increased expressions of immune markers of Th-1 type upon LA treatment in *L. donovani* infected *mφ* ([IL-12 (Figure 1C); Mean ± S. D., 3.917 ± 0.833] and iNOS ([Figure 1D]; Mean ± S. D., 4.167 ± 0.917]) as compared to control (IL-12; Mean ± S. D., 2.497 ± 0.209 and iNOS; Mean ± S. D., 1.945 ± 0.497). Treatment of LA also showed a simultaneous decrease in the Th-2 type immune markers in *L. donovani* infected *mφ* ([IL-10 (Figure 1E); Mean ± S. D., 0.7025 ± 0.0638] and Arginase-I ([Figure 1F]; Mean ± S. D., 0.816 ± 0.070]) as compared to infected but untreated *mφ* (IL-10; Mean ± S. D., 3.243 ± 0.942 and Arginase-I; Mean ± S. D., 2.807 ± 0.195). For calculation of relative fold change in expressions, uninfected *mφ* were taken as control (fold change = 1, shown as a dashed line in figures). The significant difference between two groups was analyzed using paired *t*-test.

**LA Reduced the Release of Membranous Bodies in the Supernatant of *L. donovani* Culture**
Supernatants of *L. donovani* promastigote cultures (LA treated and untreated) were analyzed for the presence of membrane bodies using diphenylhexatriene (DPH) dye (10 µM) ([Figures 2A,B](#fig2ab){refig}). DPH dye binds to cell membranes and demonstrates strong fluorescence when intercalated into lipid membranes. We have observed high fluorescence intensity of DPH in the supernatant of untreated culture (Mean ± S. D., 0.223 ± 0.020) as compared LA treated culture (Mean ± S. D., 0.121 ± 0.019) ([Figure 2B](#fig2b){refig}), which suggests a low number of membranous bodies in treated samples.

The presence of fatty acid (FA) in the culture supernatant of promastigote culture was analyzed by GC-HRMS, as it also indicates the presence of membranous bodies ([Chromatograph-Supplementary Figure 1](#figsup1){refig}). Internal standard (Heptadecanoic acid) was identified at 15.4 min. GC-HRMS results revealed that the concentrations of Palmitic acid (PA, 16:0; 14.3 min) and Stearic acid (SA, 18:0; 16.5 min) were decreased in LA treated *L. donovani* culture supernatant (Mean ± S. D., 442,600 ± 182,800 and 20,800 ± 153,200, respectively) as compared to untreated samples ([Figures 2C,D](#fig2cd){refig}; Mean ± S. D., 1,056,000 ± 141,600 and 691,600 ± 205,000, respectively). Hence, our GC-HRMS data also points toward the presence of membranous bodies in the *L. donovani* promastigote culture supernatant, which are decreased upon LA treatment.

Our SEM data showed the release of membranous bodies/extracellular vesicles by *L. donovani* promastigotes under appropriate culture conditions, which was characterized...
FIGURE 1 | Presence of Linoleic acid (LA) at the time of Leishmania donovani (Ld) infection to macrophages (mφ) decreases the parasite load and modulates the immune response. (A) The absolute expression ($2^{-\Delta\Delta C_{T}}$) of kDNA i.e., parasite load, is shown in Ld infected mφ with and without supplementation of LA. The treatment of LA has been given simultaneously at the time of infection. (B) The bar diagram shows the relative expressions ($2^{-\Delta\Delta C_{T}}$) of kDNA in Ld promastigotes upon treatment with LA. Relative fold change ($2^{-\Delta\Delta C_{T}}$) in mRNA expressions of IL-12 (C), iNOS (D), IL-10 (E), and Arginase-I (F) being shown under three different conditions i.e., mφ (J774A.1) alone (uninfected control; horizontal dotted line, fold change = 1), Ld infected mφ and Ld infected mφ + LA. The results are representative of three independent experiments and each experiment was performed in triplicate. Data are expressed as Mean ± S. D. and significant differences are shown as the $p$-value on the graph.
by vesicular blebbing of the cell membrane (Figure 2E). The same was not observed upon treatment with LA (Figure 2F). To characterize whether these membranous bodies are exosomes (30–100 nm) or and microvesicles (0.1–1 μm) (Marcilla et al., 2014), excreted membranous bodies were characterized using SEM and particle size analysis (PSA). The findings of SEM showed the presence of vesicles of sizes ranging from 0.5 to 1 μm in size (Figures 3A, B; Mean ± S. D., 0.819 ± 0.200 μm) which characterize these vesicles as microvesicles (Muralidharan-Chari et al., 2010; Raposo and Stoorvoel, 2013; Marcilla et al., 2014). Results obtained from PSA also suggested the same and the average size of vesicles was also ranging between 0.5 and 1 μm (Figure 3C; Mean ± S. D., 0.729 ± 0.269 μm).

**Immuno-Modulatory Properties of \( L. \) donovani Derived Microvesicles (LdMv)**

Intrigued by our observation, we were interested to understand the immunomodulatory properties of *Leishmania donovani* derived microvesicles (LdMv) and its effects on parasite load within mφ. LdMv were first generated from \( 5 \times 10^6/\text{ml} \) of parasite culture and used in *in vitro* experiments as per the plan. In the presence of LdMv, *Ld* infected mφ showed decreased expressions of pro-inflammatory immune markers [IL-12 (Figure 4A; Mean ± S. D., 0.834 ± 0.242) and iNOS (Figure 4B; Mean ± S. D., 0.622 ± 0.198)] as compared to *Ld* infected mφ without treatment (infected mφ only) (IL-12: Mean ± S. D., 2.095 ± 0.305 and iNOS: Mean ± S. D., 2.618 ± 0.285).
Mean ± S. D., 1.458 ± 0.404). Simultaneously, expressions of anti-inflammatory markers (IL-10 and Arginase-1) were increased after LdMv treatment [IL-10 (Figure 4C; Mean ± S. D., 5.470 ± 1.011) and Arginase-1 (Figure 4D; Mean ± S. D., 3.236 ± 0.405)] as compared to infected control (IL-10: Mean ± S. D., 2.036 ± 0.700 and Arginase-1: Mean ± S. D., 1.886 ± 0.370). Uninfected mφ (i.e., without Ld infection) were taken as control (fold change = 1, dashed line). Interestingly, we observed an increase in parasite load (absolute gene expression; 2−ΔΔT) when mφ were infected with Ld + LdMv (Figure 4E; Mean ± S. D., 8.696 ± 1.254) as compared to only infected mφ as control (Mean ± S. D., 106.2 ± 8.921). This should also be noted that culture supernatant without LdMv (from LA treated culture supernatant) has no effect on immune response against Ld infection (Supplementary Figure 2).

**DISCUSSION**

At the cellular level, the deficiency of LA i.e., ω-6 PUFA, an essential fatty acid, impairs cell-to-cell interaction by modifying cell adhesion (Jiang et al., 2000) and possibly leading to the poor synapse formation and thus compromised activation and antigen presentation (Courtney et al., 2003). LA is the dietary precursor of arachidonic acid (AA) and precursor of the long-chain PUFAs in the mammals. It is evident that mφ enriched with AA showed ~50% enhancement of phagocytic and adhesion capacity (Calder et al., 1990). AA gives rise to various bioactive molecules e.g., prostaglandins (PGs) and leukotrienes (LTs). Various groups have emphasized on roles of PGs and LTs in the immune response against *Leishmania* infection (Morato et al., 2014; Saha et al., 2014; Chaves et al., 2016; Saini et al., 2019). However, the possibilities of their therapeutic applications are limited, as these molecules are transient in nature as well as not cost-effective. Instead of them, using its dietary precursor i.e., LA may have a beneficial role in the containment of the disease. Our previous observation showed that the preventive, as well as therapeutic usage of LA, significantly contains the parasite load in infected mφ (Saini et al., 2020b). Before taking these leads further, we were interested to observe the establishment of Ld infection in mφ under LA sufficient condition.

In the present study, LA enriched macrophages successfully eliminated Ld infection. We measured the expressions of pro- and anti-inflammatory markers in LA treated and Ld infected mφ. Our finding clearly showed the strengthening of pro-inflammatory and weakening of anti-inflammatory markers, when Ld infection was being established in presence of LA (Figure 1). We conclusively demonstrated that the parasite load (i.e., kDNA expression) was also decreased under the same condition, suggesting the poor establishment of infection of *L. donovani* in an LA sufficient condition. However, we failed to observe the killing of Ld promastigotes when treated with LA. Summarily, there is no direct leishmanicidal activity of LA and the observed decrease in parasite load in LA treated infected mφ is possibly due to protective switching from anti- to pro-inflammatory type of immune response. Similar results were obtained when LA was given to either prior to Ld infection (preventive) or after Ld infection (therapeutic) (Saini et al., 2020b).

Our findings suggest the release of membranous bodies in the Ld culture, which is clearly visible on the surface of the parasite in SEM i.e., vesicular blebbing. These bodies which are released under normal culture conditions gets inhibited in the presence of LA and these categorically belong to microvesicles (0.5–1.0 μm) (Muralidharan-Chari et al., 2010; Raposo and Stoorvogel, 2013; Marcilla et al., 2014). The presence of palmitic and stearic acid in the culture supernatant of parasite suggest the same and these fatty acids contribute to ~98% of phospholipids (constituents of the cell membrane) in *L. donovani* promastigotes (Messaoud et al., 2017). Szempruch et al. (2016) summarizes the immunomodulatory activities of membranous bodies by protozoan parasites. Previous studies show the release of exosomal membranous bodies by amastigotes of *L. donovania* parasite and their immunomodulatory properties are also shown. Silverman et al. (2009) proposed an exosome-based pathway from the *Leishmania* parasite that is responsible for protein secretion and communication with mφ. *L. donovani* exosomes are known to modulate human monocyte
cytokine responses by promoting Th-2 type immune response (IL-10) and inhibiting Th-1 type immune response (TNF-α, IL-12p70). Simultaneously, BALB/c mice exposed to *L. major* exosomes showed increased IL-4 production and decreased IFN-γ production at disease sites (spleen and draining lymph node) and exacerbation of *L. major* infection (Silverman et al., 2010).
Hassani (2013) provide evidence about protein release from L. mexicana via exovesicles (40–100 nm, size range of exosome) during early moments of interaction with the mammalian host in order to modulate signaling and functioning of the mφ. It was further showed that GP63 bearing L. major exosomes have inflammatory properties and are capable of immune modulation at both signaling and gene expression levels in mφ (Hassani et al., 2014). All these studies focused on exosomes, however other types of secreted extracellular vesicles like microvesicles (up to 1 µm) remain unexplored. Our study is the first of its kind which suggests the immunomodulatory activity of Ld secreted microvesicle (LdMv). Our findings indicated that the treatment with LdMv tilts the immune response toward M-1 type (IL-12 ↑ and iNOS ↑; IL-10 ↓ and Arginase-1 ↓). Possibly, the release of LdMv tunes the mφ and sets a conducive platform (Th-2 ↑) in the mφ for the establishment of infection (Figure 4). This must also be noted that culture supernatant without LdMv had no effect on immune response against Ld infection (Supplementary Figure 2). Not only in the human host, but Leishmania parasite also secretes exosomes in the midgut of sand fly and are the part of sandfly’s infective inoculum (Atayde et al., 2015). However, the release of Mv by the parasite and its role in the establishment of infection was not yet established. To the best of our knowledge, our study first time demonstrates the release and role of Leishmania donovani promastigote derived microvesicles in the establishment of infection. Moreover, its release is inhibited by the presence of LA i.e., a nourished condition, in the culture. We are suggesting that LA plays a dual role in the protective immune response against VL infection. It decreases the release of LdMv from the promastigote form of Leishmania parasite and promotes the pro-inflammatory response inside mφ via the 5-lipoxygenase pathway to eliminate parasite present inside. This study highlights the immunomodulatory properties of LdMv and the possible role of nutrient i.e., LA in inhibiting their release which leads to the containment of L. donovani infection (Figure 5). Conclusively, it is believed that Leishmania secreted microvesicles exert modulation of immune responses.

**DATA AVAILABILITY STATEMENT**

The datasets generated for this study are available on request to the corresponding author.
AUTHOR CONTRIBUTIONS

SS performed all the experiments, analyzed as well as interpreted the data, and wrote the manuscript. AR conceptualized, designed the work, managed funding, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00406/full#supplementary-material
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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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