**Leishmania** infection-derived extracellular vesicles drive transcription of genes involved in M2 polarization

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Although it is known that the composition of extracellular vesicles (EVs) is determined by the characteristics of the cell and its environment, the effects of intracellular infection on EV composition and functions are not well understood. We had previously shown that cultured macrophages infected with *Leishmania* parasites release EVs (LiEVs) containing parasite-derived molecules. In this study we show that LdVash, a molecule previously identified in LiEVs from *L. donovani* infected RAW264.7 macrophages, is widely distributed in the liver of *L. donovani* infected mice. This result shows for the first time that parasite molecules are released in EVs and distributed in infected tissues where they can be endocytosed by cells in the liver, including macrophages that significantly increase numbers as the infection progresses.

To evaluate the potential impact of LiEVs on macrophage functions, we show that primary peritoneal exudate macrophages (PECs) express transcripts of signature molecules of M2 macrophages such as arginase 1, IL-10, and IL-4R when incubated with LiEVs. In comparative studies that illustrate how intracellular pathogens control the composition and functions of EVs released from macrophages, we show that EVs from *Salmonella* Typhimurium activate PECs to express transcripts of signature molecules of M1 macrophages such as iNOS, TNF alpha, and IFN-gamma and not M2 signature molecules. Finally, in contrast to the polarized responses observed in *in vitro* studies of macrophages, both M1 and M2 signature molecules are detected in *L. donovani* infected livers, although they exhibit differences in their spatial distribution in infected tissues.

In conclusion, EVs produced by macrophages during *Leishmania* infection lead to the gene expression consistent with M2 polarization. In contrast, the EVs produced during *S. Typhimurium* infection stimulated the transcription of genes associated with M1 polarization.

**KEYWORDS**

*Leishmania*, salmonella, EVs, liver, macrophage polarization, extracellular vesicle, exosome
Introduction

There is ample evidence that eukaryotic cells constitutively release extracellular vesicles (EVs) to their environment. EVs include exosomes that range in size from 50 - 150 nm, microvesicles that are 50 - 1000 nm, and apoptotic bodies from 500 - 5000 nm (Raposo and Stoorvogel, 2013; Zaborowski et al., 2015). In addition to the size differences between EVs, the vesicles also differ in their biogenesis and cargo content (Raposo and Stoorvogel, 2013). Although all EVs are likely to contribute to contactless cell-to-cell communication, numerous studies have focused on exosomes and their functions in these processes. There is increasing evidence that the cellular environment, including such conditions as infection with an intracellular pathogen (Hui et al., 2021) (Gioseffi et al., 2020a), or growth under low oxygen (hypoxic conditions) (Leblond et al., 2016), determines the composition of the exosome. We are still in the early stages of understanding how prokaryotic or eukaryotic intracellular pathogens modulate exosome content and the functions of the exosomes released from the infected cells.

Macrophages are functionally plastic cells with multiple distinct phenotypes regulated by microenvironmental stimuli. The macrophage function diversification called polarization assigns specific roles to macrophages for such vital processes as an antimicrobial response or tissue repair. While the dichotomy of polarization separates macrophages into classically activated (M1) and alternatively activated (M2) macrophages, the M2 macrophages are further subdivided into M2a, M2b, M2c, and M2d phenotypes that can be distinguished by their expression of different surface markers and differential production of cytokines with discrete biological functions [as reviewed in (Murray, 2017)]. Prior studies have revealed that the mechanism(s) controlling the initiation of polarization is complex. However, many of these processes rely on changes in metabolism (Haschemi et al., 2012; Jha et al., 2015). A critical pathway that renders M1/M2 polarity relates to the arginine metabolism, where M1 macrophages generally produce iNOS-dependent citrulline and nitric oxide from arginine, in contrast to M2-like macrophages that depend on the arginase metabolic pathway, yielding ornithine and urea as metabolites of the arginine (Jha et al., 2015). The critical molecules that influence this polarization in most infections are unknown, although lipopolysaccharides (LPS) and specific Th1 cytokines such as IFN-gamma or TNF promote M1 macrophage activation, while other cytokines such as IL-4 or IL-10 promote M2 macrophage activation or differentiation (Mantovani et al., 2004). Therefore, one could anticipate that the infection from many Gram-negative bacteria is more favorable to initiating M1-like macrophages due to the presence of LPS. However, this polarization might depend on the pathogen and the type of mouse model used, as studies have shown for *Yersinia enterocolitica* (Tunultan et al., 2007) or the pathogen’s growth rate as shown with *Salmonella enterica* serovar Typhimurium (S. Typhimurium) (Saliba et al., 2016).

Some studies on the infection of macrophages with the eukaryotic pathogen, *Leishmania*, have explored some of the underlying metabolic contributions that promote macrophage differentiation to M1 or M2 types (Kane and Mosser, 2000; Saunders and McConvill, 2020; Mamani-Huanca et al., 2021). *Leishmania* parasites are the causative agents of leishmaniasis that can present as cutaneous lesions, diffuse or mucocutaneous lesions, or visceral disease, dependent on the infecting *Leishmania* species. Infection with *L. donovani* causes a visceral disease that presents as splenomegaly, hepatomegaly, and infection of bone marrow cells. Increases in the volume of these organs are suggestive of tissue remodeling characterized in part by an increase in cellularity (Yurdakul et al., 2011; de Melo et al., 2021). As discussed above, macrophages, which are the primary host cells of *Leishmania*, dependent on their activation state, can initiate processes that contribute to tissue remodeling. In addition to the direct effects of the parasite on their host cell, the infection can trigger the release of factors such as cytokines that activate other cells in their vicinity, including bystander macrophages (Terrazas et al., 2015; Davis et al., 2016). Apart from soluble factors, molecules enclosed in vesicles secreted from cells may also be involved in cell-to-cell communication during infection. Our previous studies indicated that EVs obtained from *Leishmania donovani*-infected macrophages (LiEVs), when incubated with endothelial cells, can induce pathways associated with angiogenesis (Gioseffi et al., 2020b). We have also performed studies on *S. Typhimurium* infections, showing that macrophage responses to infection contribute to the course of infection (Hui et al., 2018). Salmonellae are the causative agents of salmonellosis and typhoid fever. Our previous studies described a novel function for EVs in *S. Typhimurium* infection, where EVs from infected macrophages drive M1 polarization in naïve macrophages, leading to the increase in cytokines such as IL-1 beta or TNF-alpha (Hui et al., 2021). Following intranasal delivery of EVs from *S. Typhimurium*-infected cells, the EVs accumulate in the mucosal tissue in the lungs, increasing specific macrophage and DC subpopulations in these areas (2).

To commence comparative studies on the effects of intracellular infections on EV biogenesis and composition, we considered the functions of EVs derived from macrophage infections with either *L. donovani* or *S. Typhimurium*. In an experimental model of visceral leishmaniasis, we show that in the liver of mice infected with *L. donovani*, there is an increase in the macrophage count as the infection progresses. In addition, LvVash a protein that had previously been characterized in LiEVs was found to be widely distributed in *L. donovani*-infected livers. This observation prompted studies to assess the effects of LiEVs on macrophage polarization. In parallel studies, the activation of macrophages by *S. Typhimurium* infection-derived EVs produced by macrophages was evaluated to underscore infection’s effect on EV biogenesis and EV composition.
Results

An increase in macrophages in the liver of Leishmania-infected animals

*L. donovani* infection leads to a progressive increase in the weight of the liver and spleen that can be used as a surrogate indicator of the increase in the parasite burden within these organs (Engwerda and Kaye, 2000; Murray, 2001). In the liver, resident macrophages identified by their expression of F4/80 (Kinoshiba et al., 2010) are the primary host cells of *Leishmania* parasites. Within three weeks of infection, granulomas form in the liver, which precedes the eventual drop in the parasite burden in the liver (Murray, 2001). The reduction in the parasite burden aside, other outcomes of the infection are the remodeling of the liver characterized by an increase in liver volume and cellularity, which are sustained for many more weeks. Beattie and colleagues (Beattie et al., 2010) observed morphological and other changes in infected and uninfected macrophage populations in infected livers. They then acknowledged that the factors that promoted dynamic changes to the macrophage populations in the infected liver are still unknown. In this study, we sought to gain further insight into the role of the macrophage in this experimental system. Mice were infected with *L. donovani* parasites and monitored over 42 days. Livers were recovered from uninfected mice and mice infected for 20 and 42 days. Livers from these mice were weighed (Figure 1A) and analyzed by histological examination While there are varying-sized granulomas in the 20-days infected livers, fewer granulomas were seen in the 42-days infected livers (Figure 1B), consistent with Murray’s observations (Murray, 2001). There were no granulomas in the livers of uninfected mice. Next, changes to F4/80+ cells in the liver of infected and uninfected mice were evaluated in sections from the uninfected liver or 20-day, and 42-day infections. There was a sparse distribution of F4/80+ labeled cells in the uninfected liver (Figure 2A). In contrast, there was intense F4/80+ labeling of the granulomas and a greater density of F4/80+ labeled cells in livers from 20-days and 42-days infection. Enumeration of F4/80+ cells showed that there was a 3–4-fold increase in F4/80+ cells at 20- and 42-days post-infection compared to livers from uninfected animals (Figure 2B).

**EVs are released from infected cells into the tissue environment**

It is likely that infection-derived molecules, including parasite-derived molecules in EVs, play a role in the dynamic changes of macrophages in infected tissues. We had shown that *Leishmania*-infected cells release EVs that contain parasite-derived molecules into the culture medium. To determine whether infected cells in tissues release EVs with parasite-derived molecules, tissue sections from 20- and 42-days post-infection were evaluated for LdVash distribution. LdVash is a parasite-derived molecule incorporated in LiEVs (Gioseffi et al., 2020b). Paraffin-embedded livers from uninfected, 20-days, and 42-days old infections were sectioned and labeled with a custom-made peptide antibody to LdVash, as described in the Methods section. Representative images of labeled liver sections are shown (Figure 3). In contrast to livers from infected mice with no LdVash label, there was a widespread distribution of LdVash in the 20-days post-infection liver. Intense LdVash labeling of granulomas confirmed that infected macrophages, which are the source of LdVash are at the center of granulomas. Interestingly, there was also LdVash labeling of the lining of liver sinusoids, which suggested that EVs and their cargo were widely distributed in the infected liver. In liver sections from 42-days infection, there was evident LdVash labeling of cells, albeit at more reduced levels than the 20- days post-infection livers. It is to be expected that with the reduction in parasite numbers in 42-days infections, there is a reduction in LdVash levels in the liver. Overall, these studies showed that LiEVs and their contents are widely distributed in infected tissues where they can be endocytosed by liver cells, including uninfected bystander macrophages and other liver cells.

**Bioinformatic predictions of the function of molecules in LiEVs**

Considering observations that showed that LiEV molecules are widely distributed in infected tissues where macrophages and other cells endocytose these EVs, we sought to predict the potential functions of the EVs based on their composition. We had previously reported on the proteomic composition of LiEVs from RAW264.7 macrophages infected with *L. donovani* parasites (Gioseffi et al., 2020b). In that study, quantitative mass spectrometry analysis revealed distinct host-derived exosomal proteins with their abundance altered during infection compared to uninfected control. Pathway analysis tools subsequently analyzed the proteins with a protein level differentially regulated by *Leishmania* infection (fold change >2 and p-value <0.05) to identify the mechanisms that could explain the putative function of these EVs. The downstream pathway analysis identified the upregulation of CD36, CTSD, MYH9, CLTC, MFGE8, SLCS1A5, LGALS3 and downregulation of CORO1A are specific molecules in LiEVs predicted to stimulate the metabolism of cells, represented by the M2 marker ARG1 (Figure 4). Arg1 is a metabolic enzyme that catalyzes the hydrolysis of arginine into urea and ornithine (Wu and Morris, 1998). ARG1 is considered a marker of M2 macrophages. In resting macrophages, several stimuli, including cytokines such as IL4 and IL-13, activate STAT6, which in turn upregulates the mRNA and protein levels of ARG1 (Odegaard and Chawla, 2011). This bioinformatic analysis led to the testable hypothesis that LiEVs stimulated alternative activation of macrophages. A similar analysis of exosomes derived from *S. Typhimurium* -infected macrophages identified that the EVs generated by this bacterial infection led to increased M1 macrophage polarization (Hui et al., 2021).
FIGURE 1
The course of L. donovani infection in BALB/c mice. Mice were infected by tail vein injection with metacyclic promastigote forms. Mice were sacrificed after 20 or 42 days. The liver was recovered and weighed before formalin fixation. (A) Plot of liver weights. (B) Representative H&E sections of liver tissue that was paraffin-embedded and processed for histochemical analyses. White arrows point to granulomas. Each group was composed of 3 animals. *** denotes statistically significant difference p<0.005. Experimental L. donovani infections were performed 2 times.

FIGURE 2
F4/80+ macrophages increase in the liver after L. donovani infection. BALB/c mice were infected by tail vein injection of metacyclic L. donovani promastigotes. At the indicated times after infection, the livers of infected and uninfected mice were recovered and fixed in formalin and paraffin embedded. Livers sections were prepared and processed for immunohistochemical labeling. F4/80 positive macrophages were labeled with a primary antibody detected with an HRP-conjugated secondary antibody followed by activity of diaminobenzidine (DAB). The sections were counterstained with hematoxylin. (A) White arrows point to representative F4/80+ labeled cells; red arrows points to a granuloma. (B) F4/80+ cells were enumerated in regions randomly selected through the entire liver and plotted in GraphPad. Data was compiled from three mice per group. Student t-test was used to establish statistical significance. ** p<0.01; ns (not significant) p>0.05.
Differential activation of primary macrophages by EVs, depending on the pathogen infection

Macrophages exhibit plasticity in their responses to a variety of stimuli. Some stimuli promote macrophages to exhibit gene transcription consistent with classical or M1-type characteristics, while others promote alternatively activated or M2-type characteristics (Cohen and Mosser, 2013; Saliba et al., 2016). The specific stimuli in each infection promoting such macrophage polarization are still being studied. As discussed above, the bioinformatic analysis of the LiEVs proteome had led to the prediction that these EVs would likely promote alternative activation of macrophages, based on the predicted increase in the Arg-1 protein (Figure 3). Hence, we aimed to determine the effect of EVs on macrophage activation. LiEVs from RAW264.7 macrophage infected with L. donovani parasites were incubated with peritoneal exudate cells prepared from BALB/c mice. Macrophage expression of prototypic M1-type and M2-type activation markers was determined after 24-hrs. LiEVs and EVs from uninfected cells did not induce the expression of IFN-gamma, iNOS, or TNF-alpha, which contrasted with LPS that induced conspicuous expression of these markers (Figure 5). Instead, LiEVs and not EVs from uninfected cells induced a statistically significant increase in the M2 markers, Arg-1, IL-10 compared to the treatment with exosomes from uninfected cells. Disruption of LiEVs before incubation with macrophages mitigated their capacity to activate the induction of Arg-1 and IL-10. Although LPS did not activate Arg-1 expression, we observed induction of IL-10 expression in response to LPS, which is consistent with observations by others (Hobbs et al., 2018).

To further address the critical role of the composition of EVs on the macrophage responses that are elicited, EVs prepared from RAW264.7 macrophages infected with S. Typhimurium were evaluated alongside LiEVs. As expected, EVs derived from S. Typhimurium-infected cells induced significant iNOS and TNF-alpha levels, comparable to levels of these markers induced by LPS treatment of cells. EVs from S. Typhimurium-infected cells did not increase Arg-1 or IL-4R transcripts. This is consistent with observations on macrophage activation in bystander cells or in cells harboring non-growing S. Typhimurium (Saliba et al., 2016). In contrast, LiEVs did not induce iNOS or TNF-alpha transcription but increased Arg-1 and IL-4R (Figures 6A–D). As another finding, the vesicles derived from S. Typhimurium-infected macrophages did not cause increased expression of iNOS when the vesicles were disrupted by sonication, but the effect on TNF-alpha transcript expression was less pronounced (Figures 6E, F). Measurement of soluble cytokine levels confirms this observation (Supplemental Figure 1). It is worth noting that differences in responses elicited by these EVs underscore the effects of their composition on macrophage activation.

M1 and M2 macrophages in Leishmania-infected liver

Studies described above showed that LdVash, a prototypic LiEV molecule, is conspicuously distributed in infected mouse livers, especially at 20 days after infection. As was described earlier, LdVash is not only detected within infected F4/80+ cells but also in bystander F4/80+ cells. Next, F4/80+ cells in the liver were examined in the extent to which they displayed...
characteristics of classically activated or alternatively activated cells. Towards this goal, the expression of M1 or M2 signature molecules was ascertained in these cells. Tissue sections from 20-days infected livers were evaluated for expression of iNOS (M1 signature) or IL-4Rα (CD124) (M2 signature). Although iNOS labeling was sparse, it was primarily detected in the center of granulomas (Figure 7A). In contrast, IL-4Rα labeled F4/80+ cells were more widely distributed in the infected livers (Figure 7B).

Discussion

In infections of Leishmania donovani, the liver, spleen, and bone marrow are parasitized. There is progressive increase in the weight of the liver and spleen that can be used as a surrogate indicator of the increase in the parasite burden within these organs (Engwerda and Kaye, 2000; Murray, 2001). In the liver, resident macrophages identified by their expression of F4/80 (Kinoshita et al., 2010) are the primary hosts of Leishmania parasites. Within three weeks of infection, granulomas form in the liver, which precedes the eventual drop in the parasite burden in the liver (Murray, 2001). The reduction in the parasite burden aside, other outcomes of the infection are the remodeling of the liver characterized by an increase in liver volume and cellularity, which are sustained for many more weeks. Beattie and colleagues (Beattie et al., 2010) observed morphological and other changes in infected and uninfected macrophage populations in infected livers. They acknowledged that the factors that promoted dynamic changes to the macrophage populations in the infected liver are still unknown. Several studies have shown that parasitemia in the liver eventually drops in experimental infections after achieving peak levels. This disease progress contrasts with the situation in the spleen (McElrath et al., 1988; Faleiro et al., 2014). Granuloma formation in the liver precedes and foretells the drop in parasite numbers even though liver volume and cellularity do not change significantly. Kupffer cells, the liver’s resident macrophages, are the primary hosts of Leishmania (Cummings et al., 2010). Kupffer cells in the liver are characterized by the expression of F4/80 (Kinoshita et al., 2010). The current understanding of the contributions of Kupffer cells to the progress of the infection in the liver is incomplete. Both infected and uninfected macrophages may play complementary roles in the infection. Our studies found a 3-to-4-fold increase in F4/80+ macrophages.
in the livers of infected mice over a 42-days infection course. Although the number of granulomas was significantly reduced by 42-days post-infection, macrophage numbers remained high. Several studies have reported on the cytokine milieu in Leishmania-infected liver and spleen tissues, and some experiments have shown the role of cytokines in the remodeling of the infected tissues (Cummings et al., 2010; Faleiro et al., 2014; Montes de Oca et al., 2020). However, it is not known what role infection-derived molecules, including parasite molecules, may play in promoting cell activation in infected tissues.

To follow up on our previous studies that characterized EVs derived from LiEVs and identified several parasite molecules in these EVs (Gioseffi et al., 2020b), we sought to determine whether LdVash, a prototypic parasite molecule in LiEVs, was released into infected livers. Immunolabeling with a custom-made anti-peptide antibody to LdVash revealed that it was widely distributed in the liver in 20-day-old infections. In addition to labeling granulomas that contain infected macrophages at their center, LdVash was also seen in other cells, including cells lined livers sinusoids. This result suggested that infected F4/80+ Kupffer cells are potentially modulated by parasites and that infection-derived products could functionally modulate uninfected bystander macrophages and other non-immune cells that ingest LiEVs. Our previous studies showed that LiEVs could activate endothelial cells in surrogate angiogenesis assays, including tube formation and the release of angiogenesis-promoting factors (Gioseffi et al., 2020b). Macrophages are critical cells of the innate immune system, serving as primary phagocytic cells that produce nitric oxide (NO) for pathogen destruction, cells that present antigens on MHC I and MHC II receptors to T-cells (Guerriero, 2019), and releasing cytokines that signal to other cells to infiltrate into the infected region (Atri et al., 2018). However, macrophages are plastic cells that can modify their metabolism and hence the functional properties, depending on the stimulants they encounter (Mantovani et al., 2004). As such, macrophages can affect the immediate environment by producing cytokines, leading to the induction of proinflammatory responses in the case of M1 macrophages or enhanced phagocytosis and tissue healing properties in the case of M2 macrophages (Atri et al., 2018). Signals such as LPS, IFN-γ, TNF-α, or IL-1β induce differentiation into M1 macrophages, displaying increased proinflammatory gene transcription and bactericidal properties (Liu et al., 2018). For example, infection with S. Typhimurium leads to an LPS-dependent M1 macrophage profile in murine macrophages (Miflin et al., 2002). Such M1 polarized macrophages release IFN-λ, TNF-α, or IL-1β and upregulate inducible nitric oxide synthase (iNOS) in naïve cells to promote bacterial killing [reviewed in (Bogdan, 2001)].

**FIGURE 5**

The effect of EVs derived from L. donovani-infected macrophages on macrophage polarization. RAW264.7 macrophages were infected with L. donovani (or not), and the EVs were obtained from these cells using the ultracentrifugation method. A portion of the vesicles was disrupted by sonication. Naïve macrophages were then exposed to these EVs for 24 hours. Media, EVs from uninfected cells, and LPS (100 ng/ml) were used as control treatments. The cells were collected, and RNA purified for RT-qPCR analysis of IFN-γ (A), iNOS (B), TNF-α (C), Arg1 (D), and IL-10 (E) transcripts. One-way ANOVA was used to establish statistical significance. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. **Summary of the results indicating up- or down-regulation of M1 and M2 markers. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001.
Polarization to M2, or alternatively activated macrophages, is mediated by IL-4 (Liu et al., 2016) and generally occurs after M1 responses to decrease inflammation (Liu et al., 2020) and maintain homeostasis (Liu et al., 2014). IL-4 induces M2 type gene transcription such as Arg-1, which mediates cell growth via metabolizing L-arginine into urea and L-ornithine, which are crucial for generating proline and polyamines necessary for collagen production represses ROS generation by downregulating iNOS expression (Rutschman et al., 2001; Junttila et al., 2008). IL-10 is typically secreted from M2 macrophages and acts as an anti-inflammatory cytokine (Riley et al., 1999; Banerjee et al., 2017), restricting tissue damage during infection (Ouyang et al., 2011). The balance between M1 and M2 macrophages appears critical for proper immune responses by balancing proinflammatory and anti-inflammatory responses (Liu et al., 2014; Atri et al., 2018; Liu et al., 2020). It is unknown whether the EVs produced by macrophages during Leishmania infection lead to M2 polarization, but these vesicles certainly stimulate the gene expression consistent with M2 polarization. In contrast, the EVs produced during S. Typhimurium infection stimulate the transcription of M1 polarization markers (Hui et al., 2021). Since the EVs can migrate to tissues far from the infection site, there is a likelihood that the vesicles produced during infection can stimulate macrophages far from the site of their generation.

Materials and methods

Bioinformatic analysis

Ingenuity Pathway Analysis software (Qiagen) was used for protein network analysis of exosomal proteins, focusing on the analysis of proteins with different abundance upon infection with L. donovani at 72 hpi compared with exosomes isolated from uninfected RAW 264.7 macrophages. Activation of specific downstream proteins and functions was identified and measured by Z-score higher than 2/-2, where relevant canonical pathways were overlayed.

Cell culture

Murine macrophage cell line RAW264.7 (ATCC# TIB-71, ATCC, USA) was cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml Penicillin/Streptomycin (Life Technologies Inc., USA) at 37°C and 5% CO2. Peritoneal exudate cells (PECs) were obtained 4 days after intraperitoneal injection of thioglycolate into BALB/c mice.

L. donovani wild type (MHOM/S.D./62/CL21) was obtained from Dr. Nakhasi’s lab (FDA) and cultivated in

FIGURE 6

The effect of EVs derived from L. donovani-infected macrophages on macrophage polarization. RAW 264.7 macrophages were infected with L. donovani (72 hpi) or S. Typhimurium (MOI 5:1, 24 hpi). The EVs obtained from these cells were used to treat primary macrophages for 24 hours. Media (Ctrl) or EVs from uninfected cells were used as negative controls, and LPS treatment 100 ng/mL was used as a control treatment. The cells were collected and RNA purified for RT-qPCR analysis of iNOS (A, F), TNFa (B, E), Arg1 (C), and IL-4R (D). One-way ANOVA for multiple comparison tests was used to establish statistical significance. N=6. Exo (-), EVs from uninfected cells; Exo (Ld), EVs from cells infected with L. donovani; Exo (St), EVs from cells infected with S. Typhimurium; Exo (St) dis, disrupted EVs from cells infected with S. Typhimurium. Ctrl, cells not treated. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001.
M199 media (Sigma M0393) containing 15% FBS, 0.1 mM Adenosine, 0.1 mg/mL folic acid, 2 mM glutamine, 25 mM HEPES, 100 units/mL penicillin/100 µg/mL streptomycin (Gibco 15140122), 1X BME vitamins (Sigma B6891), and 1 mg/mL sodium bicarbonate with pH 6.8 at 26°C.

*S. Typhimurium* strain UK-1 c3761 (wild-type) was cultured at 37°C in lysogeny broth (LB) media and shaken at 200 rpm (rotations per minute). After the overnight culture, the bacterial cultures were diluted in new LB media to reach the optical density at 600 nm (OD600) of 0.05. This culture was grown until OD600 reached 0.50, the mid-logarithmic phase. The bacteria were then washed in 1 mL of phosphate-buffered saline (PBS) and centrifuged at 6,000 x g for 10 minutes at 37°C before bacterial cultures were used for infections.

**Infection conditions**

RAW264.7 cells were washed with pre-warmed PBS, and incomplete growth media containing no FBS or antibiotics were added for 60 minutes before infection with *S. Typhimurium* at a multiplicity of infection (MOI) of 5:1. The overnight culture of *S. Typhimurium* was diluted as described above and cultivated at 37°C until it reached the mid-logarithmic phase. The bacteria were added onto the cells for 2 hours, after which the culture media were removed, cells were washed with PBS, and media containing gentamicin (100 µg/mL) were added onto the cells for 1 hour. Finally, the media containing lower gentamicin (20 µg/mL) and exosome-free heat-inactivated FBS were added to the cells for the remaining time of infection. Based on our previous studies, the total infection time for *S. Typhimurium* was 24 and 48-hours, based on our previous studies (Hui et al., 2018; Hui et al., 2021).

For *L. donovani* infections, RAW264.7 macrophages were plated on 100 mm culture dish containing sterile glass coverslips at a concentration of 5 × 10^6 cells per dish, and cells adhered overnight at 37°C with 5% CO2 before infection with metacyclic promastigotes. To enrich for metacyclic parasites, peanut agglutination (PNA) was performed on stationary-phase wild-type promastigote cultures (Sacks and Melby, 2015). Briefly, 4-d-old cultures of *L. donovani* parasites were washed twice and resuspended in incomplete DMEM at a 2 × 10^8 parasites/ml concentration. PNA was then added to the parasites at a final concentration of 50 µg/ml and incubated at room temperature for 15 min. The parasites were then centrifuged at 200g for 5 min to pellet agglutinated parasites. The supernatant was then collected, and the PNA-metacyclic parasites were washed twice, resuspended in complete DMEM, and counted for infection. Parasites were then added to macrophage dishes at a ratio of 20:1 (parasites: macrophage).

**EV purification**

As described previously, the EVs were isolated from the cell culture media by an ultracentrifugation method (Gioseffi et al., 2020b; Hui et al., 2021). Cell culture supernatant was briefly collected and filtered through a 0.22-micron polyethersulfone (PES) filter. PBS containing 1 mM PMSF and 1X protease cocktail protease inhibitor cocktail (EDTA-free; Roche, USA) was added. The filtrates were centrifuged at the following conditions: 10 min
infections were performed 2 times. 

Histology

Liver tissues were placed in cassettes, recovered and weighed on days 20 and 42 post-infection. Fresh was carried out by the Molecular Pathology Core at the

EMMA

embedded in paraf

group) were inoculated intravenously (tail vein) with 1 x 10^7

uninfected RAW 264.7 macrophages, controls for 2, 24, or 72 hours. EVs were obtained from

stationary phase

L. donovani

. The liver of each mouse was

fixed in 10% formalin, and

dec fix

incubated in paraf

xylene and graded ethanol changes. For single-stained slides, sections were subjected to heat-induced antigen retrieval in

10mM Citra pH 6 and blocked with avidin, biotin, and goat

tissue. They were incubated overnight at 4°C with a primary rat antibody against F4/80 (F4/80 Monoclonal Antibody (BM8), ThermoFisher Scientific) or LdVash (rabbit anti-LdVash) After washing, tissues were labeled with Mach2 Gt x Rabbit HRP polymer (Biocare Medical, Walnut Creek, CA), the DAB chromogen (Biocare Medical, Walnut Creek, CA), and CAT hematoxylin counterstain (Biocare Medical, Walnut Creek, CA). Whole slides were scanned using an Aperio CS2 Scanscope (Leica/ Aperio, Vista, CA). For double-stained slides, FFPE liver sections on slides were heated in EDTA buffer (10 mM Tris, 1 mM EDTA) to measure the vesicle concentration and the hydrodynamic diameter.

Treatments of cells with EVs

Peritoneal exudate cells (PECs) were treated with EVs or controls for 2, 24, or 72 hours. EVs were obtained from

media and LPS 100 ng/mL. Following treatment, supernatants were lysed

Supplemental Table 1. RNA was extracted from macrophages

were lyses

sections (4 μm) were deparaffinized with xylene, and the tissue sections were rehydrated in a graded series of ethanol solutions. The rehydrated tissues were stained with hematoxylin (Richard-Allan Scientific, 7212) for 2 minutes, incubated with clarifier 2 (Richard-Allan Scientific, 7402) for 30 seconds, followed by incubating with a bluing reagent (Richard-Allan Scientific, 7301) for 1 minute, then incubated one minute in 80% ethanol before staining with eosin (Richard-Allan Scientific, 71311) for 1 minute. In between the application of each reagent, the slides were washed with running water. Finally, the H&E stained slides were dehydrated in a graded ethanol series, dipped in xylene, and then coverslipped. For immunohistochemistry. 4 mm sections were deparaffinized and rehydrated by serially passing through xylene and graded ethanol changes. For single-stained slides, sections were subjected to heat-induced antigen retrieval in

10 mM Citra pH 6 and blocked with avidin, biotin, and goat

sections (4

sections were rehydrated using Nanoparticle Tracking Analysis (NTA, NanoSight LM10) to measure the vesicle concentration and the hydrodynamic diameter.

qPCR analysis

The transcription of murine iNOS, Arg-1, IL-4R, IFN-gamma, and TNF-alpha was analyzed using the primers described previously (Hui et al., 2021), while transcripts of IL-4R and IFN_gamma were performed using the primers described in

Supplemental Table 1. RNA was extracted from macrophages using a Qiagen RNeasy Mini Plus extraction kit. The cDNA was

generated from the isolated RNA using Maxima First Strand cDNA synthesis kit (Thermo Fisher), and the expression of genes was measured using a two-step quantitative real-time polymerase chain reaction (RT-qPCR) using MXP3005 instrument (Bio-Rad) and SYBRGreen reagents (Bio-Rad). Primer sequences were validated by melt-curve analysis.

Mouse infections and histochemistry

Six to eight-week-old groups of female BALB/c mice (3 per group) were inoculated intravenously (tail vein) with 1 x 10^7 stationary phase L. donovani. The liver of each mouse was recovered and weighed on days 20 and 42 post-infection. Fresh liver tissues were placed in cassettes, fixed in 10% formalin, and embedded in paraffin (FFPE). Studies on experimental L. donovani infections were performed 2 times. Histochemistry was carried out by the Molecular Pathology Core at the University of Florida. For hematoxylin and Eosin staining, tissue sections (4 μm) were deparaffinized with xylene, and the tissue sections were rehydrated in a graded series of ethanol solutions. The rehydrated tissues were stained with hematoxylin (Richard-Allan Scientific, 7212) for 2 minutes, incubated with clarifier 2 (Richard-Allan Scientific, 7402) for 30 seconds, followed by incubating with a bluing reagent (Richard-Allan Scientific, 7301) for 1 minute, then incubated one minute in 80% ethanol before staining with eosin (Richard-Allan Scientific, 71311) for 1 minute. In between the application of each reagent, the slides were washed with running water. Finally, the H&E stained slides were dehydrated in a graded ethanol series, dipped in xylene, and then coverslipped. For immunohistochemistry. 4 mm sections were deparaffinized and rehydrated by serially passing through xylene and graded ethanol changes. For single-stained slides, sections were subjected to heat-induced antigen retrieval in

10 mM Citra pH 6 and blocked with avidin, biotin, and goat

sections were lyses

sections were treated with EVs or controls for 2, 24, or 72 hours. EVs were obtained from

media and LPS 100 ng/mL. Following treatment, supernatants were collected and stored at -80˚C. Cell pellets were lysed

peritoneal exudate cells (PECs) were treated with EVs or controls for 2, 24, or 72 hours. EVs were obtained from

media and LPS 100 ng/mL. Following treatment, supernatants were collected and stored at -80˚C. Cell pellets were lysed

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Author contributions
LE and AG were responsible for the execution of the experiments. AS, HB, and JM performed some of the experiment in the study. MJE and PK contributed equally to developing the research question, supervision of experiments, securing funding for the studies and writing the manuscript. All authors contributed to the article and approved the submitted version.

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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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SUPPLEMENTARY TABLE 1
Primers used in the study.

SUPPLEMENTARY FIGURE 1
The effect of EVs derived from L. donovani- or S. Typhimurom -infected macrophages on TNF-alpha release. RAW 264.7 macrophages were infected with L. donovani (72 hpi) or S. Typhimurium (MOI 5:1, 24 hpi). The EVs obtained from these cells were used to treat primary macrophages for 24 hours. Media (Ctrl) or EVs from uninfected cells were used as negative controls, and LPS treatment 100 ng/mL was used as a control treatment. The cell culture supernatants were collected and processed for anti-TNF-alpha ELISA. One-way ANOVA for multiple comparison tests was used to establish statistical significance. N=3. Exo (-), EVs from uninfected cells; Exo (Ld), EVs from cells infected with L. donovani; Exo (S.t), EVs from cells infected with S. Typhimurium; Exo (S.t) dis, disrupted EVs from cells infected with S. Typhimurium; Ctrl, cells not treated.
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