Title: Co-infection with *Leptomonas seymouri* enhances macrophage survival and promotes intracellular parasite persistence during *Leishmania donovani* infection

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Running head: *Leptomonas seymouri* can infect mammalian macrophages

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Abstract

Background

Leishmania donovani (LD) is an obligate intracellular parasite that survives and replicates within macrophages. *Leptomonas seymouri* (LS), a traditionally monoxenous trypanosomatid, has been repeatedly co-isolated with LD from visceral leishmaniasis (VL) and post-kala-azar dermal leishmaniasis (PKDL) patients in India, often together with *Leptomonas seymouri* narna-like virus 1 (Lepsey NLV1). Whether LS can survive and replicate within mammalian macrophages, and how co-infection influences parasite and viral dynamics, remains unresolved.

Methods and Findings

Using murine (RAW 264.7) and human (THP-1) macrophages, we systematically evaluated intracellular survival, replication, and revival of LS alone and during co-infection with LD. Quantitative ITS1 PCR demonstrated significant increases in intracellular parasite DNA over 48-168 h post-infection, with mean intracellular loads rising up to ~7.6-fold, indicating active replication rather than persistence. Reduced extracellular parasite load suggested restricted cell lysis and enhanced macrophage survival in co-infection compared to mono-infections. In co-infection scenario, generally LS displayed higher persistence compared to LD. Giemsa staining confirmed intracellular localization of LS. Parasites recovered from infected macrophages remained viable and revived as motile promastigotes, whereas extracellular parasites failed to survive beyond 48 h, confirming macrophages as the exclusive niche for prolonged viability. Interestingly, co-infection dampened macrophage IL-12 production, suggesting altered host immune activation. Lepsey NLV1 RNA accumulated predominantly within macrophages and

persisted up to 168 h post-infection. Virus load within LS in co-infection state was ~2.5-4 and ~7.5-23 fold higher (for RAW 264.7 and THP-1 respectively) compared to LS mono-infection. Purified virus alone failed to enter macrophages, indicating LS-dependent viral delivery.

Conclusions

Our findings question the prevailing view of LS as a strictly non-infective parasite, demonstrating its capacity to replicate within mammalian macrophages and persist during mono- or LD co-infection. The identification of a stable LD-LS-virus interaction highlights a previously underappreciated "triple-pathogen" biology with potential implications for VL and PKDL pathogenesis.

Keywords: *Leptomonas seymouri*, *Leishmania donovani*, Lepsey NLV1, Co-infection, Triple-pathogen, Visceral leishmaniasis.

1. Introduction:

Leishmania parasites are obligate intracellular pathogens that rely on macrophages for their survival, replication, and differentiation **[1].** Despite the extreme challenges posed by the intracellular environment, *Leishmania* are among the few protozoa capable of surviving and multiplying under such harsh conditions [2]**.** *Leptomonas seymouri* (*L. seymouri*; LS), typically a monoxenous trypanosomatid that infects insects, is generally not capable of infecting humans [3]**.** However, several reports from India indicated that this parasite could co-infect humans alongside *Leishmania donovani* (*L. donovani*; LD) in cases of visceral leishmaniasis (VL), cutaneous leishmaniasis and post-kala-azar dermal leishmaniasis (PKDL) [3–6]**.**

An in-depth study had been previously conducted to evaluate the infective potential of LS. It was reported that despite several genetic adaptations, LS remained primarily a monoxenous organism and couldn't infect mammalian macrophages such as J774 or BMM□, either on its own or alongside LD [7]**.** Others implicated that it might infect mammals under specific conditions, for instance, when the host's immune system was compromised by another pathogen such as LD or HIV [6]**.** Interestingly, a study published in 1981 examined the interactions between LD and *Leptomonas costoris* within peritoneal macrophages from hamster. It was observed that LD differentiated into amastigotes and survived within cultured macrophages, whereas *L. costoris* also differentiated into amastigotes but failed to survive. Additionally, macrophage cultures infected with the infectious strain of LD retained 50% more *L. costoris* compared to control macrophages that were infected solely with *L. costoris* [8]**.**

Macrophages serve as both the primary niche for *Leishmania* survival and replication, and as a key effector population in parasite clearance [9]. However, the cellular consequences of LS infection on macrophages remained largely unexplored, highlighting a critical knowledge gap that warranted systematic investigation.

The presence of *Leishmania* RNA virus (LRV) is known to enhance the persistence and worsen the pathogenesis of *Leishmania sp*.[10,11]**.** Unexpectedly, LS was found to harbour a distinct protozoan virus, *Leptomonas seymouri* narna-like virus 1 (Lepsey NLV1), an RNA virus of approximately 4.5 kb **[12].** A study from India reported that >90% of LD laboratory isolates from clinical samples of VL were co-infected with LS. Lepsey NLV 1 was predominantly present in the LS-positive samples (75% of the laboratory isolates) [13]**.** Recent findings reported that LS and Lepsey NLV1 could be even detected alongside LD in archived serum samples of kala-azar or PKDL patients. It also suggested that Lepsey NLV1 might enhance the persistence

of LD and dampen immune responses by down-regulating IL-18 [14] ,which also corroborated with the results from a previously published LRV2+ *L. major* study [15]**.** Leishmania RNA viruses (LRVs) had been known to exacerbate *Leishmania* pathogenesis. In comparison, whether or not Lepsey NLV1 is linked to pathogenesis or persistence of VL/PKDL ("triple pathogen phenomenon") warrants further investigation.

2. MATERIALS AND METHODS:

2.1. Parasite culture:

Leishmania donovani (MHOM/IN/1983/AG83) and *Leptomonas seymouri* (ATCC) promastigotes were cultured at 22 °C in M199 (Sigma) with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) and 1% L glutamine-penicillin-streptomycin solution (Sigma). Promastigotes were harvested on the 6th or 7th day of the stationary phase of growth for *in vitro* infection.

2.2. Murine macrophage culture:

Murine macrophage cell line RAW 264.7 was cultured at 37 °C with 5% CO_2 in DMEM (Dulbecco 's modified Eagle's medium) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) and 1% L glutamine-penicillin-streptomycin solution (Sigma).

For infection experiments, cells were plated in a 12-well flat bottom plates (Nunclon Delta Surface™, Thermo scientific) with $2x10^5$ cells and incubated at 37 °C with 5% CO_2 for 48h. Cells were counted at $8x10^5$/well at 80% confluency.

2.3. Human monocyte culture:

Human monocyte cell line THP-1 was cultured at 37 °C with 5% CO_2 in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) and 1% L glutamine-penicillin-streptomycin solution (Sigma).

2.4. THP-1 activation:

For infection experiments, cells were plated in a 6-well flat bottom plates (Nunclon Delta Surface^{TM}, Thermo scientific) with $5x10^5$ cells /well. Before infection, cells were stimulated with 100 nM /well of 4α-phorbol 12-myristate 13-acetate (PMA) (Sigma, USA) to induce differentiation into macrophage-like cells. The cultures were then incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO□. Following differentiation, wells were gently washed twice with culture medium (without PMA) to remove any non-adherent cells.

2.5. *In vitro* infection:

Accordingly, macrophages were infected with 10^7 stationary phase promastigotes of *Leishmania donovani* (AG83), *Leptomonas seymouri* (ATCC) and 10:1 Co-culture (LD: LS) at a MOI of 10:1 (parasite: MQ ratio) and incubated at 37C with 5% CO_2 for 4 hours. Cells were then washed twice with media to remove non-adherent parasites and incubated with media for 48 hours to 168 hours. After the post-infection incubation period, the cells were processed for further analysis.

2.6. Extraction of purified Lepsey NLV1 and *in vitro* infection with virus:

For extraction of purified Lepsey NLV1 virus, approx. 10^8 LS parasites were frozen at –80 °C for 3 hours, followed by thawing at 37 °C. Sonication was performed using three pulses of 10 seconds each. The samples were then centrifuged at 3000 rpm for 10 minutes to pellet down the *Leptomonas seymouri* (LS) cells. The resulting supernatant, containing the Narna-like virus 1 (NLV1), was collected, passed through a 0.22 µm syringe filter to remove residual cellular debris, and stored as 500 µl aliquots for downstream applications.

For *in vitro* infection, RAW 264.7 macrophage cells were infected with purified Lepsey NLV1 at a MOI =10 and incubated at 37C with 5% CO_2 for 4 hours. After 4 hours, the inoculum was not removed and supplemented with DMEM-1% FBS for further incubation. After 48, 72 and 96 h.p.i., cell supernatants and lysates were collected for further assays.

2.7. Macrophage infected parasite revival assay:

To check the if the parasites are viable and can revive from infected macrophages, 10^6 RAW 264.7 macrophages were infected with only LD, LS or a 2:1 and 10:1 (LD:LS) Co-culture at a MOI of 1:10. After 4 hours of infection, non-adhered parasites were removed by twice washing the cells twice with 1X PBS. Cells were maintained in DMEM-10 at 37C with 5% CO2 for 48 to 168 hours. After every incubation period, the sup was collected and 500 ul cell lysates (in DMEM-10) were added to 5 ml Scneiders medium with 20% FBS and incubated at 22°C and incubated for at least 48 hours to check the transformation of parasites from infected macrophages.

To exclude any possibility of parasite viability in DMEM10, 10^7 parasites /well were maintained in DMEM-10 at 37 °C for 48-168h and subsequently 2 x 10^6 parasites/time point were transferred to Schneider's medium at 22 °C to assess revival capacity at different time points. Parasite titre was quantified 9 days post inoculation in Schneider's media using ITS1 qPCR. In another set of experiment, 10^6 viable LS parasites /well were inoculated into 12-well plates and incubated at 37°C in DMEM-10 for 8 days. For further verification, 10^6 LD and 10^6 LS parasites were cultured in both DMEM-10 and RPMI-10 at 37°C for a period of 24, 72- and 168-hour time points. Both microscopic and molecular analysis (ITS1 PCR) were performed to check the replication of parasites. Parasite titre was determined from densitometric analysis of specific bands on gel using ImageJ software.

2.8. DNA extraction and ITS1 gene PCR:

DNA was extracted from 200 ul of parasite infected cell supernatant or cell lysate using a QIAamp DNA Mini kit (Qiagen The quality and concentration of DNA was assessed using a NanoDrop spectrophotometer (Thermo Scientific, USA).

An rDNA-ITS1 fragment was directly amplified from the isolated DNA using ITS1-FP (5' CTGGATCATTTTCCGATG 3') and ITS1-RP (5' TGATACCACTTATCGCACTT 3') primers. The PCR protocol included an initial denaturation at 94°C for 10 minutes, followed by 40 cycles consisting of denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute, and extension at 72°C for 1 minute. A final elongation step was performed at 72°C for 10 minutes. PCR products were analysed using 1.0% agarose gel containing SYBR safe for nucleic acid staining. DNA from the *Leishmania donovani* strain MHOM/IN/1983/AG83 (320 bp) and *Leptomonas seymouri* (ATCC) (418 bp) was used as a positive control, while nuclease-free water served as the non-

template control (NTC). For *L. seymouri* specific PCR, primers and conditions were followed as described previously (13).

2.9. Real Time PCR and Image J Analysis:

A quantitative real-time PCR assay was designed to quantify the parasite DNA copy number in the samples using a SYBR Green dye-based quantitative (q)PCR with the Luna Universal PCR reagent (New England Biolabs). Equal amounts of DNA from each experimental condition were used for qPCR analysis. A 320 bp and 418 bp fragment of the ITS-1 gene was amplified using the same primers as previously described (Table 1). The reactions were carried out on a StepOne™ system (Applied Biosystems, Thermo).

The presence of bands of the expected size was further verified through 1% agarose gel electrophoresis of qPCR products. To determine the LD:LS ratio in Co-infected samples, specific bands (both standard and samples) from digital images of the gel were subjected to densitometric analysis using ImageJ software.

2.10. RNA Extraction and cDNA synthesis:

Total RNA was extracted from infected macrophage cell supernatant or cell lysates using standard TRIzol method following manufacturer's kit protocol (PureLink RNA Minikit, Invitrogen). For detecting virus load in vivo, total RNA was extracted similarly from whole blood (100-200 ul), urine (100 ul) and Splenic tissue cultures (500 ul). RNA from spleen and liver tissue was isolated using RNeasy Kit (Qiagen). The concentration and purity of RNA was determined using a Nanodrop One spectrophotometer (Thermo Scientific, USA).

The extracted RNA was subjected to cDNA synthesis and virus specific nested PCR using primers and protocol as previously described in (14). PCR products were separated using 1% agarose gel electrophoresis. Bands of the expected size were either gel-purified using the QIAGEN Gel Extraction Kit (Germany) or PCR-purified using the QIAGEN PCR Purification Kit (Germany) before DNA sequencing.

2.11. Real Time PCR:

The viral RNA copy number in the nucleic acid isolated from the samples were ascertained using qRT-PCR. The primers CT2-F and CT2-R were used to amplify a 338 bp segment of the Lepsey NLV1 gene using protocol as described elsewhere [14]. Specific amplified products were visualized in 1% agarose gel electrophoresis with SYBRsafe™ for nucleic acid staining.

2.12. Giemsa Staining:

Cells were plated in a 4-well chamber (Genetix Biotech) tissue culture slide with $5x10^4$ cells (RAW 264.7) or 10^5 (THP-1) and incubated at 37 °C with 5% CO2 overnight. Macrophages were infected with the stationary phase of *Leishmania donovani* (AG83), *Leptomonas seymouri* (ATCC) or 10:1 Co-culture (LD:LS) promastigotes at a MOI of 10:1 (parasite: MQ ratio) and incubated at 37C with 5% CO_2 for 4 hours. Cells were then washed twice with media to remove non-adherent parasites and incubated with media for 72 hours. After the incubation period, cells were fixed with methanol and stained with Giemsa. The stained slides were visualized under an optical microscope (EVOS microscope) for determining the infection of macrophages at 1000x magnification.

2.13. IL-12 (p40) ELISA:

Quantitative IL-12 p40 ELISA (BD OptEIA™, Cat. No.- 555165) was performed as per manufacturer's protocol to determine cytokine levels from cell supernatants of infected RAW 264.7 cells at 72 h.p.i. Standard curve was prepared using dilutions of the respective recombinant cytokine provided with the kit.

2.14. Statistical Analysis:

Statistical analysis was performed using GraphPad Prism version 8.0 (GraphPad Software, USA). Data are expressed as mean ± SD unless otherwise indicated. Statistical tests were selected based on experimental design and data distribution. Differences between two groups were evaluated using unpaired two-tailed Student's t-test. For comparisons involving more than two groups, one-way ANOVA followed by Tukey's post hoc test was used. Each experiment was performed using at least three independent biological replicates. A p value of < 0.05 was considered statistically significant. Exact p values are reported where applicable.

3. RESULTS:

3.1. Monoxenous parasite *Leptomonas seymouri* harbouring Lepsey NLV1 could efficiently infect and replicate in mammalian macrophage cells

3.1.1. LD-LS co-infection led to ~300-2500-fold higher extracellular *L. seymouri* load compared to *L. donovani*

ITS-1 PCR–based quantification revealed that extracellular parasite DNA from LD, LS, and co-culture (2:1) infected RAW 264.7 macrophage cells showed a modest, non-significant (~1.6-fold) rise between 48 and 168 h.p.i. Only 10:1 co-culture demonstrated a significant (~2.9-fold) increase (Fig 1A). The mean intracellular (IC) DNA levels significantly increased ~7.6-fold over the same period, indicating sustained replication within macrophages (Fig 1B). Total parasite DNA count (LD, LS, co-cultures 2:1 and 10:1) inside macrophages (IC) increased by approximately 1.1 and 5.0-fold, compared to total parasite DNA in supernatant (EC) at 48- and 168 h.p.i. respectively. The mean combined parasite load (EC+IC) at 168 h.p.i. increased ~4.7-fold compared to 48 h.p.i. These data supported active proliferation rather than persistence of LS inside macrophages alone or when present with LD over 168 hours. The ~2-fold rise in IC LS DNA at 168 h.p.i. compared to the initial inoculum (10^7 LS; though two-times washed after 4 h of cellular internalization) confirmed true intracellular replication rather than residual carry-over (S1 Table). ImageJ-based band intensity and standard-curve analysis further demonstrated that LS replicated far more efficiently thereby rupturing and getting released in the extracellular milieu at higher proportions up to $2x10^3$-fold at 48 h and $2.5x10^3$-fold at 168 h compared to LD in co-culture infected cells. Despite higher initial LD abundance (2:1 or 10:1), the IC LD: LS ratio stabilized at ~1:150 to 1:66 at 48- and 168 h.p.i time points respectively. Interestingly, the EC ratio shifted from 1:333 to 1:2500, suggesting rapid and more pronounced lysis of LS-infected macrophage cells. (S2 Table).

3.1.2. Temporal quantification revealed differential replication and population dynamics of LD and LS in co-infected human macrophages

Similar to findings in murine macrophages, both LD and LS successfully infected and replicated within differentiated human monocyte–derived macrophages (THP-1). The mean extracellular (EC) parasite DNA count (LD, LS and co-culture 10:1) increased by ~1.17 fold and ~1.71- fold at 96 and 168 h.p.i, respectively compared to 48 h.p.i (Fig 1C). The mean intracellular (IC) parasite DNA levels increased significantly by approximately 1.77 and 4.3-fold at 96 and 168 h.p.i compared to 48 h.p.i (Fig 1D). The mean parasite DNA count (LD, LS and co-culture 10:1) inside macrophages (IC) increased by approximately 1.76, 2.66 and 4.46-fold, compared to mean parasite DNA in supernatant (EC) at 48, 96 and 168 h.p.i. respectively. Combined parasite load (EC+IC) increased by approximately 1.55 and 3.37-fold at 96 and 168 h.p.i compared to the initial time point of 48 h.p.i. These results further validated the replication of monoxenous parasite LS inside mammalian macrophages (S3 Table). Image J analysis of the relative ITS-1 PCR products revealed that in case of co-culture, LD load was more than LS in supernatant at 48 h.p.i. However, this scenario was reversed at 96 and 168 h.p.i. Inside macrophages (IC), the LD: LS DNA ratio was 67:1 at 48 h.p.i, which decreased to 1:1.3 at 168 h.p.i. Interestingly, the LD: LS ratio in supernatant (EC) reversed from 2.5:1 at 48 h to 1:50 at 168 h.p.i. This suggested that the LS parasites were released rapidly from macrophages at later time points (upon infected cell lysis) which resulted in greater to at least equal LD: LS ratio inside macrophages from 48- to 168 h time points respectively (S4 Table).

3.2. Macrophage internalized parasites transformed to promastigotes when cultured in Schneider's medium

3.2.1. Parasites remained viable after infecting murine macrophage cells RAW 264.7

For every time point yield (72–168 h), transformation of amastigotes to promastigotes (reactivation) was carried out in sub-culture flasks containing supplemented Schneider's Drosophila medium. These subcultures were examined at 48 h post-transfer under the microscope to check for any viable and motile parasites i.e. transformed promastigotes. Four representative microscopic fields were observed per flask and motile cells were enumerated (Data in S5 Text). Sub-cultures were further incubated, and two representative fields were observed per flask again at 11 days p.i. (Data in S6 Text)

The 48 h counts upon transfer from each time-point harvest showed that the number of parasites increased progressively in the sub-culture flasks from earlier to later time-point harvests, indicating increasing amastigote production within macrophages over 7 days. Increased parasite counts at 11 days p.i. in the subculture flasks compared to corresponding 48 h counts indicated successful replication of transformed promastigotes upon revival of amastigotes in supplemented Schneider's Drosophila medium. For instance, LS subculture from the 120-h original harvest showed an increase in parasite count between early (2 days post subculture) and later observations (11 days subculture), confirming parasite viability and proliferation in Schneider's medium. At 12 days p.i., viable, motile cells in the total subcultures were also quantified using a haemocytometer (Fig 1E and S7 Table), supporting the microscopic counts.

To compare the intrinsic growth properties of the revived parasites, 10^6 viable parasites from the 12th day harvest of the subculture flasks was further cultured and monitored up to 168 h in Schneider's medium. Haemocytometer counts showed that LS-containing cultures (alone or in mixtures) grew to higher titres at 96 h and 168 h compared to LD-only cultures (Data in S8 Text).

Importantly, additional observations revealed that adhered, non-phagocytosed parasites could remain static but viable in DMEM-10 up to 48 h during macrophage infection, and when transferred to Schneider's medium they regained the capacity to proliferate. However, such revival did not occur in case of later harvests of infected macrophages (>48 h). Non-internalised parasites could neither persist beyond 48 h in DMEM-10, nor regained viability when transferred to Schneider's medium at 22 °C (Fig 1F). This clarified that any apparent revival beyond 48 h observed in Schneider's medium, was only from intracellular amastigotes that transformed in the subculture flasks.

3.2.2. LD or LS could not replicate to higher titres in DMEM-10 or RPMI-10 at 37°C

In order to test whether free parasites could replicate in macrophage culture conditions, viable LS parasites were inoculated into 12-well plates at 10^6/well and incubated at 37°C in DMEM and RPMI-1640 supplemented with 10% FBS for 7 days. No motile or viable parasites were observed at the end of the experiment.

It was further evident from both molecular analysis (PCRs) as well as microscopic analysis neither LD nor LS could not grow in DMEM-10 or RPMI-10 at 37°C when tested over a time

point of 168 h. The titre was estimated (IMAGE J analysis) to stay around 10^4-10^5 parasites per 5ml (10^4 for LD and 10^5 for LS) culture over a period of 24-168 h post-inoculation in the aforesaid growth media (Fig 1G).

As mentioned earlier, LS stayed static but viable up to 48 h but then became non-viable and could not be revived beyond 48 h of culture. Hence, it can be concluded that LS detected in the sub-cultures (in Schneider's medium at 22 °C) from macrophage-infected yields at 72h time point onwards, must had replicated inside the macrophages and revived as promastigotes in the subculture media.

3.3. IL-12 cytokine profiling reflected immune modulatory role of LD, LS and co-infective states

IL-12 (p40) ELISA from 72 h supernatant of infected RAW 264.7 cells revealed that LD-infected cells induced the highest IL-12 protein expression by approximately ~2.62-fold compared to the uninfected control cells. LS-infected cells did not show any significant change in IL-12 levels compared to LD-infected cells. In contrast, co-culture-infected cells showed reduced IL-12 expression, approximately ~1.43-fold lower compared to LD-infected cells (Fig 1H). It may be an indication of dampened pro-inflammatory signalling, possibly linked to reduced macrophage activation, higher parasite load, and prolonged persistence.

3.4. Lepsey NLV1 was detected more (10-100 times) inside mammalian macrophages compared to extracellular milieu

3.4.1. Intracellular persistence of Lepsey NLV1 without temporal proliferation in murine macrophages

RT-PCR analysis at 48 h.p.i. confirmed the presence of Lepsey NLV1 RNA within RAW 264.7 macrophages. Intracellular (IC) viral level was approximately ~20-130-fold higher than the extracellular (EC) fraction (Fig 2A). Lepsey NLV1 titre was further estimated from 48-168 h.p.i. in a time point assay. 14-fold greater viral load was detected in IC than EC at 48 h.p.i. IC viral titres were found to remain stable at 7.0–8.0 × 10^7 gE/well, whereas EC titres were maintained at lower levels (4.0–6.0 × 10^6 gE/well) (Figs 2B and 2C). Although, the total LS count decreased in co-infections as compared to the LS mono-infection, the total viral titre remained static in both the scenarios (S9 Table). Overall results suggested no progressive increase in virus titre inside the host macrophages over 7 days (168 h.p.i). LS carrying Lepsey NLV1 efficiently entered RAW 264.7 macrophages, confirming strong intracellular localization.

3.4.2. Human monocyte-derived macrophages supported progressive intracellular replication of Lepsey NLV1 unlike murine macrophages

Similar analysis confirmed the presence of Lepsey NLV1 RNA in differentiated human monocyte-derived macrophages (THP-1) at multiple post-infection time points. Extracellular (EC) titres in LS infection for Lepsey NLV1 declined modestly from 48 h.p.i. to 168 h.p.i by ~1.45-fold. Viral titre in co-culture 10:1 infected cell supernatant increased by ~1.36-fold from 48 h.p.i. to 96 h.p.i (Fig 2D). The mean intracellular (IC) titres in LS and co-culture infected

cells significantly increased from 48 h.p.i. to 96 h.p.i. and 168 h.p.i by ~4.33-fold and ~4.15-fold respectively (Fig 2E). IC viral titre increased compared to EC levels by ~10.3-fold, ~46-fold and ~57.6-fold at 48, 96 and 168 h.p.i., respectively, indicating strong intracellular retention. Total viral load (EC + IC) increased over time and peaked at 96 h.p.i. ($1.74 \pm 0.53 \times 10^7$ gE), possibly due to increase in total LS count over time from 48-168 h.p.i (S10 Table). These findings showed that Lepsey NLV1 persisted at ~10 –100 -fold higher levels intracellularly than in the extracellular environment.

3.4.3. Lepsey NLV1 extracted from LS, could not alone infect macrophage cells

In contrast, purified Lepsey NLV1 (obtained by sonication, sedimentation and filtration of LS cultures) failed to infect RAW 264.7 macrophages. Viral RNA was detected from 48 to 96 h.p.i. It was limited to the supernatant and remained comparable to inoculum levels, with no intracellular detection (Figs 2F and 2G). These findings indicated that purified Lepsey NLV1 alone could not enter or replicate in macrophages independently. Thereby, it highlights LS as a necessary carrier, likely delivering the virus via phagocytosis of infected parasites rather than direct cellular entry.

3.5 Microscopic observation of mammalian macrophages infected with *L. donovani* or *L. seymouri*

Spherical shaped amastigote images were observed under microscope at 72 h.p.i. inside Giemsa stained macrophages. LS amastigotes were visible within both RAW 264.7 (Figs 3A-C) and THP-1 cells (Figs 3D-F), albeit less conspicuously compared to LD.

Discussion:

Here, we conducted a comprehensive assessment of the infectivity of LS, a parasite frequently co-isolated with LD from kala-azar patients in India [13]. *In vitro* results from this study, suggested that LS could infect murine as well human macrophage cells such as RAW 264.7 and THP-1 respectively. Although the two parasites showed increased intracellular DNA over time, LS was abundantly detected in intracellular (IC) and extracellular (EC) compartments, both alone and as co-infecting parasite with LD. In co-infection scenario, upon progressive infection, the markedly higher extracellular LS burden suggested efficient intracellular amastigote replication coupled with enhanced LS-induced host-cell rupture, thereby increasing the extracellular release of LS amastigotes. The 2-fold increase in LS DNA in IC compartment at 168 h.p.i. compared to the initial inoculum (10^7 LS; though two-times washed after 4 h of cellular internalization) confirmed true intracellular replication of LS rather than residual carry-over. Moreover, co-infection experiments revealed that the presence of LD did not adversely affect LS replication. On the contrary, in murine macrophage cells, LS replicated faster

compared to LD resulting in 1:66 ratio in IC milieu at 168 h.p.i. despite 2 or 10-fold more abundance of LD in the original inoculum (LD: LS=2:1 or 10:1). A comparable earlier study, likewise, demonstrated critical preliminary evidence of the varying ability of LD strains to alter macrophage function, notably impairing the digestion of subsequently internalized *Leptomonas* sp and facilitating *Leptomonas* sp. survival [8]**.**

The THP-1 macrophage infection model further corroborated our observations in murine RAW 264.7 cells, confirming that LS could infect and replicate within differentiated human monocyte–derived macrophages. Although both LD and LS showed increasing intracellular parasite DNA over time, intracellular accumulation was greater than extracellular levels. Thus, suggesting active intracellular replication of LS rather than extracellular release. Importantly, the increase in combined parasite load (EC+IC) over time further supported active replication of LS within a human macrophage environment, thereby extending our murine findings to a clinically relevant host cell system.

In co-infection, a marked temporal shift in LD:LS ratio was observed. While LD predominated in the supernatant at 48 h.p.i., LS became the dominant extracellular parasite at 96 and 168 h.p.i., coinciding with a sharp reduction in the intracellular LD:LS ratio. This reciprocal redistribution suggested progressive intracellular accumulation of LS followed by enhanced release into the extracellular milieu at later time points, likely associated with increased rupture of LS-infected macrophages, consistent with observations in RAW 264.7 cells.

Interestingly, the intracellular persistence of LS in the presence of LD, as reflected by the stabilization of LD: LS ratios within macrophages over time, suggested that co-infection did not impose a competitive disadvantage on LS. Instead, LD might have provided a permissive

intracellular niche that supported LS survival and replication in human macrophages. This phenomenon mirrored our murine macrophage findings and argued against the notion that LS replication is typically restricted to insect hosts (Fig 4).

Our observations didn't align with a previous report that LS, as a monoxenous kinetoplastid, could not be replicated in mammalian cells (although different macrophage cell lines, J774 and BMM were used) [6]. Taken together, the RAW 264.7 and THP-1 infection models collectively supported a broader host adaptability of LS, highlighting its capacity for sustained intracellular replication, enhanced cyto-pathogenicity, and efficient dissemination from mammalian macrophages especially under co-infection conditions.

This is further consistent with earlier hypotheses that LS may act as a commensal or co-endosymbiont in LD infections, potentially influencing disease outcomes [16]. The viability assays demonstrated that parasites harvested from macrophage infections could be successfully revived in serum-supplemented Schneider's medium across multiple time points (72h-168h), confirming their persistence in intracellular environments. The progressive increase in parasite counts during sub-culturing underscored that LS retained their proliferative potential post-macrophage passage. However, the inability of free, non-internalised parasites to persist or replicate in macrophage culture conditions indicated that parasite survival beyond 48 h was exclusively attributable to an intracellular niche. Transfer of known number of parasites (3×10^6) from macrophage culture media to Schneider's medium beyond 48 h up to 168 h post-inoculation didn't result in any restoration of motility of parasites even after 9 days incubation and the final yields came less than inoculum. Only static parasites at 48 h in macrophage media gained motility and replicated in Schneider's growth medium. These observations ruled out

extracellular contamination as a confounding factor and strengthened the conclusion that parasite revival from cultures beyond 72 h.p.i. represented genuine intracellular survival and release. Conversely, neither LS nor LD parasites were able to replicate in DMEM-10 or RPMI-10 at 37°C media used during macrophage infections where parasite counts remained static and no motile forms were detected up to 8 days post-inoculation.

These data convincingly ruled out extracellular replication in culture medium, implicating intracellular macrophage environments as the primary niche for LS persistence and propagation during infection, consistent with previous suggestion of LS adaptation under host-induced selective pressures [5,16]**.** Taken together, these results emphasized the importance of the host cellular environment in sustaining parasite viability and suggested that LS possessed enhanced capacity to endure intracellular stress, a feature that might redefine its ecological persistence and pathogenic potential.

Dampened IL-12 secretion upon co-infection compared to mono-infections suggested a possible evasion mechanism from host immune system, consistent with the frequent detection of LD-LS co-infection in clinical VL samples. These observations aligned with our previously published observation of reduced IL-18 levels in human serum samples in case of co-infection cases compared to LD only samples [14]. Taken together, these results emphasized the importance of the host cellular environment in sustaining parasite viability. It suggested that LS possessed enhanced capacity to endure intracellular stress, a feature that might redefine its ecological persistence and pathogenic potential.

Microscopic evaluation of Giemsa-stained RAW 264.7 and THP-1 cells revealed amastigote-like forms of both LD and LS at 72 h.p.i. LD-infected cells exhibited more distinct amastigote forms.

In contrast the presence of LS in intracellular compartments, albeit less prominently, suggested a morphologically adaptive, possibly amastigote-like transformation of LS under intracellular stress. Notably, these findings diverged from the results reported by Kraeva et al., possibly due to differences in murine host cell lines employed, such as J774 or BMM□, which might not have supported LS persistence [6]**.** The replication and persistence of LS in mammalian macrophage cells further supported our molecular and growth-based observations of transient intracellular survival.

This study substantiated that LS, carrying Lepsey NLV1, could enter and persist within mammalian macrophages. RT-PCR revealed higher intracellular viral loads than extracellular milieu at 48h.p.i. This indicated efficient LS uptake and potential viral retention or amplification inside macrophages. Detected intracellular titres (10^6-10^7 gEs) contrasted with ~10^5-10^6 gEs in supernatants. This indicated robust intracellular localisation. This pattern remained stable over 168 h in murine macrophages, implying a non-replicative or tightly controlled viral state inside macrophage cells. The lack of increasing titre post-infection possibly reflected macrophage-mediated restriction or a steady-state balance of replication and degradation. Interestingly, in THP-1, a slight increase of ~4 fold in mean viral titre was observed in the tested time points compared to the initial time point of 48 h.p.i. (in case of mono- or co-infection). In THP-1, the reduction in total viral titre in the co-infection scenario compared to LS infection alone was negligible (~1.6-6.5-fold). This decrease was minimal relative to the significant reduction in LS parasite counts (~4-15-fold in EC versus ~50-1000-fold in IC) observed from 48 to 168 h.p.i. Therefore, co-infection with LD possibly appeared to favor virus replication and persistence within LS, although LS count was lower compared to only LS infection. The schematic highlights enhanced viral replication and improved LS persistence in the presence of LD across

murine and human macrophage models. It was evident from our calculations that the parasite: virus ratio in case of co-infection was significantly higher (2.5-23-fold) compared to LS mono-infection in case of both types of macrophages tested (Fig 4). .

Notably, purified Lepsey NLV1 could not enter or replicate in macrophages independently, thereby highlighting LS as a necessary carrier, likely delivering the virus via phagocytosis of infected parasites rather than direct cellular entry. Recent LD cases in Himachal Pradesh reported LS co-infection, raising questions about the virus's role in atypical cutaneous manifestations [3]**.** The exact impact of Lepsey NLV1 dynamics and host-pathogen interaction, however, remained to be fully explored.

Taken together, our findings presented a thought-provoking re-evaluation of LS, particularly in co-infection scenarios with LD. This study critically redefined the biological and pathogenic profile of LS, challenging its long-held classification as a non-infective, monoxenous parasite, but as a potentially replicative parasite capable of persisting within mammalian macrophages. The association of LS with the Lepsey NLV1 virus introduced a significant paradigm shift, highlighting a complex LD-LS-virus (triple pathogen) biology. It is noteworthy that *Leptomonas* sp. had been previously associated with infections in immunosuppressed patients [17]. These findings underscored the underestimated pathogenic potential of LS. It warrants a detailed mechanistic study into host-pathogen dynamics and its influence on *Leishmania* disease outcomes, particularly in co-infection contexts.

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AUTHORS' CONTRIBUTION

S.B., S.D. and P.D.S. conceived and designed the experiments. S.D. and P.D.S performed the experiments equally. S.D. and P.D.S. performed data analysis and S.B. performed critical analysis of the data. S.B. provided funding, reagents, materials and analysis tools. S.B., S.D. and P.D.S. jointly wrote the original draft of the manuscript. All authors critically reviewed and modified and agreed on the current version of the manuscript.

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DECLARATION OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY

Further information and resource requests should be directed to and will be fulfilled by the corresponding author, Dr. Subhajit Biswas (subhajit.biswas@iicb.res.in; subhabiscam@gmail.com).

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Figure Legends

Fig 1. Quantification of parasite load from infected macrophage cells.

Parasite load was assessed in culture **(A)** supernatants and **(B)** cell lysates from parasite infected RAW 264.7 cells at 48- and 168 h post-infection. Parasite load quantified from infected THP-1 macrophage **(C)** supernatants and **(D)** lysates at 48-,96- and 168- h.p.i. **(E)** Motile parasite counts from transformation/subculture flasks at 12 days post-transfer, derived from infected macrophage harvests collected at 48, 96, and 168 h.p.i and monitored using a hemocytometer.**(F)** Approx. 10^7 parasites /well were maintained in DMEM-10 at 37 °C for 48-168h and subsequently 2 x 10^6 parasites/time point were transferred to Schneider's medium at 22 °C to assess revival capacity at different time points.**(G)** Representative ITS-1 PCR image from DNA extracted from total parasite pellets harvested after exposure to macrophage growth conditions. PCR amplicons were resolved on a 1% agarose gel. L1: Ladder; L2- L4: LD PCR bands from parasites inoculated in DMEM-10 and totally pelleted after 24-, 72- and 168 h. L5-L7: LD PCR bands from parasites inoculated in RPMI-10 for 24-, 72- and 168 h, L8: 10^6 LD DNA as positive control; L9-L11: LS PCR bands from parasites inoculated in DMEM-10 and totally pelleted after 24-, 72- and 168 h. L12-L14: LS PCR bands from parasites inoculated in RPMI-10 and totally pelleted after 24-, 72- and 168 h, L15: 10^6 LS DNA as positive control (where L represents Lane number). (H) IL-12p40 cytokine level was measured by ELISA from cell supernatant infected with LD, LS and co-culture at 72 h.p.i. For (A-F and H), the data were represented as mean ± SD of a minimum of three independent experiments. Statistical comparisons were made using an unpaired two-tailed Student's t-test or one way-Anova where $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), ns = non- significant.

Fig 2. Quantification of viral load from parasite infected macrophage cells.

Viral titres were assessed in culture **(A)** supernatants and cell lysates from parasite infected RAW 264.7 cells at 48 h.p.i. Viral load quantified from infected RAW 264.7 macrophage. **(B)** supernatants and **(C)** lysates at 48- and 168- h.p.i. Quantification of viral titres from culture. **(D)** supernatants and **(E)** cell lysates of parasite infected THP-1 cells at 48-, 96- and 168 h.p.i. representative images of semi nested RT-PCR analysis for detection of Lepsey NLV1 from virus infected RAW 264.7 **(F)** supernatant and **(G)** lysate at 48-, 72- & 96 h.p.i. M: marker, L1-L6: represents 1st round of PCR and L7-L12: represents 2nd round of PCR. L1 and L7: cell control, L2-L4 and L8-L10: 48-72-96 h.p.i. samples respectively. L5 and L11: positive control, L6 and L12: negative control (where L represents lane number). For (A-E), the data are represented as mean ± SD of a minimum of three independent experiments. Statistical comparisons were made using an unpaired two-tailed Student's t-test or one way-Anova where $p < 0.05$ (*), $p < 0.001$ (***), $p < 0.0001$ (****), ns = non-significant.

Fig 3: Microscopic examination of Giemsa-stained infected macrophage cells at 72 h.p.i.

(A) uninfected RAW 264.7 macrophage cells; **(B)** RAW 264.7 macrophages infected with LD; **(C)** RAW 264.7 macrophages infected with LS. **(D)** Uninfected THP-1 macrophage cells; **(E)** THP-1 cells infected with LD; **(F)** THP-1 cells infected with LS. All images were acquired under a light microscope at 1000× magnification. Scale bar: 10 µm.

Fig 4: Schematic representation of parasite and viral load dynamics during mono-infection and co-infection in murine and human macrophage models.

Supporting Information Legends

S1 Table. Quantitative analysis of ITS1 qPCR-derived parasite load in RAW 264.7 cells.

Parasite load was quantified from supernatant and lysate samples collected at 48 and 168 h post-infection (h.p.i.). Data are presented as mean ± SD from a minimum of three independent experiments.

S2 Table. ImageJ-based relative band intensity analysis of ITS1 PCR products from infected RAW 264.7 cells.

Relative band intensities of ITS1 PCR products were quantified from supernatant and lysate samples of parasite-infected RAW 264.7 cells at 48 and 168 h post-infection (h.p.i.).

S3 Table. Quantitative analysis of parasite load from infected THP-1 cells.

Parasite load was quantified from supernatant and lysate samples collected at 48, 96, and 168 h post-infection (h.p.i.). Data are presented as mean ± SD from a minimum of three independent experiments.

S4 Table. ImageJ-based relative band intensity analysis of ITS1 PCR products from parasite-infected THP-1 cells.

Relative band intensity of ITS1 PCR products was analyzed from supernatant and lysate samples of parasite-infected THP-1 cells at 48, 96, and 168 h post-infection (h.p.i.).

S5 Text. Supplementary microscopic enumeration of motile parasites and corresponding graphical representation.

This file contains **(S5 Table)** showing representative counts of motile parasites per four microscopic fields and **(S1 Fig)** showing graphical representation of the same data.

S6 Text. Microscopic enumeration of motile parasites and corresponding graphical analysis at 11 days post-transfer.

This file contains **(S6 Table)** showing representative microscopic parasite counts from two different fields and **(S2 Fig)** showing graphical representation of motile parasite counts.

S7 Table. Representative counts of motile and transformed parasites in subculture flasks at different time points -12 days post-transfer.

Motile and transformed parasites were quantified using a haemocytometer following transfer of harvested cells into subculture flasks at the indicated time points. Data are presented as mean ± SD from a minimum of three independent experiments.

S8 Text. Supplementary data showing viable and motile parasite counts and graphical representation after subculture.

This file contains **(S8 Table)** showing hemocytometer-based parasite counts and **(S3 Fig)** showing graphical representation of parasite counts.

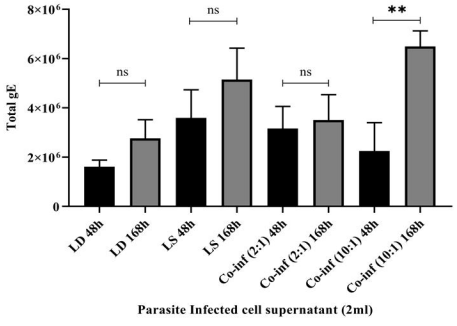
S9 Table. Quantitative analysis of viral load in infected RAW 264.7 macrophages.

Viral load was quantified from infected RAW 264.7 cell culture supernatant and cell lysate at 48 and 168 h post-infection (h.p.i.). Data are presented as mean ± SD from a minimum of three independent experiments.

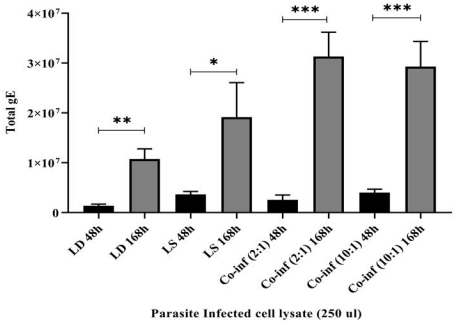
S10 Table. Quantitative analysis of viral load in infected THP-1 macrophages.

Viral load was quantified from infected THP-1 cell culture supernatant and cell lysate at 48, 96, and 168 h post-infection (h.p.i.). Data are presented as mean ± SD from a minimum of three independent experiments.

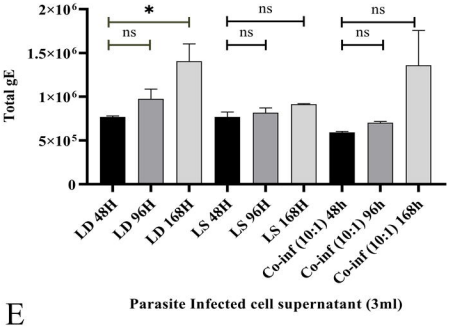
A


Total gE
8×10^6
6×10^6
4×10^6
2×10^6
0
ns
ns
ns
**
LD 48h
LD 168h
LS 48h
LS 168h
Co-inf (2:1) 48h
Co-inf (2:1) 168h
Co-inf (10:1) 48h
Co-inf (10:1) 168h
Parasite Infected cell supernatant (2ml)

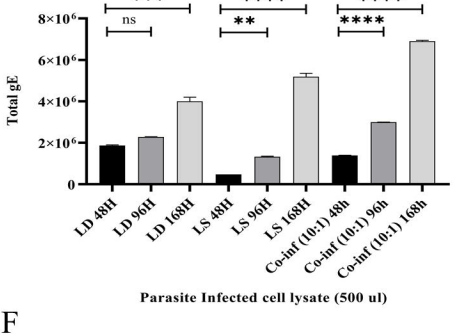
B


Total gE
4×10^7
3×10^7
2×10^7
1×10^7
0
**
*
***
***
LD 48h
LD 168h
LS 48h
LS 168h
Co-inf (2:1) 48h
Co-inf (2:1) 168h
Co-inf (10:1) 48h
Co-inf (10:1) 168h
Parasite Infected cell lysate (250 ul)

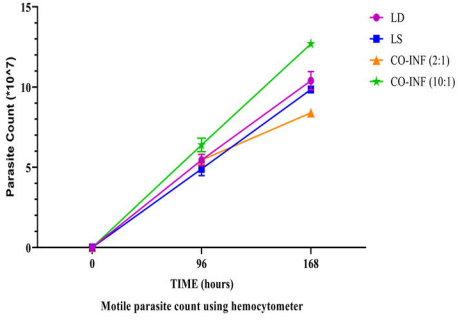
C


Total gE
2×10^6
1.5×10^6
1×10^6
5×10^5
0
*
ns
ns
ns
ns
ns
LD 48H
LD 96H
LD 168H
LS 48H
LS 96H
LS 168H
Co-inf (10:1) 48h
Co-inf (10:1) 96h
Co-inf (10:1) 168h
Parasite Infected cell supernatant (3ml)

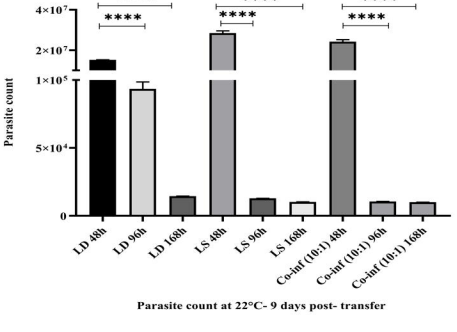
D


Total gE
8×10^6
6×10^6
4×10^6
2×10^6
0
***
ns
****
**
****
****
LD 48H
LD 96H
LD 168H
LS 48H
LS 96H
LS 168H
Co-inf (10:1) 48h
Co-inf (10:1) 96h
Co-inf (10:1) 168h
Parasite Infected cell lysate (500 ul)

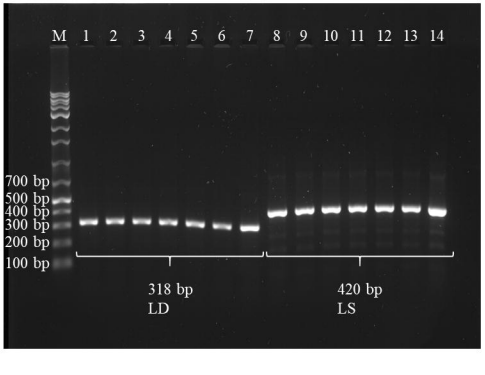
E


Parasite Count (*10^7)
15
10
5
0
0
96
168
TIME (hours)
LD
LS
CO-INF (2:1)
CO-INF (10:1)
Motile parasite count using hemocytometer

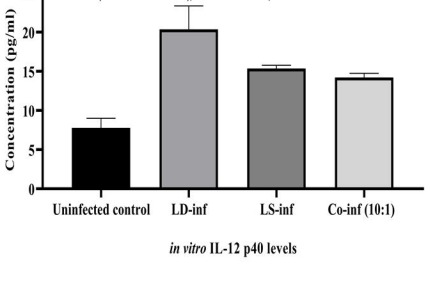
F

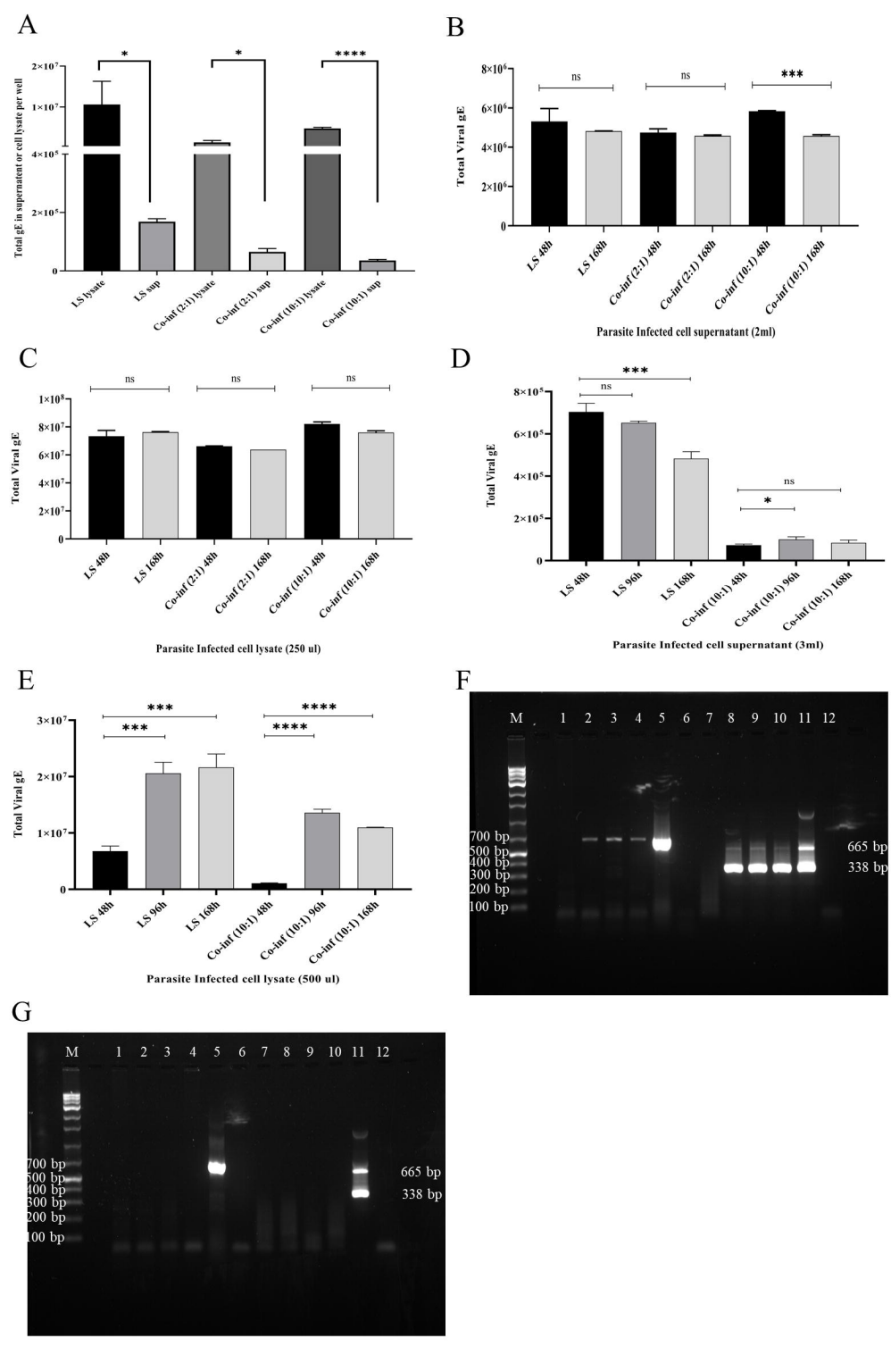

Parasite count
4×10^7
2×10^7
1×10^5
5×10^4
0
****
****
****
****
****
****
LD 48h
LD 96h
LD 168h
LS 48h
LS 96h
LS 168h
Co-inf (10:1) 48h
Co-inf (10:1) 96h
Co-inf (10:1) 168h
Parasite count at 22°C- 9 days post- transfer

G

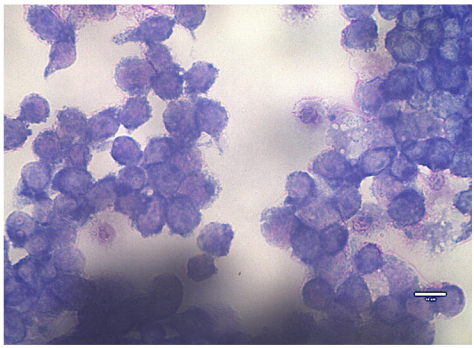

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14
700 bp
500 bp
400 bp
300 bp
200 bp
100 bp
318 bp
LD
420 bp
LS

H

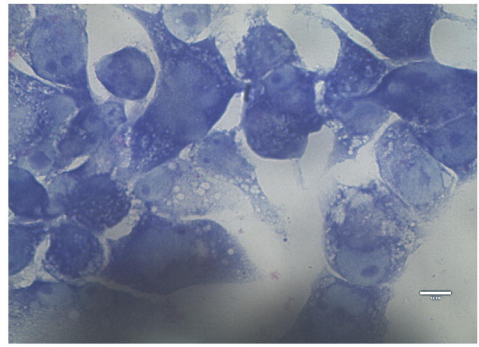

Concentration (pg/ml)
25
20
15
10
5
0
**
ns
*
Uninfected control
LD-inf
LS-inf
Co-inf (10:1)
in vitro IL-12 p40 levels


A
Total gE in supernatent or cell lysate per well
LS lysate
LS sup
Co-inf (2:1) lysate
Co-inf (2:1) sup
Co-inf (10:1) lysate
Co-inf (10:1) sup
B
Total Viral gE
LS 48h
LS 168h
Co-inf (2:1) 48h
Co-inf (2:1) 168h
Co-inf (10:1) 48h
Co-inf (10:1) 168h
Parasite Infected cell supernatant (2ml)
C
Total Viral gE
Parasite Infected cell lysate (250 ul)
D
Total Viral gE
LS 48h
LS 96h
LS 168h
Co-inf (10:1) 48h
Co-inf (10:1) 96h
Co-inf (10:1) 168h
Parasite Infected cell supernatant (3ml)
E
Total Viral gE
Parasite Infected cell lysate (500 ul)
F
M
700 bp
500 bp
400 bp
300 bp
200 bp
100 bp
665 bp
338 bp
G
M
700 bp
500 bp
400 bp
300 bp
200 bp
100 bp
665 bp
338 bp

RAW 264.7 **THP-1**



(A)



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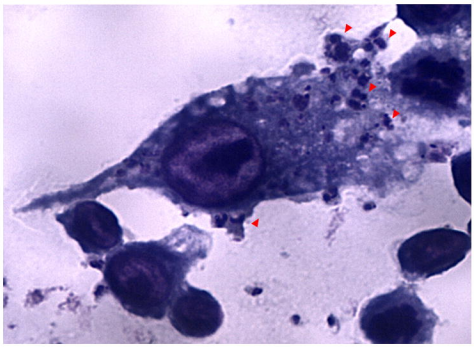
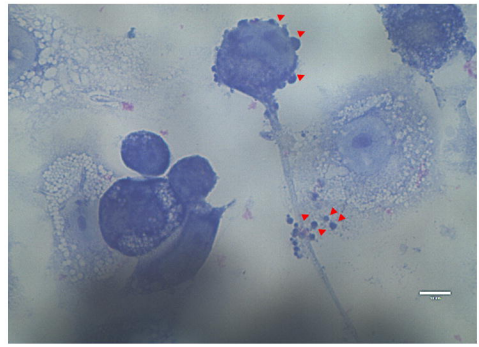
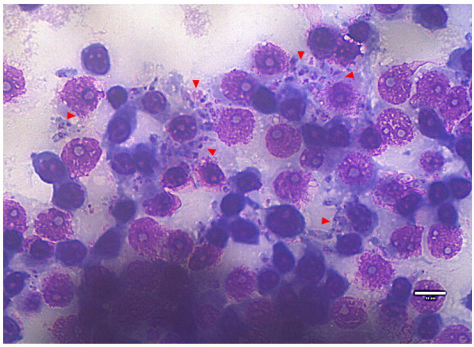


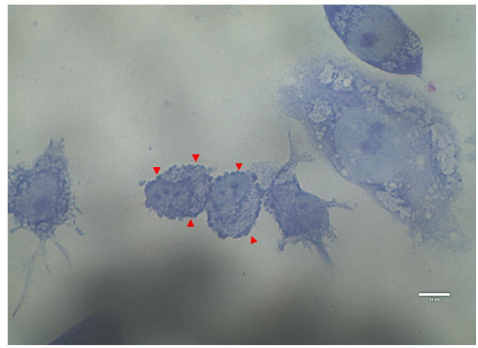
(B)



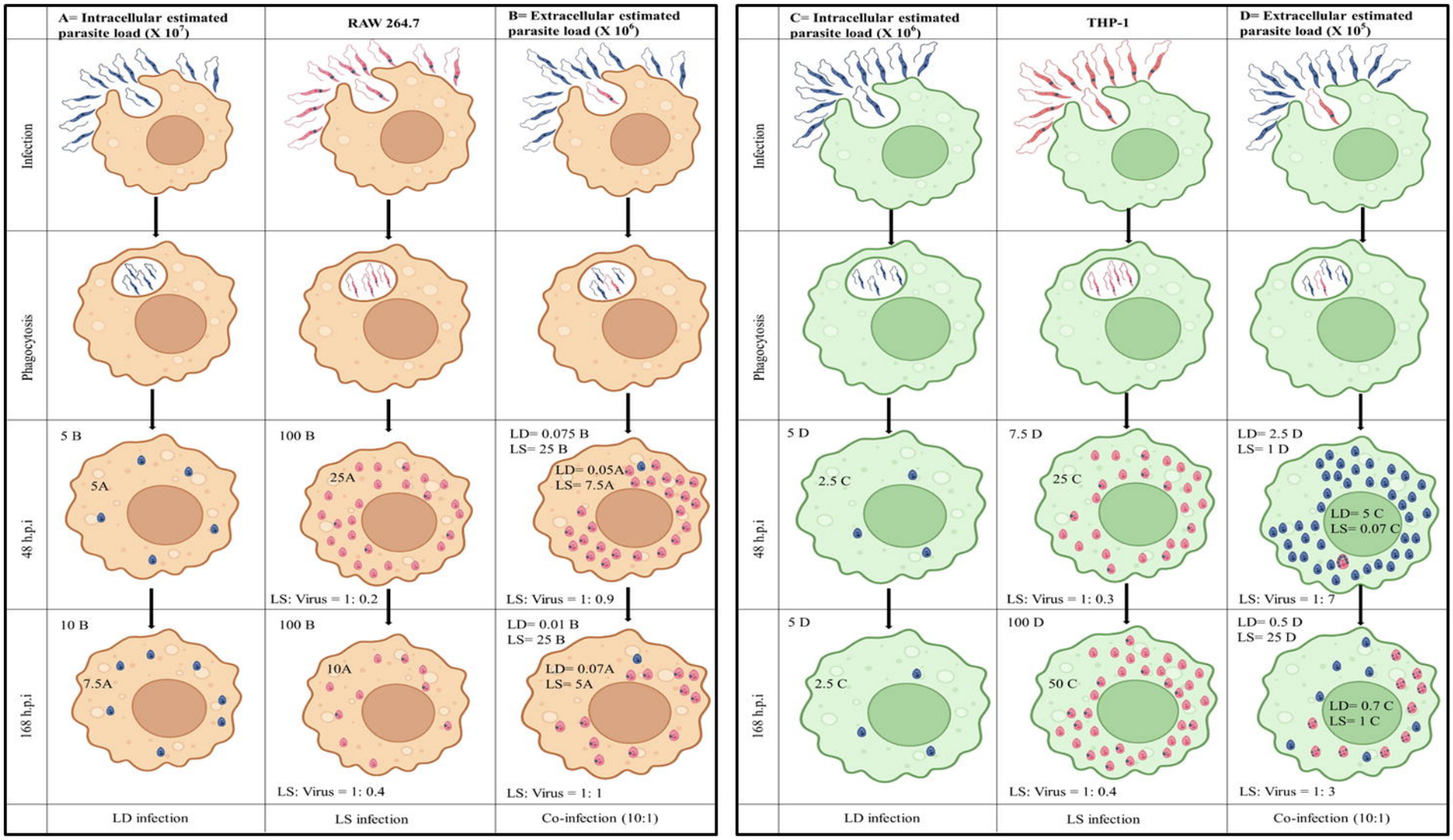
(E)



(C)



(F)



• Co-infection with LD **reduces host cell lysis** compared to mono-infection, thereby creating a more permissive intracellular environment that **supports LS persistence**.

• In co-infection scenario, the intracellular burden of virus increased compared to LS mono-infection,

- **RAW 264.7 macrophages: ~2.5–4.5-fold** increase in viral load.
- **THP-1 cells: ~7.5–23-fold** increase in viral load.
- **Enhanced intracellular viral survival or replication** in the presence of LD during co-infection.

* LD: LS relative quantity in co-infection was assayed by ImageJ analysis.