Adjuvanted *Leishmania infantum* extracellular vesicles induce protective immunity in experimental visceral leishmaniasis

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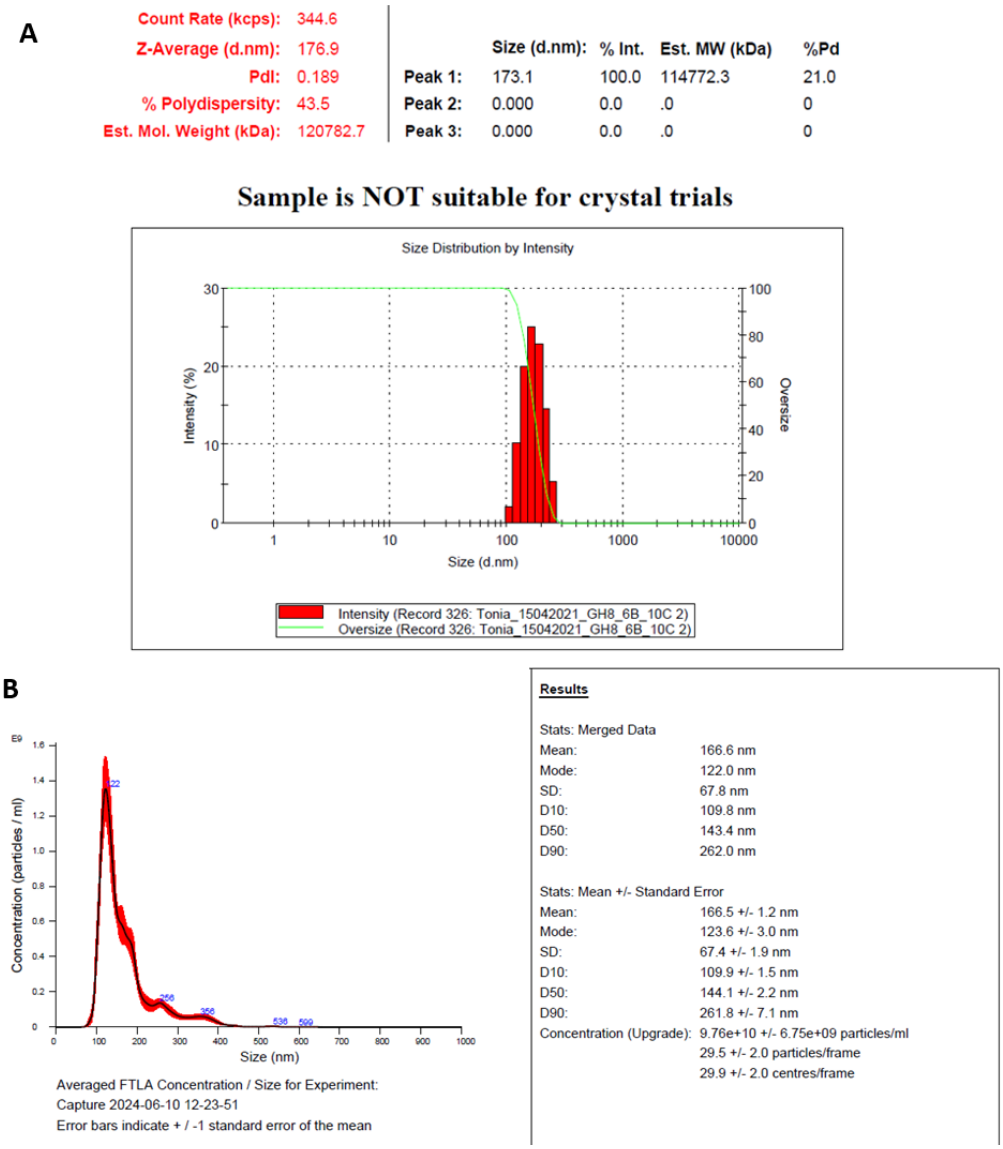
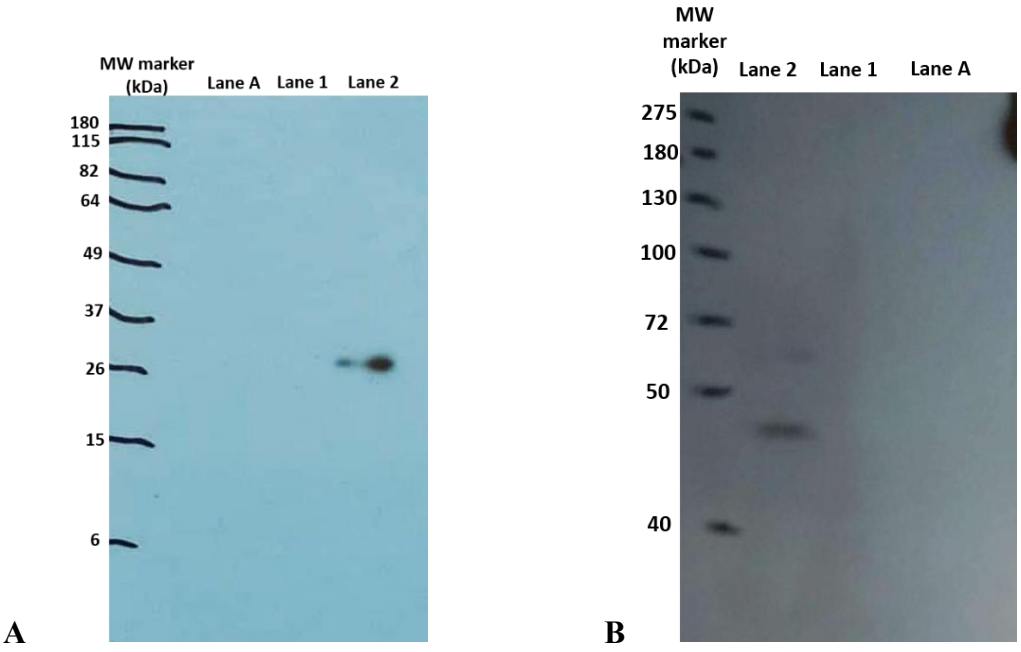
Supplementary Figures

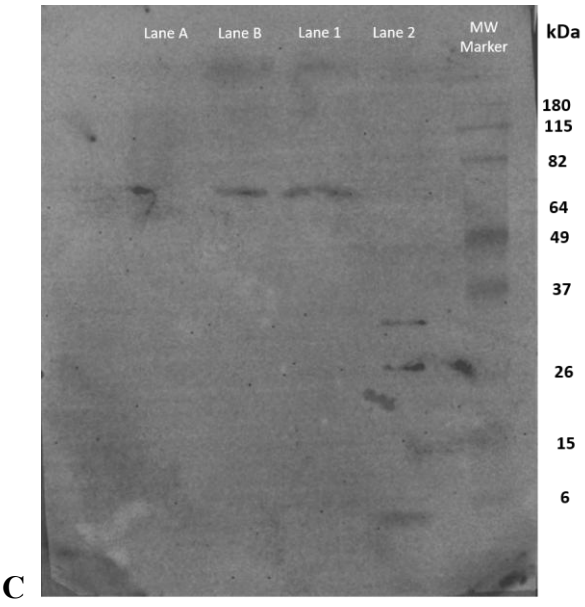


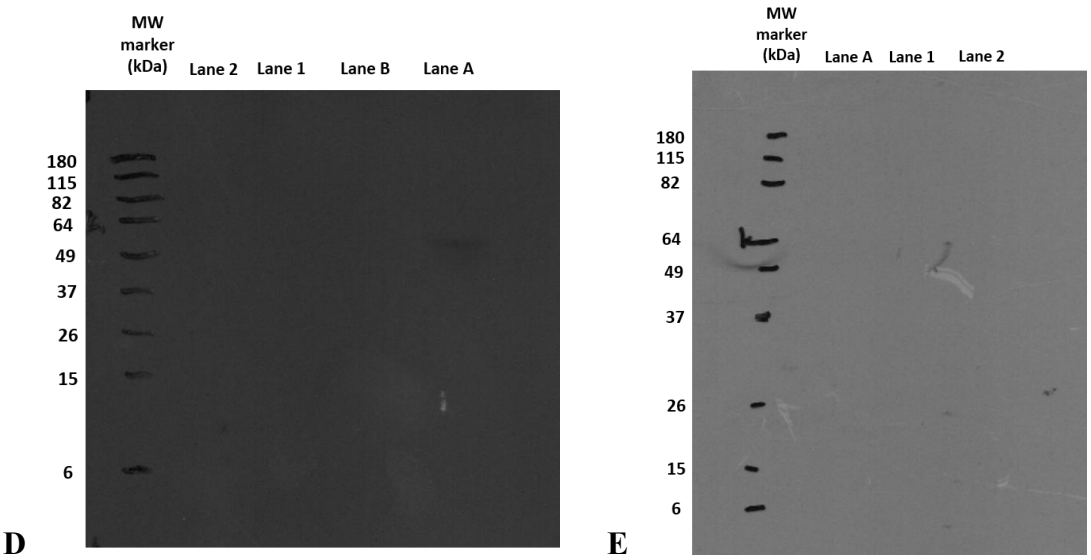
Supplementary Figure 1 | Characterization of extracellular vesicles.

A) Representative dynamic light scattering (DLS) output of extracellular vesicles. Intensity-weighted size distribution of EV preparations including Z-average and polydispersity index (PDI). Measurements were performed in triplicate, and reported values reflect technical variability across repeated acquisitions of the same sample. B) Representative nanoparticle tracking analysis (NTA) of extracellular vesicles. Averaged particle size distribution and concentration profile of isolated EV preparations measured using NanoSight analysis. Samples were diluted 1:500 prior to analysis, and values are presented as mean ± standard error derived from repeated video acquisitions.

Supplementary Movie 1 | Representative NanoSight recording demonstrating the dispersion and movement characteristics of isolated extracellular vesicles within the analyzed preparation.

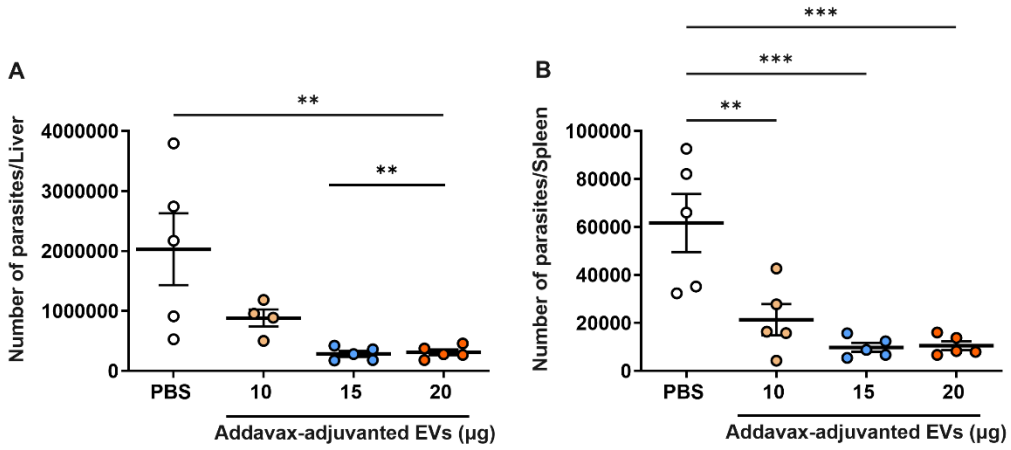






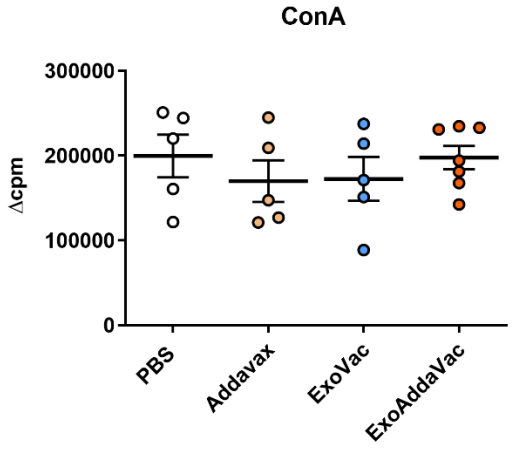
Supplementary Figure 2 | Western blot images. A) Scanned and unprocessed CD9 immunoblot corresponding to Figure 1. The CD9 immunoblot was detected by chemiluminescence and recorded on film. Molecular weight positions are indicated and were assigned by aligning the developed film with the corresponding Ponceau-stained membrane and protein marker. CD9 is expected to migrate at approximately 25–26 kDa. Lane A contains a low-concentration whole *Leishmania infantum* parasite lysate. Lane 1 corresponds to pooled EV-negative fractions, while Lane 2 corresponds to the pooled EV immunization fraction used for downstream characterization and vaccination studies. Lanes 1 and 2 are the samples presented in Figure 1. **B)** Scanned and unprocessed CD63 immunoblot corresponding to Figure 1. The CD63 immunoblot was detected by chemiluminescence and recorded on film. Molecular weight positions are indicated and were assigned by aligning the developed film with the corresponding Ponceau-stained membrane and protein marker. CD63 is expected to migrate at approximately 30–60 kDa, depending on glycosylation status. Lane A contains a low-concentration whole *Leishmania infantum* parasite lysate. Lane 1 corresponds to pooled EV-negative fractions, while Lane 2 corresponds to the pooled EV immunization fraction used for downstream characterization and vaccination studies. Lanes 1 and 2 are the samples presented in Figure 1. **C)** Scanned and unprocessed CD81 immunoblot corresponding to Figure 1. Molecular weight marker positions are indicated. CD81 is expected to migrate between approximately 23–35 kDa. Lane A and Lane B contain low-concentration whole *Leishmania infantum* parasite lysates. Lane 1 corresponds to pooled EV-negative fractions, while Lane 2 corresponds to the pooled EV

immunization fraction used for downstream characterization and vaccination studies. Lanes 1 and 2 are the samples presented in Figure 1. The CD81 immunoblot was acquired using a ChemiDoc imaging system. **D)** Scanned and unprocessed albumin immunoblot corresponding to Figure 1. The albumin immunoblot was detected by chemiluminescence and recorded on film. Molecular weight positions are indicated and were assigned by aligning the developed film with the corresponding Ponceau-stained membrane and protein marker. Albumin is expected to migrate at approximately 66 kDa. Lane A and Lane B contain a low-concentration whole *Leishmania infantum* parasite lysate. Lane 1 corresponds to pooled EV-negative fractions, while Lane 2 corresponds to the pooled EV immunization fraction used for downstream characterization and vaccination studies. Lanes 1 and 2 are the samples presented in Figure 1. **E)** Scanned and unprocessed cytochrome c immunoblot corresponding to Figure 1. The cytochrome c immunoblot was detected by chemiluminescence and recorded on film. Molecular weight positions are indicated and were assigned by aligning the developed film with the corresponding Ponceau-stained membrane and protein marker. Cytochrome c is expected to migrate at approximately 12 kDa. Lane A contains a low-concentration whole *Leishmania infantum* parasite lysate. Lane 1 corresponds to pooled EV-negative fractions, while Lane 2 corresponds to the pooled EV immunization fraction used for downstream characterization and vaccination studies. Lanes 1 and 2 are the samples presented in Figure 1.

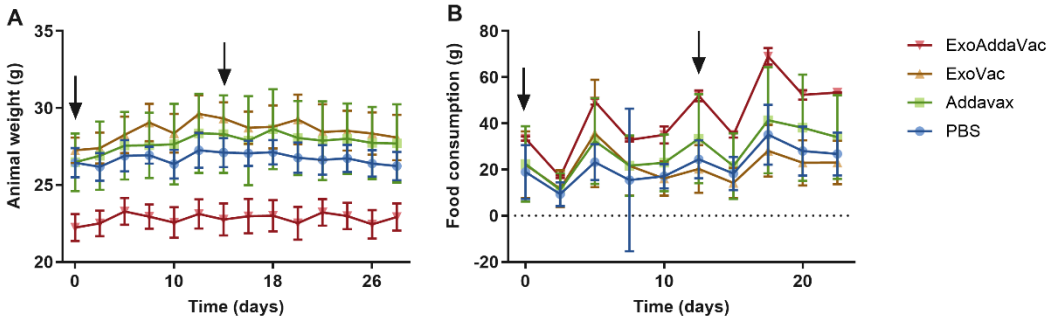


Supplementary Figure 3 | Preliminary dose-selection analysis of EV immunization in mice.

BALB/c mice were immunized twice at two-weeks interval with 10, 15, or 20 µg of Addavax-adjuvanted *L. infantum* EVs, corresponding to approximately 3.67×10^9, 5.51×10^9, and 7.34×10^9 particles, respectively, based on NTA characterization of the pooled EV preparation. Two weeks after the booster immunization, mice were intravenously challenged with stationary-phase *L. infantum* promastigotes and 30 days post-infection (acute phase) parasite burden was determined in (A) liver and (B) spleen. Data are representative of one independent experiment with n = 4-5 mice per group. Points represent mean values ± SEM. Comparable reductions in parasite burden were observed in the 15 and 20 µg EV-immunized mice groups; therefore, the 15 µg dose was selected for all subsequent experiments.

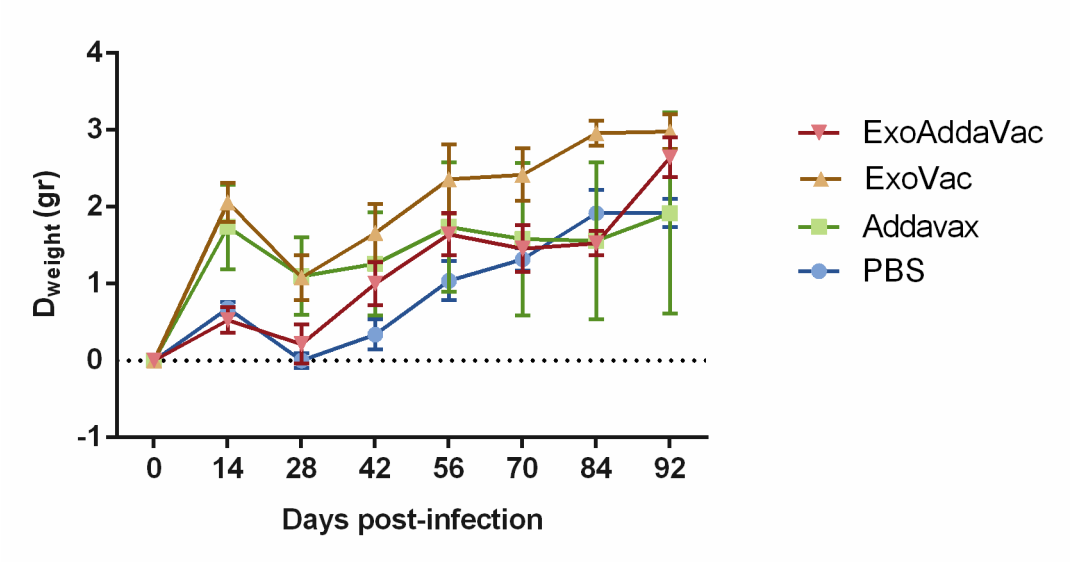


Supplementary Figure 4 | Lymphoproliferative responses in the presence of ConA. Mice were intramuscularly immunized twice a two-weeks interval with 15 µg EVs (ExoVac) or 15 µg EVs adjuvanted with Addavax (ExoAddaVac). Mice injected with equal volumes of PBS or Addavax served as control groups. Spleens were collected two weeks after the booster immunization. Splenocytes were stimulated with ConA and lymphoproliferative responses were expressed as Δcpm. Data are representative of one independent experiment with n = 5-6 mice per group. Points represent mean values ± SEM.



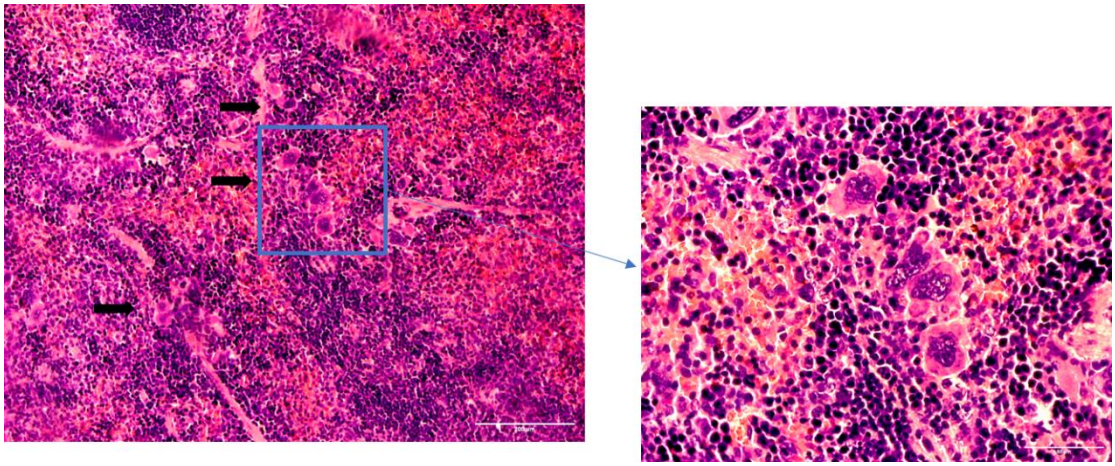
Supplementary Figure 5 | Changes in body weight of PBS-, Addavax-, ExoVac-, and ExoAddaVac-immunized mice.

Mice were intramuscularly immunized twice a two-weeks interval with 15 µg EVs (ExoVac) or 15 µg EVs adjuvanted with Addavax (ExoAddaVac). Mice injected with equal volumes of PBS or Addavax served as control groups. (A) Body weight was monitored (B) Food consumption was monitored every two days. Black arrows indicate the time points of the first and second immunizations. Data are representative of one independent experiment with n = 4–7 mice per group. Points represent mean values ± SEM.



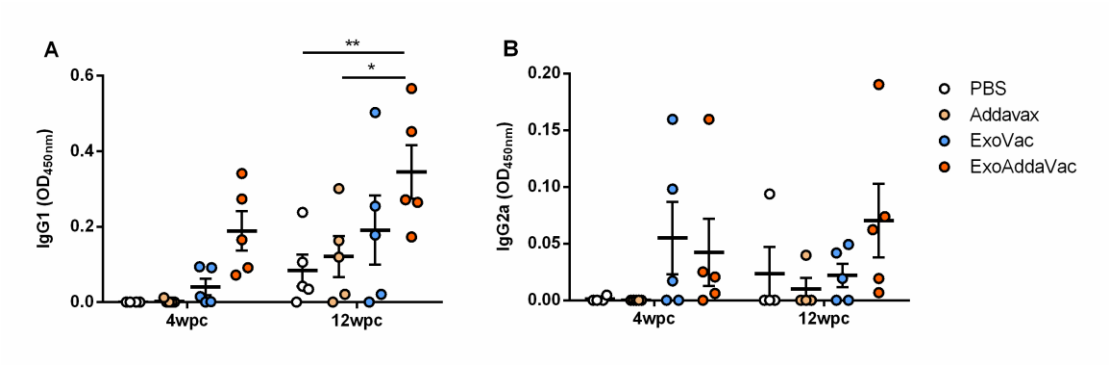
Supplementary Figure 6 | Changes in body weight of immunized mice following challenge with *Leishmania infantum* promastigotes.

Mice were intramuscularly immunized twice at a two-week interval with 15 µg EVs (ExoVac) or 15 µg EVs adjuvanted with Addavax (ExoAddaVac). Mice injected with equal volumes of PBS or Addavax served as control groups. Two weeks after the booster immunization, mice were intravenously challenged with stationary-phase L. infantum promastigotes, and body weight was monitored every two weeks up to 12 weeks post challenge (92 days). Data are representative of one independent experiment with n = 4–7 mice per group. Points represent mean values ± SEM.



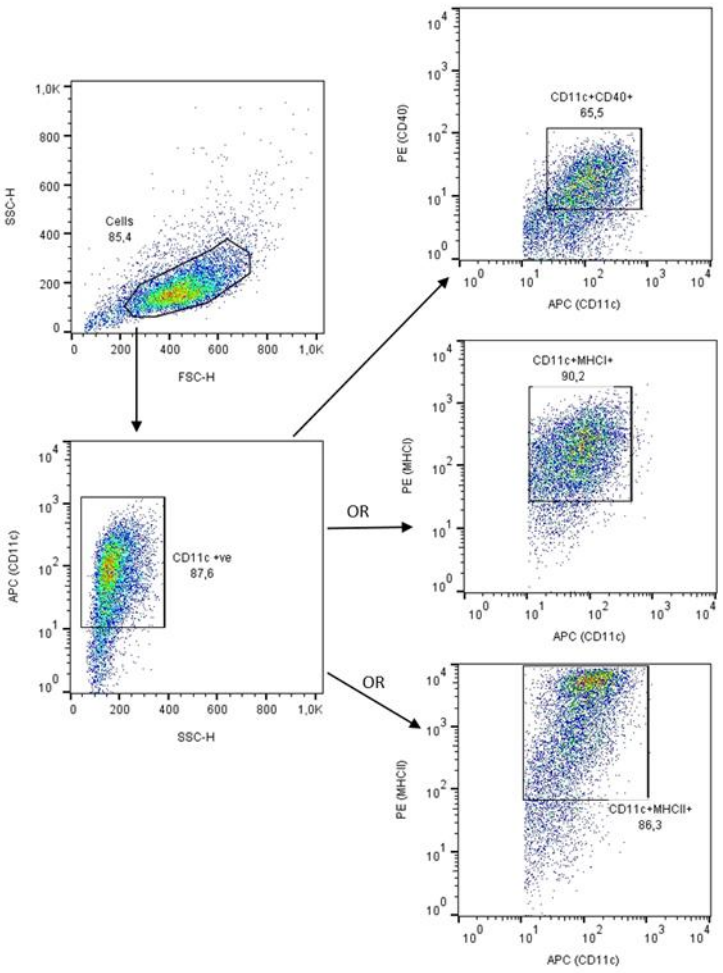
Supplementary Figure 7 | Detection of multinucleated giant cells (MGCs) in infected control mice.

Representative image showing the presence of multinucleated giant cells (MGCs), a hallmark of *Leishmania* infection and inflammation, in the spleen of the non-immunized, infected (PBS) mice at 12 weeks post challenge. Black arrows indicate MGCs. The spleen section (magnification: 20×; scale bar: 200 µm) is shown together with higher-magnification view (60×; scale bar: 50 µm) of the region indicated by the red arrow.



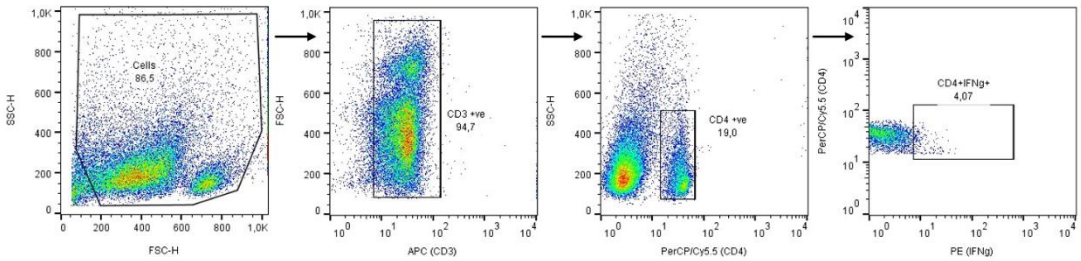
Supplementary Figure 8 | Detection of parasite-specific IgG isotypes during *L. infantum* infection.

(A and B) Mice were intramuscularly immunized twice at a two-week interval with 15 µg EVs (ExoVac) or 15 µg EVs adjuvanted with Addavax (ExoAddaVac). Mice injected with equal volumes of PBS or Addavax served as control groups. Two weeks after the booster immunization, mice were intravenously challenged with L. infantum promastigotes, and immune responses were evaluated during the acute (4 weeks post challenge) and chronic (12 weeks post challenge) phases of infection. Parasite-specific IgG1 (A) and IgG2a (B) antibodies were measured by ELISA. Data are representative of one independent experiment with n = 5 mice per group. Each symbol represents an individual animal; bars indicate mean values ± SEM. *$p < 0.05$, **$p < 0.01$ by two-way ANOVA with Tukey's multiple-comparison test.



Supplementary Figure 9 | Identification and analysis of BMDCs by flow cytometry, related to Methods.

Flow cytometry gating strategy used for the identification of BMDCs ($CD11c^+$) expressing CD40, MHCI or MHCII after stimulation with EVs in the presence or not of Cytochalasin D or LPS or left in medium alone.



Supplementary Figure 10 | Identification and analysis of EVs-specific CD4$^+$ T cells by flow cytometry, related to Methods.

Flow cytometry gating strategy used for the identification of cytokine-producing CD4$^+$ T cells following stimulation with EVs or SLA.