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Supplementary Materials: Supplementation with serum-derived extracellular vesicles reinforces antitumor immunity induced by cryo-thermal therapy

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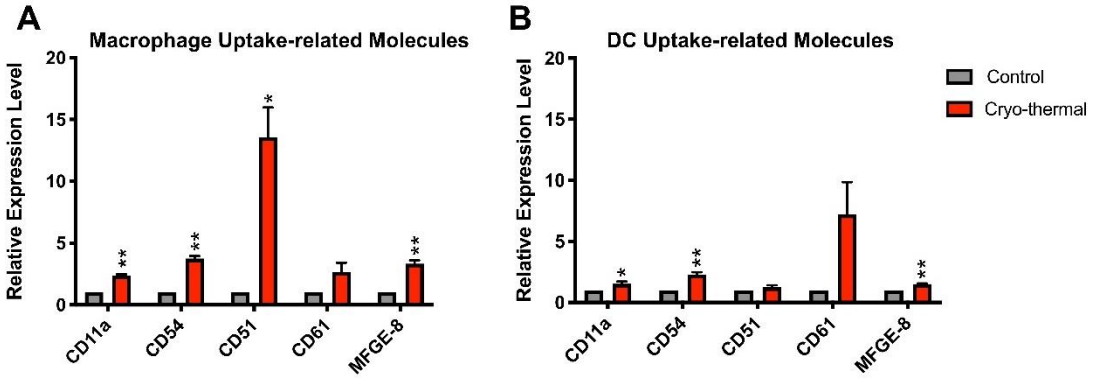
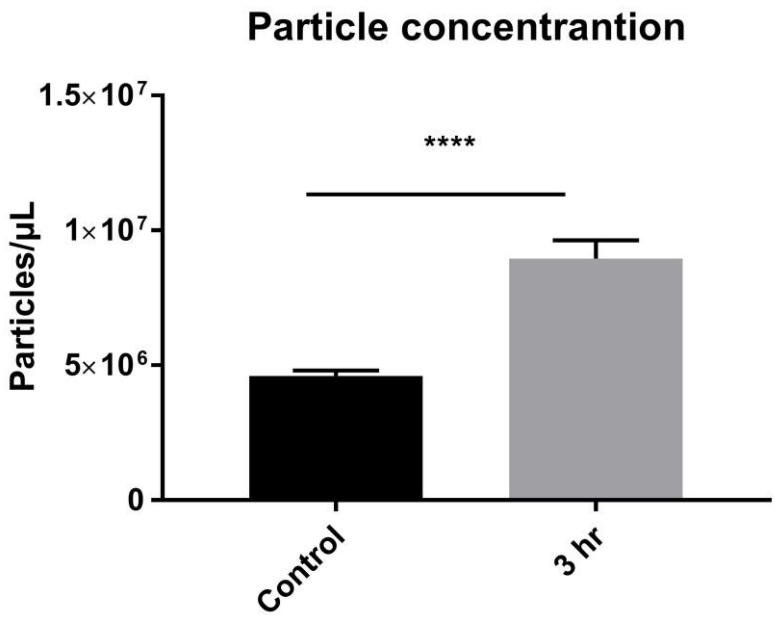
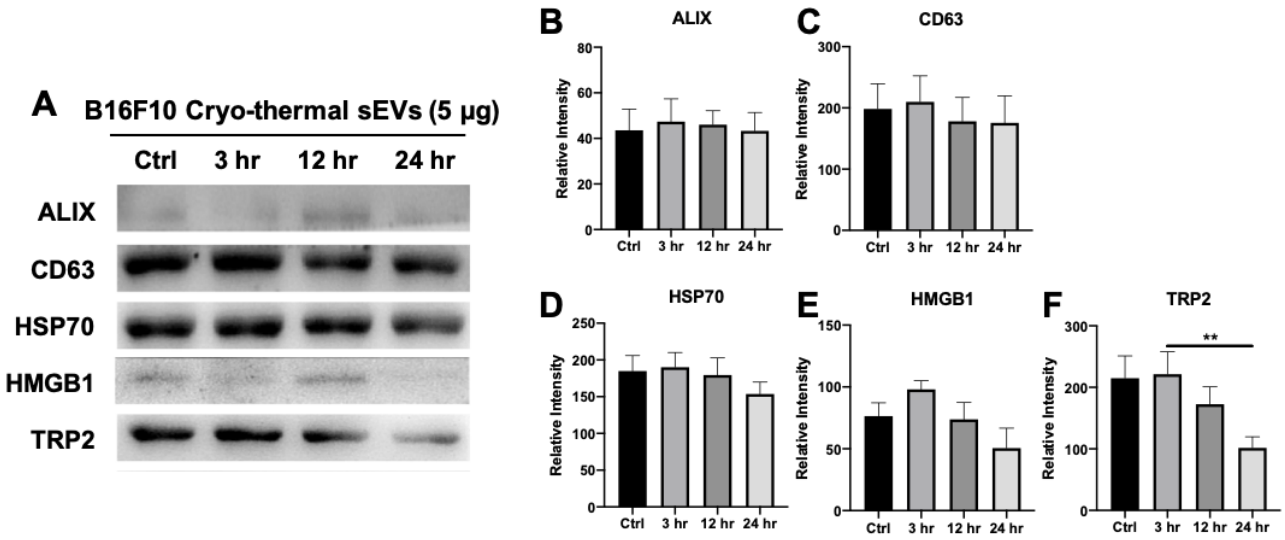


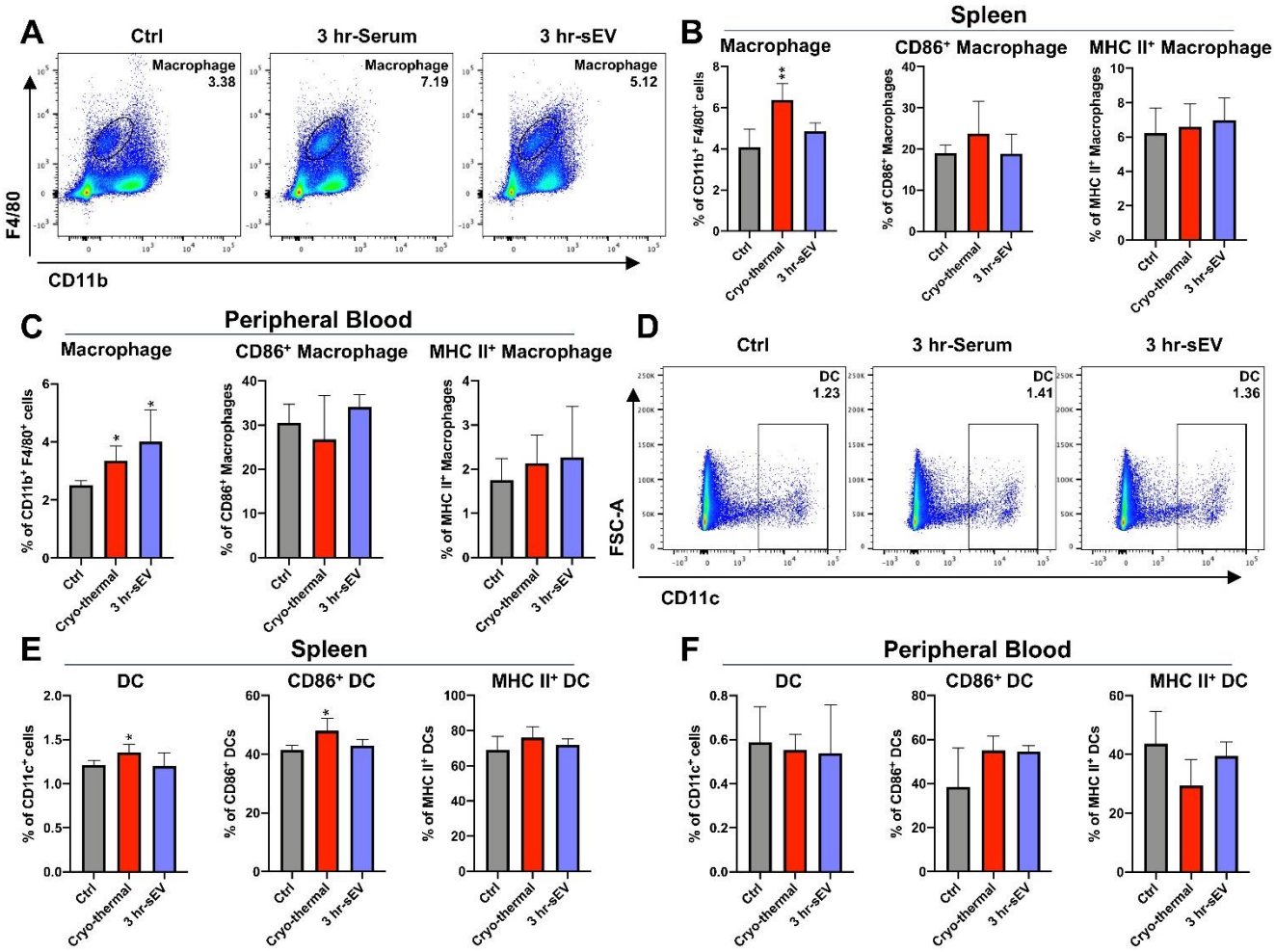
Supplementary Figure S1 The up-regulated expression of EV uptake-related genes in spleen macrophages and DCs on day 3 after cryo-thermal therapy in 4T1 subcutaneous tumor model. Total RNA of spleen macrophages and DCs were isolated on day 3 after treatment. RT-qPCR was used to detect mRNA levels of EV phagocytosis-related protein of macrophages (**A**) and DCs (**B**) on day 3 after treatment. Data were shown as mean±SD, and the student's t-test was used for statistical analysis. n = 4, * $p < 0.05$, ** $p < 0.01$ compared with the control group.



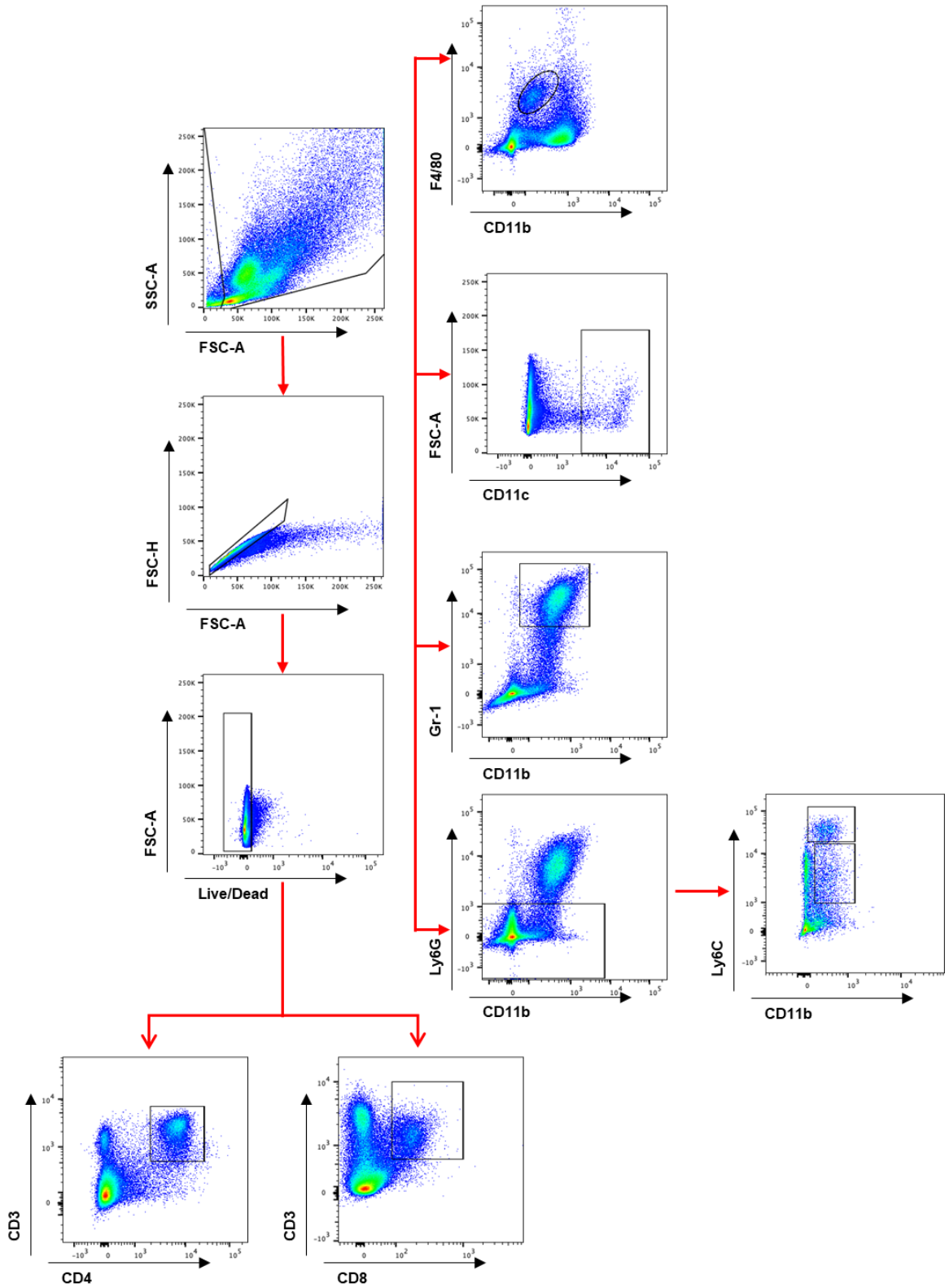
Supplementary Figure S2 Cryo-thermal therapy induced the release of sEVs. sEVs concentration (standardized by serum volume) 3hr after cryo-thermal therapy were measured by Nanoparticle Tracking Analysis (NTA). Data were shown as mean±SD, and the student's t-test was used for statistical analysis. n = 5, **** $p < 0.0001$ compared with the control group.



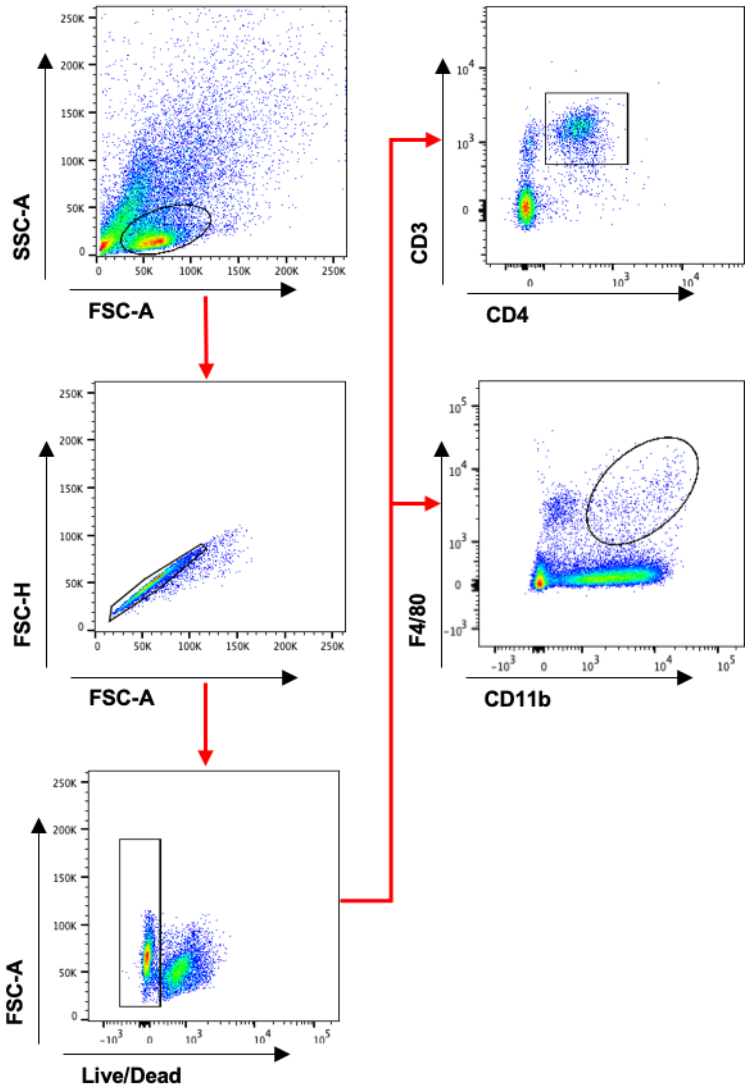
Supplementary Figure S3 Western blotting of sEVs (5 μg) from the serum in B16F10 model at 3, 12, and 24 hr after cryo-thermal therapy with individual densitometry analysis. ALIX, CD63: Exosomal protein markers; HSP70, HMGB1: danger signal; TRP2: known B16 tumor antigen. Data were shown as mean±SD, and the student's t-test was used for statistical analysis. n = 3, ** $p < 0.01$.



Supplementary Figure S4 Supplement of sEVs after cryo-thermal therapy upregulated the proportion of macrophages and DCs. (**A**) Flow cytometry gating strategy for the determination of $CD11b^+$ $F4/80^+$ macrophages. (**B**-**C**) Proportions of macrophages, $CD86^+$ macrophages, and MHC II^+ macrophages in spleen (**B**) and peripheral blood (**C**)were determined by using flow cytometry (n = 4 per group). (**D**) Flow cytometry gating strategy for the determination of $CD11c^+$ DCs. (**E**-**F**) Proportions of DCs, $CD86^+$ DCs, and MHC II^+ DCs in spleen (**E**) and peripheral blood (**F**) were determined by using flow cytometry (n = 4 per group). Data were shown as mean±SD, and the student's t-test was used for statistical analysis. * $p < 0.05$, ** $p < 0.01$ compared with the control group.



Supplementary Figure S5 Flow cytometry gating strategy for the determination of CD11b$^+$ F4/80$^+$ macrophages, CD11c$^+$ DCs, CD11b$^+$ Gr1$^+$ MDSCs, CD11b$^+$ Ly6G$^-$ Ly6C$^+$ Monocytes, CD3$^+$ CD4$^+$ T cells and CD3$^+$ CD8$^+$ T cells in in vivo analyses.



Supplementary Figure S6 Flow cytometry gating strategy for the determination of CD11b^{+} F4/80^{+} macrophages and CD3^{+} CD4^{+} T cells in in vitro analyses.