Supplementary Information

Elucidating Mechanisms of Antitumor Immunity Mediated by Live Oncolytic Vaccinia and Heat-Inactivated Vaccinia

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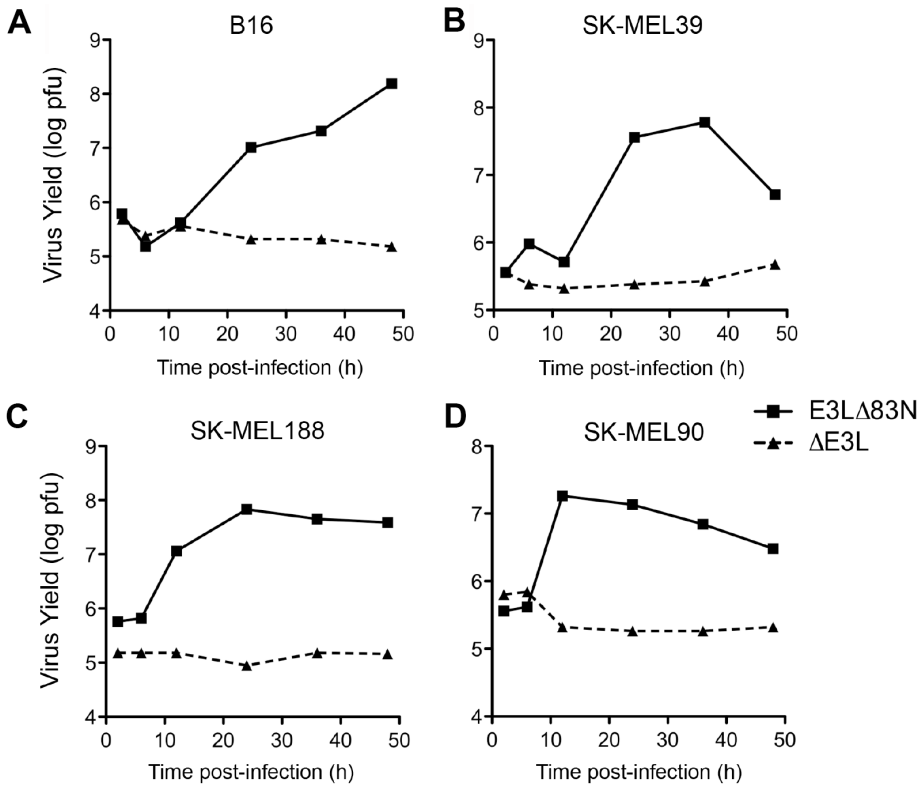

Figure S1 Virus replication of vaccinia E3LΔ83N virus in multiple tumor cell lines. Cells were infected with vaccinia E3LΔ83N or vacciniaΔE3L virus at MOI of 10. Samples were collected at different time points post infection and virus yields were determined by titration on BSC40 cells. Results from B16-F10 (A), SK-MEL39 (B), SK-MEL188 (C), SK-MEL90 (D) cells are shown.

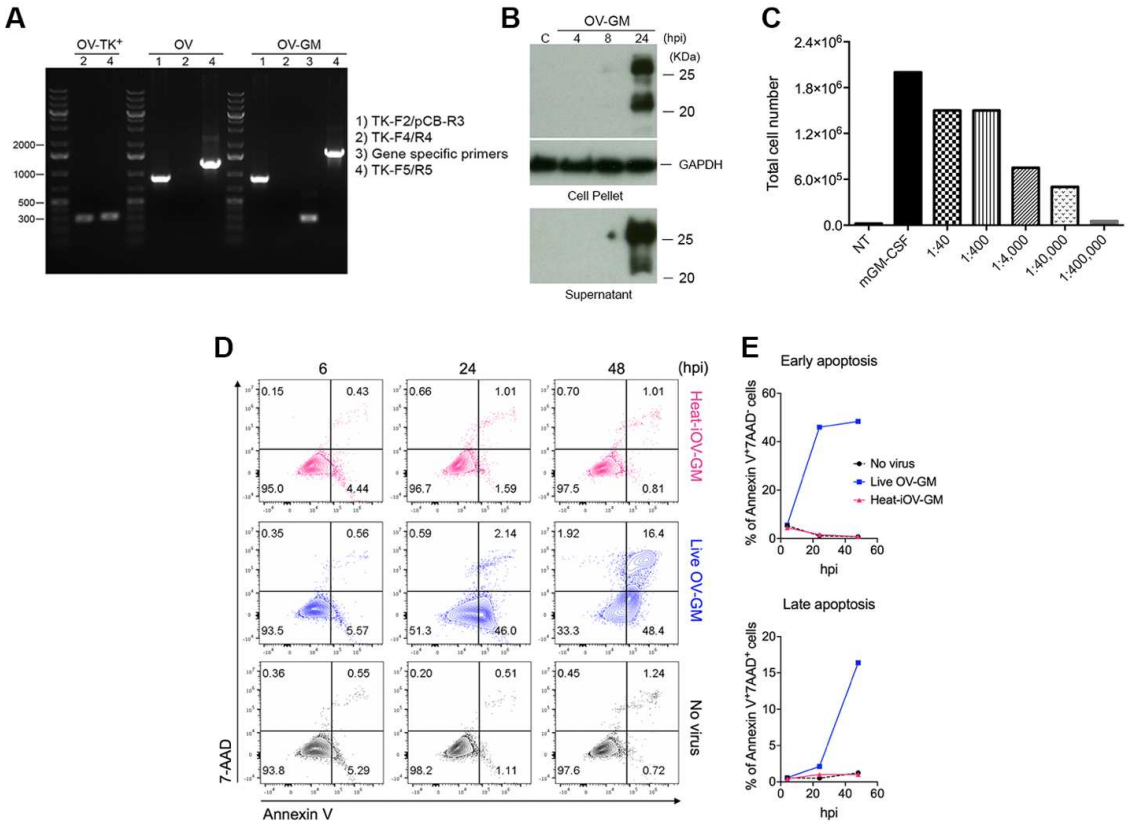

Figure S2 Expression of GM-CSF by B16-F10 cells infected with live OV-GM and cell viability after virus infection on B16-F10 cells. (A) PCR verification of recombinant OV and OV-GM. (B) Western blot analysis of mGM-CSF expression in OV-GM-infected murine B16-F10 cells. mGM-CSF protein was detected in both the cell pellet and the culture supernatant. (C) Bioactivity of secreted mGM-CSF protein produced by B16-F10 cells infected with OV-GM. Bone marrow cells (2.5×10^5) from C57BL/6J mice were cultured in the presence of recombinant GM-CSF at 20 ng/ml or with serial dilutions of supernatants from OV-GM-infected B16-F10 melanoma cells for 7 days, and they were subjected to flow cytometry analysis. The total numbers of $CD11c^+$ DCs in various culture conditions are shown. (D, E) Cell apoptosis induced by live OV-GM or heat-iOV-GM infection. B16-F10 cells were infected by indicated viruses at a MOI of 10. Cells were collected at different time points and stained for apoptotic markers. Samples were analyzed by flow cytometry. (D) Frequencies of Annexin $V^+7\text{-}AAD^-$, Annexin $V^+7\text{-}AAD^+$ of each sample are shown in the dot plots. (E) The percentages of early and late apoptotic cell out of total cells are shown.

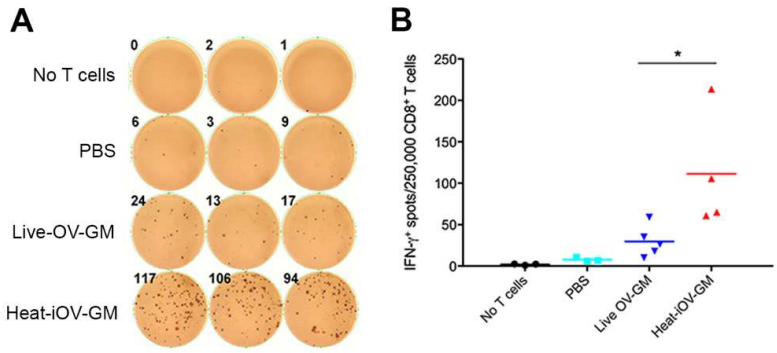

Figure S3 Higher anti-tumor IFN-γ^+CD8$^+$ T cells in the spleen from mice treated with heat-iOV-GM compared with live OV-GM in MC38 murine colon cancer model. MC38 tumor cells were intradermally implanted into both flanks of C57BL/6J mice. Established tumors were treated with either Live-OV-GM or heat-iOV-GM. PBS was used as a control. IFN-γ^+CD8$^+$ T cells from spleens of MC38 tumor-bearing mice treated with different viruses were analyzed using ELISPOT assay. (A) Representative images from an ELISPOT assay. (B) IFN-γ^+ spots per 250,000 purified CD8$^+$ T cells from the spleens of the mice treated with IT PBS, OV, live OV-GM, or heat-iOV-GM (n=5, *$P < 0.05$; **$P < 0.01$).

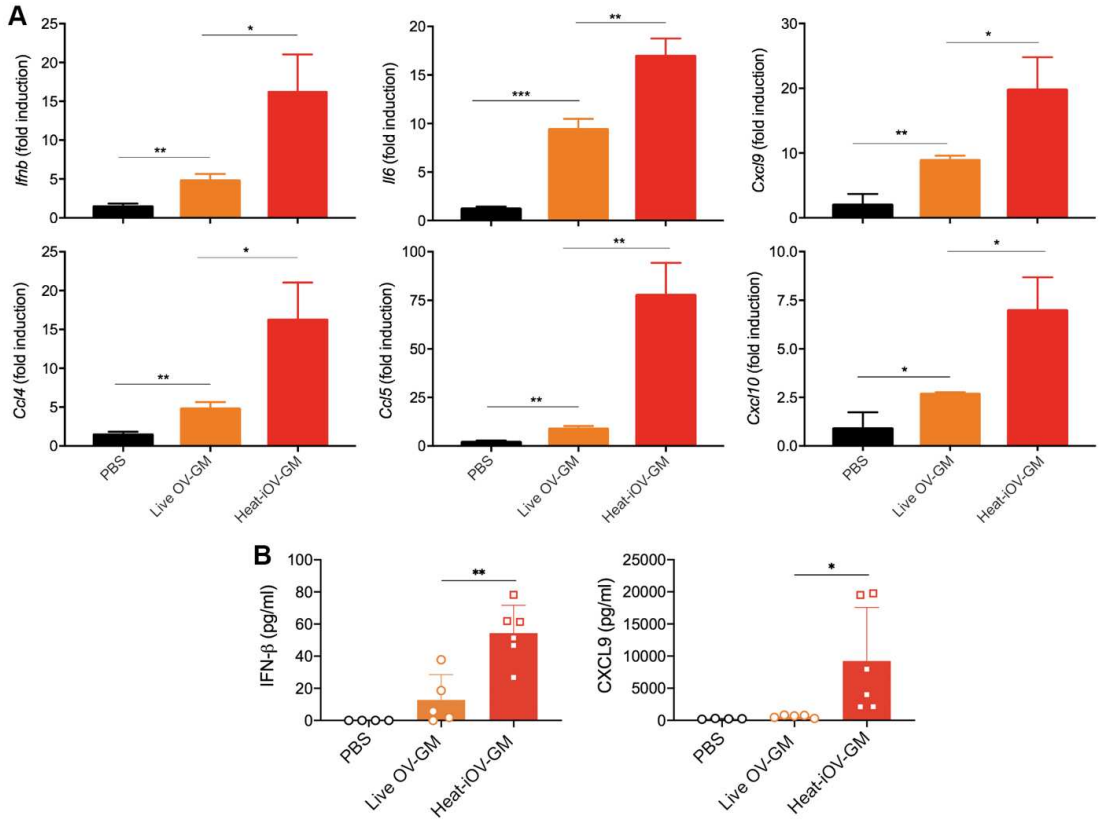

Figure S4 IT heat-iOV-GM induces higher levels of IFN and proinflammatory cytokines and chemokines in the injected tumors compared with IT live OV-GM. B16-F10 melanoma cells were implanted intradermally on the right flank of C57BL/6J mice. Once tumors reach 3-4 mm in diameter, they were injected with either PBS or live OV-GM (2 x 10^7 pfu), or with equivalent amounts of heat-iOV-GM. The tumors were harvested one day after injection and mRNAs were extracted. (A) Shown here are quantitative real-time PCR analyses of *Ifnb*, *Ccl4*, *Il6*, *Ccl5*, *Cxcl9*, and *Cxcl10* gene expression in the injected B16-F10 tumors from mice treated with either PBS, live OV-GM, or heat-iOV-GM (n=4-5, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *t* test). (B) Tumors were harvested one day post injection and homogenized by GentleMACS Dissociator in PBS in the presence of proteinase inhibitor. The levels of IFNβ and CXCL9 were determined by ELISA (n=5-6, **P* < 0.05, *t* test).

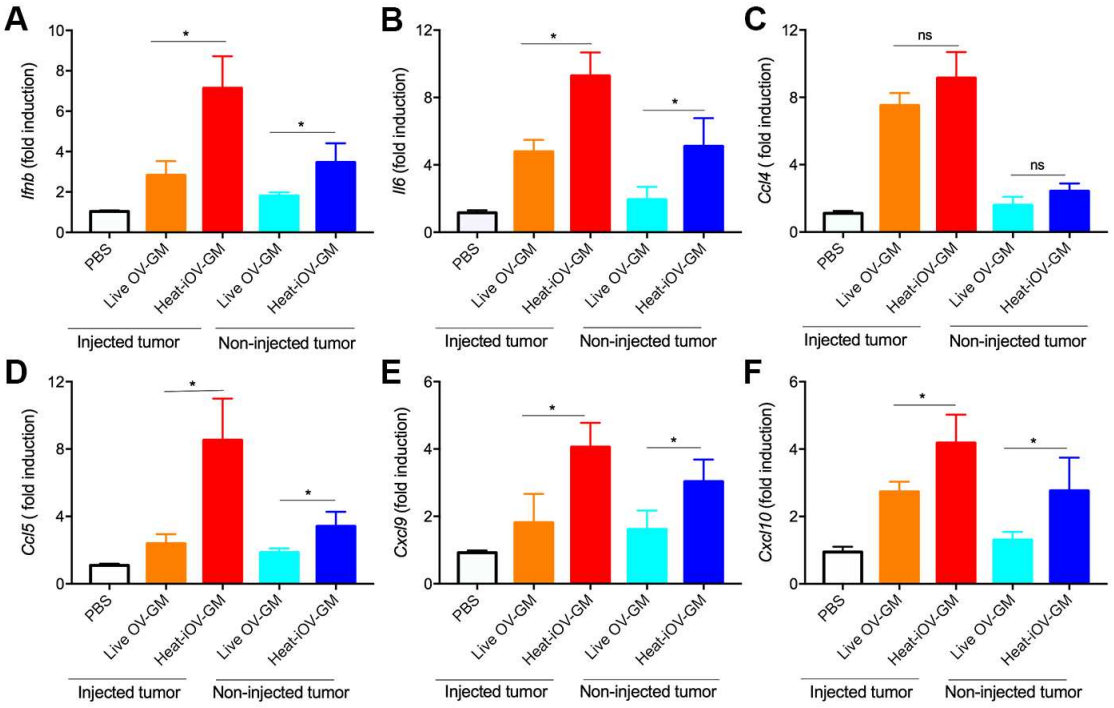

Figure S5 IT heat-iOV-GM induces higher levels of IFN and proinflammatory cytokines and chemokines in both injected and non-injected tumors in MC38 tumor model. MC38 tumor cells were intradermally implanted into both flanks of C57BL/6J mice. Established tumors on the right flanks were treated with either live-OV-GM or heat-iOV-GM. PBS was used as a control. The right flank tumors were harvested one day after first injection. To investigate the innate immunity of the left flank tumors, mice were treated with IT viruses to the right flank tumors twice three days apart. The left flank tumors were harvested one day after the second injection. (A-F) Shown here are quantitative real-time PCR analyses of *Ifnb* (A), *Il6* (B), *Ccl4* (C), *Ccl5* (D), *Cxcl9* (E), and *Cxcl10* (F) gene expression in the injected MC38 tumors from mice treated with either PBS, live OV-GM, or heat-iOV-GM. (n=4-5, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *t* test).

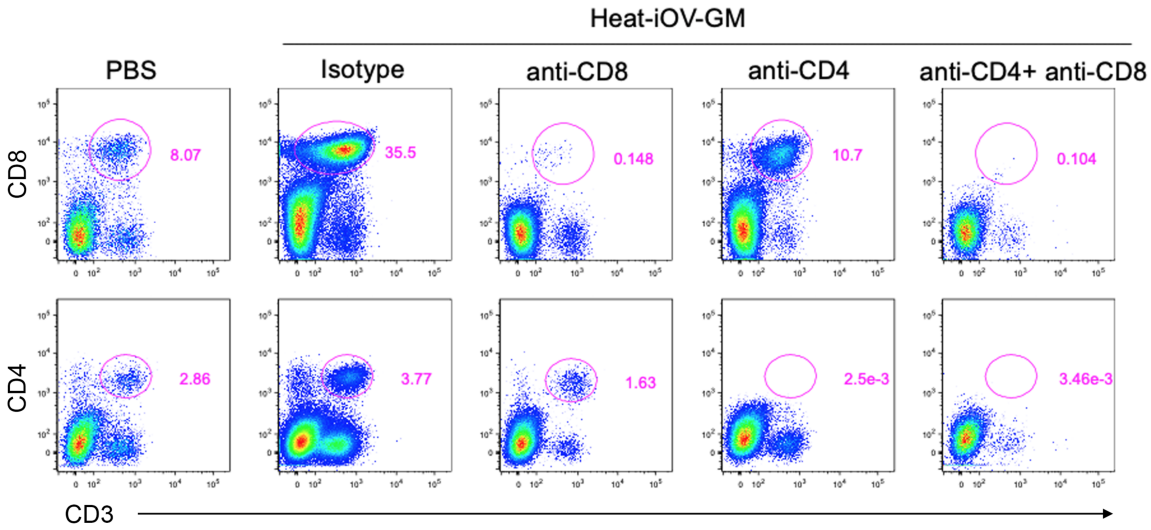

Figure S6 Verification of $CD4^+$ and $CD8^+$ T cell depletion in mice B16-F10 implantation model by flow cytometry. C57BL/6J mice were intradermally implanted with B16-F10 tumors on both the left and right flanks. Established tumors were injected with heat-iOV-GM or PBS control at day 8 and 11 post implantation. Depletion antibody was injected intraperitoneally at day 7, 10, and 12 post implantation. Tumors were harvested at day 13 for TIL analysis. Frequencies of $CD4^+CD3^+$ and $CD8^+CD3^+$ T cells in tumor samples from each treatment group are shown.