Supplementary Materials

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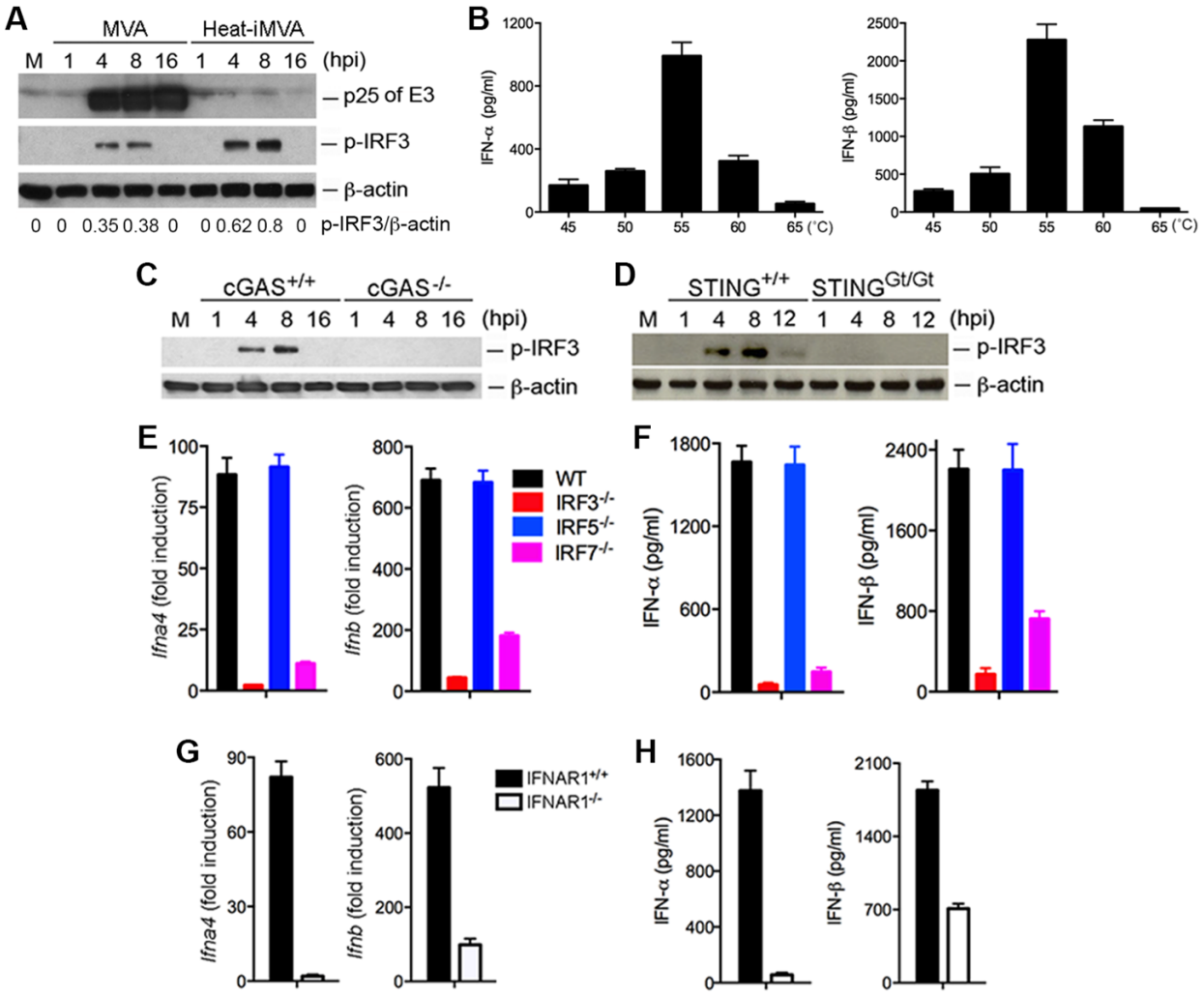


Fig. S1. **Induction of type I IFN in murine cDCs by Heat-iMVA requires cGAS, STING, IRF3, IRF7 and IFNAR1. (A)** Western blot showing protein levels of p-IRF3 and vaccinia E3 in Heat-iMVA or MVA-infected cDCs. The ratios of p-IRF3/β-actin were shown. (**B**) The effect of heating temperature on the abilities of heat-iMVA to induce IFN-α and IFN-β secretion in infected cDCs. (**C, D**) Heat-iMVA infection of cDCs induces phosphorylation of IRF3 that is dependent on cGAS (**C**) and STING (**D**). Western blot showing protein levels of p-IRF3 and β-actin in Heat-iMVA-infected cDCs from $cGAS^{+/+}$ and $cGAS^{-/-}$ mice (**C**) and from $STING^{+/+}$ and $STING^{Gt/Gt}$ mice (**D**). "hpi", hours post infection. "M", mock infection control. (**E**) *Ifna4* and *Ifnb* relative mRNA expression compared with no virus control in cDCs generated from WT, $IRF3^{-/-}$, $IRF7^{-/-}$, or $IRF5^{-/-}$ mice and infected with Heat-iMVA. Data are means ± SEM (n=3). (**F**)

Concentrations of secreted IFN-α and IFN-β in the medium of cDCs generated from WT, IRF3$^{-/-}$, IRF7$^{-/-}$, or IRF5$^{-/-}$ mice and infected with Heat-iMVA. Data are means ± SEM (n=3). (**G**) *Ifna4* and *Ifnb* relative mRNA expression compared with no virus control in cDCs generated from IFNAR1$^{+/+}$ and IFNAR1$^{-/-}$ mice and infected with Heat-iMVA. Data are means ± SEM (n=3). (**F**) Concentrations of secreted IFN-α and IFN-β in the medium of cDCs generated from IFNAR1$^{+/+}$ and IFNAR1$^{-/-}$ mice and infected with Heat-iMVA. Data are means ± SEM (n=3).

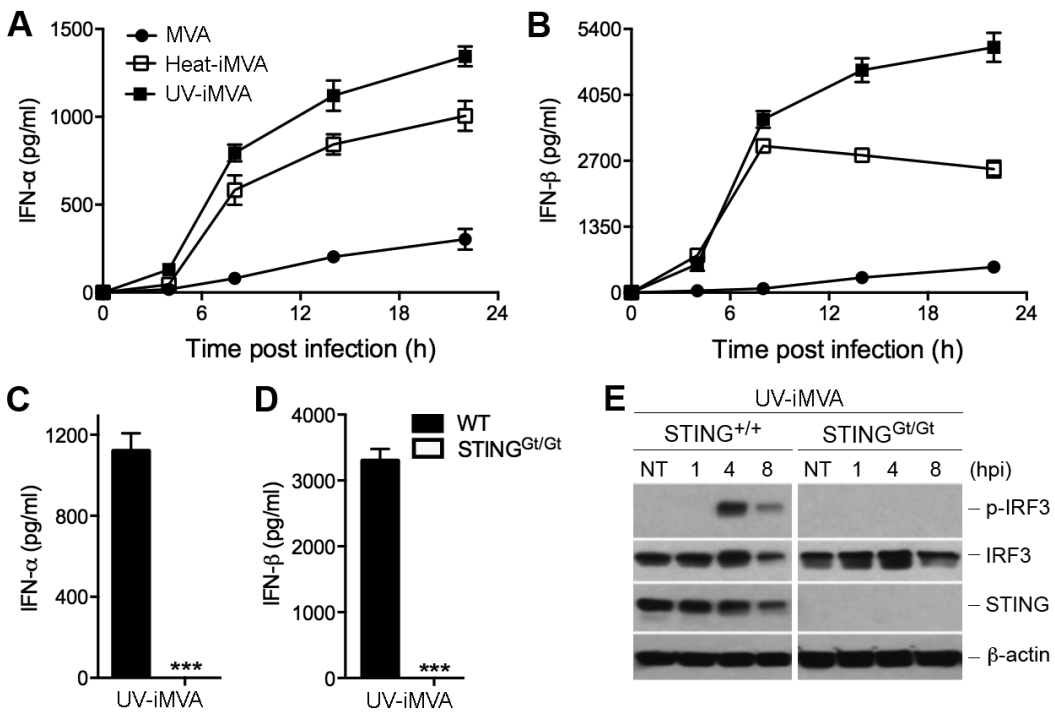


Fig. S2. **Induction of type I IFN in cDCs by UV-iMVA requires also requires STING.** (**A, B**) The concentrations of secreted IFN-α (**A**) and IFN-β (**B**) in the medium over time following MVA, Heat-iMVA, or UV-iMVA infection of cDCs. Data are means ± SEM (n=3). (**C, D**) Concentrations of secreted IFN-α (**C**) and IFN-β (**D**) in the medium of cDCs generated from WT and STING$^{Gt/Gt}$ mice and infected with UV-iMVA. (n=3; ***$P < 0.001$; *t* test). (**E**) Western Blot showing protein levels of p-IRF-3, IRF3, STING, and β-actin. "hpi", hours post infection. "NT", no treatment control.

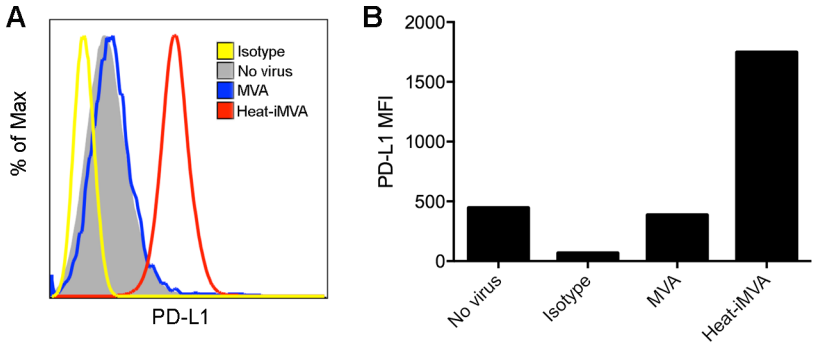


Fig. S3. **Induction of PD-L1 expression on Heat-iMVA-infected B16-F10 cells.** (**A**) Representative flow cytometry plot of B16-F10 cells infected with either MVA at a MOI of 10 or with an equivalent amount of Heat-iMVA. No virus infection and isotype control were also included. (**B**) The mean fluorescence intensity (MFI) of PD-L1 expression on B16-F10 cells infected with either MVA, Heat-iMVA, or mock infection control is shown.

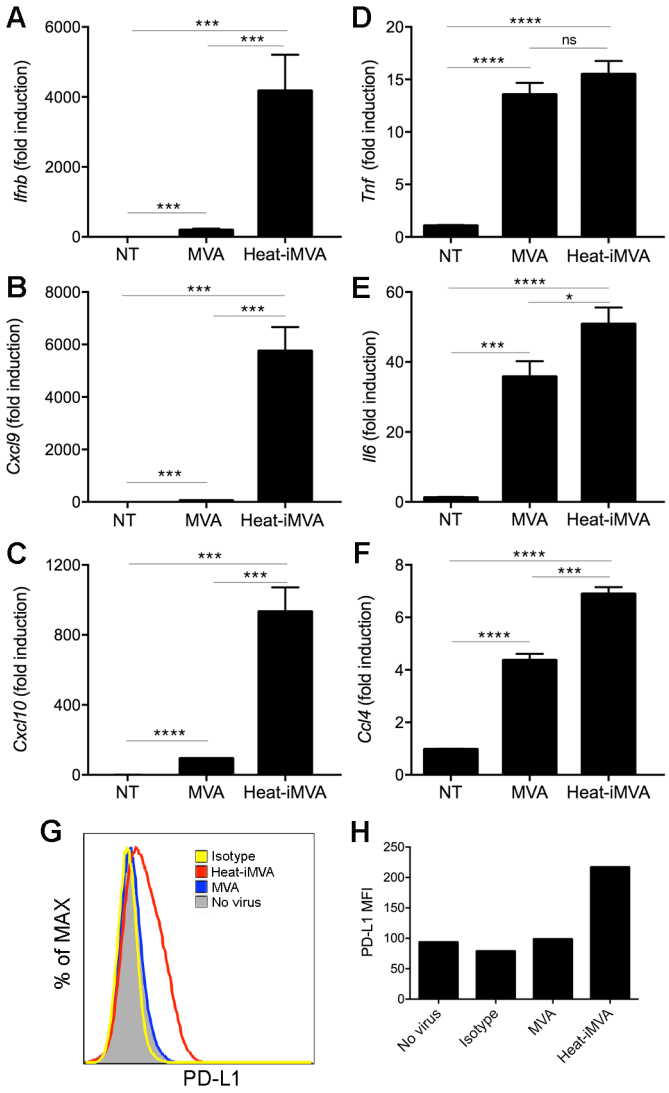


Fig. S4. **Induction of type I IFN, proinflammatory cytokines and chemokines, and PD-L1 expression in human melanoma cell line infected with Heat-iMVA.** (**A-F**) Human melanoma cell line SK-MEL-146 cells were infected with MVA at a MOI of 10 or with an equivalent amount of Heat-iMVA. Cells were collected at 6 h post infection and RNAs were extracted. Quantitative real-time PCR was performed. The relative mRNA expression of *Ifnb*, *Cxcl9*, *Cxcl10*, *Tnf, Il6*, *Ccl4*, and *Ccl5* in B16-F10 cells infected with either MVA or Heat-iMVA. (n=3; ***$P < 0.001$; ****$P < 0.0001$; *t* test). (**G, H**) Expression of PD-L1 on SK-MEL-146 cells infected with either MVA, Heat-iMVA or mock infection control. Representative flow cytometry plot is shown in (**G**), repeated once. (**H**) The mean fluorescence intensity (MFI) of PD-L1 expression on SK-MEL-146 cells infected with either MVA, Heat-iMVA, or mock infection control is shown.

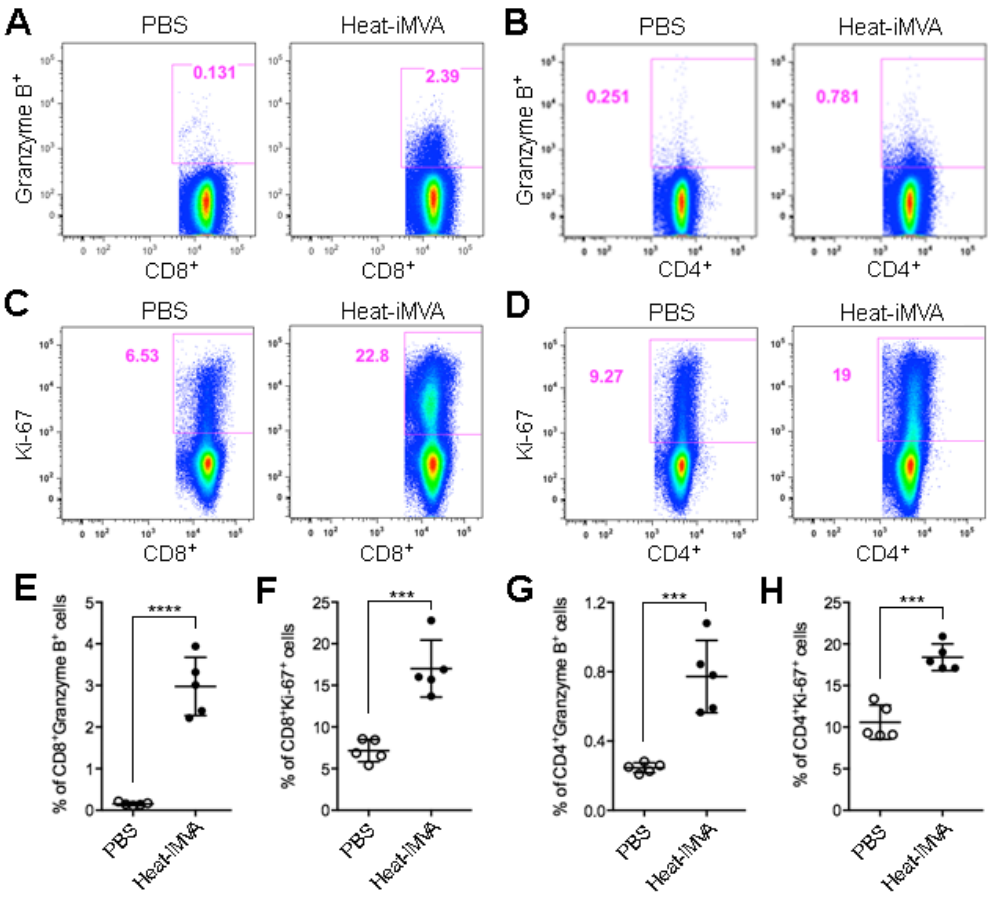


Fig. S5. **Intratumoral injection of Heat-iMVA results in the generation of Granzyme B$^+$ and Ki67$^+$ CD8$^+$ and CD4$^+$ T cells in the TNDLs.** 5 x 10^5 B16-F10 melanoma cells were implanted intradermally to the right flank of the mice. Seven days post implantation, either Heat-iMVA (an equivalent of 2x 10^7 pfu of MVA) or PBS were injected into the tumors on the right flank. The injections were repeated three days later. TDLNs were harvested 3 days post last injection and cell suspensions were generated. Immune cells were stained with various markers and analyzed by FACS. (**A-D**) Representative flow cytometry plot of CD8$^+$ cells expressing Granzyme B$^+$ (**A**) or Ki-67 (**C**), CD4$^+$ cells expressing Granzyme B (**B**), or Ki-67 (**D**). (**E-H**) Percentages of CD8$^+$Granzyme B$^+$ (**E**), CD8$^+$Ki-67$^+$ (**F**), CD4$^+$Granzyme B$^+$ (**G**), and CD4$^+$Ki67$^+$ (**H**) cells within TNLNs of mice treated with PBS (n=5) or Heat-iMVA (n=5; ***P < 0.001; ****P < 0.0001; t test). A representative experiment is shown, repeated once.

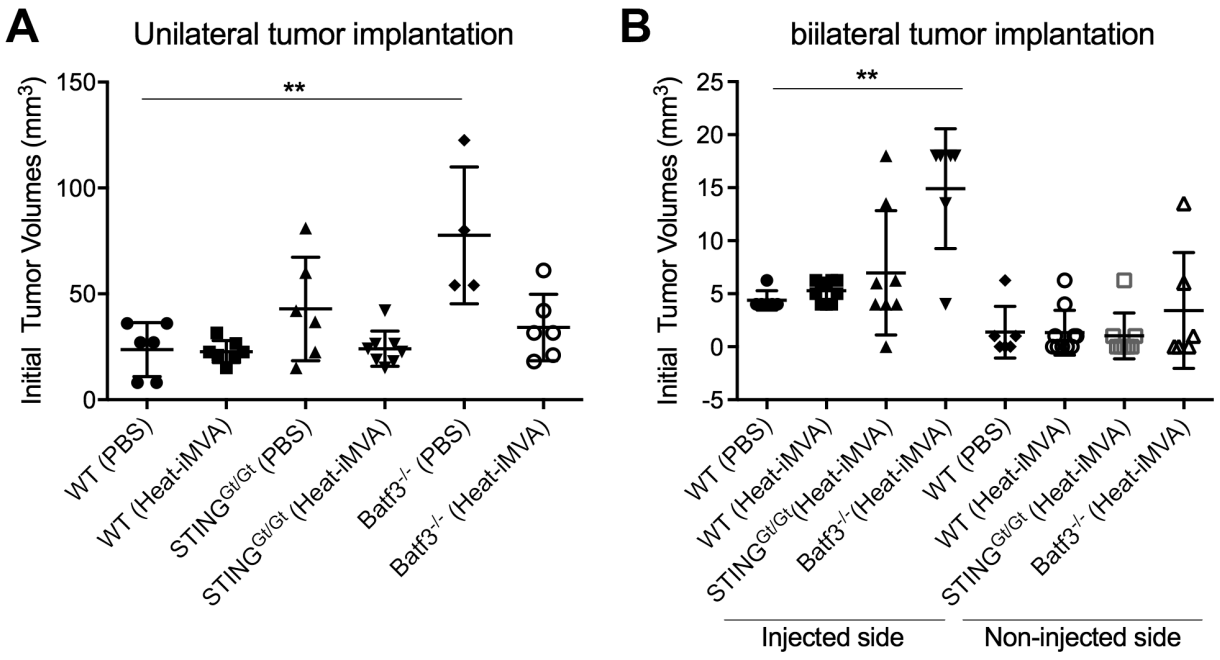


Fig. S6. **Initial B16-F10 tumor volumes at the time of the first injection.** (**A**) Unilateral tumor implantation model. B16-F10 melanoma cells (1 x 10^5 cells) were intradermally implanted into the right flank of WT C57B/6, $STING^{Gt/Gt}$, or $Batf3^{-/-}$ mice. At 11 days post-implantation, the tumors were injected with either Heat-iMVA (equivalent of 2 x 10^7 pfu) or PBS twice weekly. The initial tumor volumes at the time of first injection are shown (n=5; $^{**}P < 0.01$; *t* test). (**B**) Bilateral tumor implantation model. B16-F10 melanoma cells were implanted intradermally to the left and right flanks of C57B/6 mice (5 x 10^5 to the right flank and 1 x 10^5 to the left flank). 8 days after tumor implantation, Heat-iMVA or PBS was injected to the larger tumors on the right flank. The initial tumor volumes at the time of first injection are shown (n=5, 6; $^{**}P < 0.01$; *t* test).

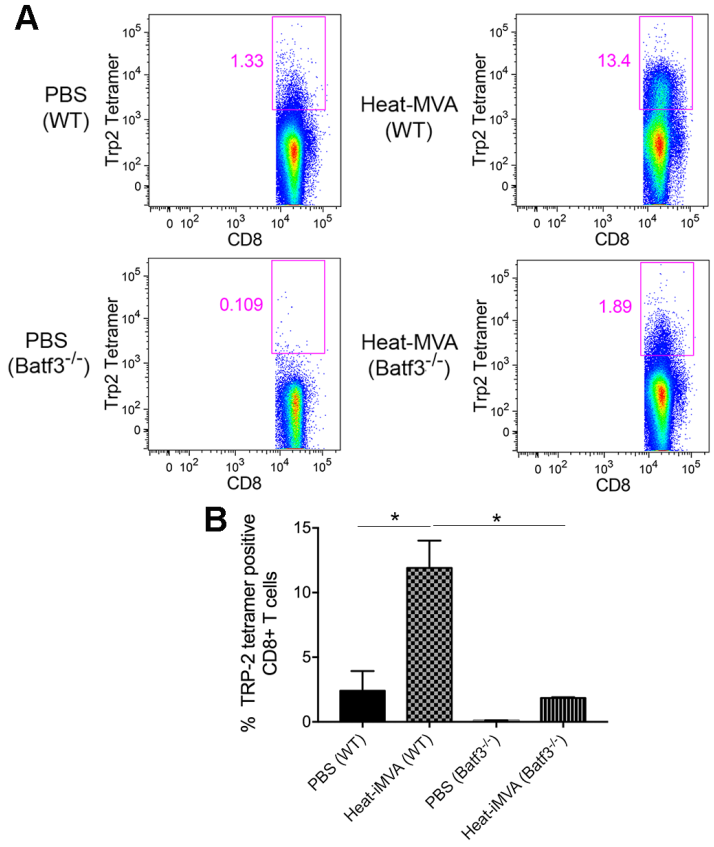


Fig. S7. **Intratumoral injection of Heat-iMVA leads to the generation of anti-tumor specific CD8$^+$ T cells in the TDLNs in a Batf3-dependent manner.** (A) Representative flow cytometry plots of TRP-2 tetramer positive CD8$^+$ T cells in TDLNs in a B16-F10 melanoma model treated with either PBS or Heat-inactivated MVA. (B) Percentages of TRP-2 tetramer positive CD8$^+$ T cells in WT and Batf3$^{-/-}$ mice with B16-F10 melanomas treated with either PBS or Heat-MVA. Each sample was from lymph nodes pooled from 2-3 mice treated with the same condition (n=3; *P < 0.05; *t* test).

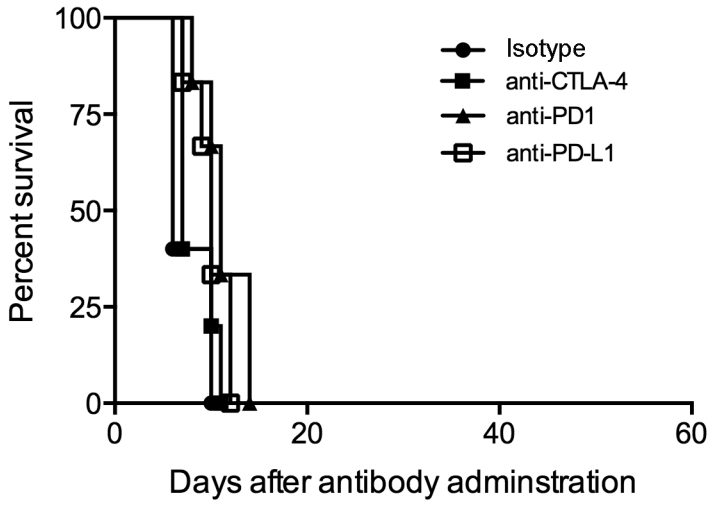


Fig. S8. **Intraperitoneal delivery of anti-CTLA-4, anti-PD1, or anti-PD-L1 antibody has minimum survival benefit in a unilateral B16-F10 melanoma implantation model.** B16-F10 melanoma (1x 10^5 cells) were implanted intradermally into the shaved skin on the right flank of WT C57BL/6J mice. 8 days post implantation, mice were treated with intraperitoneal delivery of anti-CTLA-4 (100 µg), anti-PD1 (250 µg), anti-PD-L1 (250 µg), or isotype control twice a week (every 3-4 days). Kaplan-Meier survival curve is shown for isotype control (n=5), anti-CTLA-4-treated mice (n=6), anti-PD1-treated mice (n=6), and anti-PD-L1-treated mice (n=6).

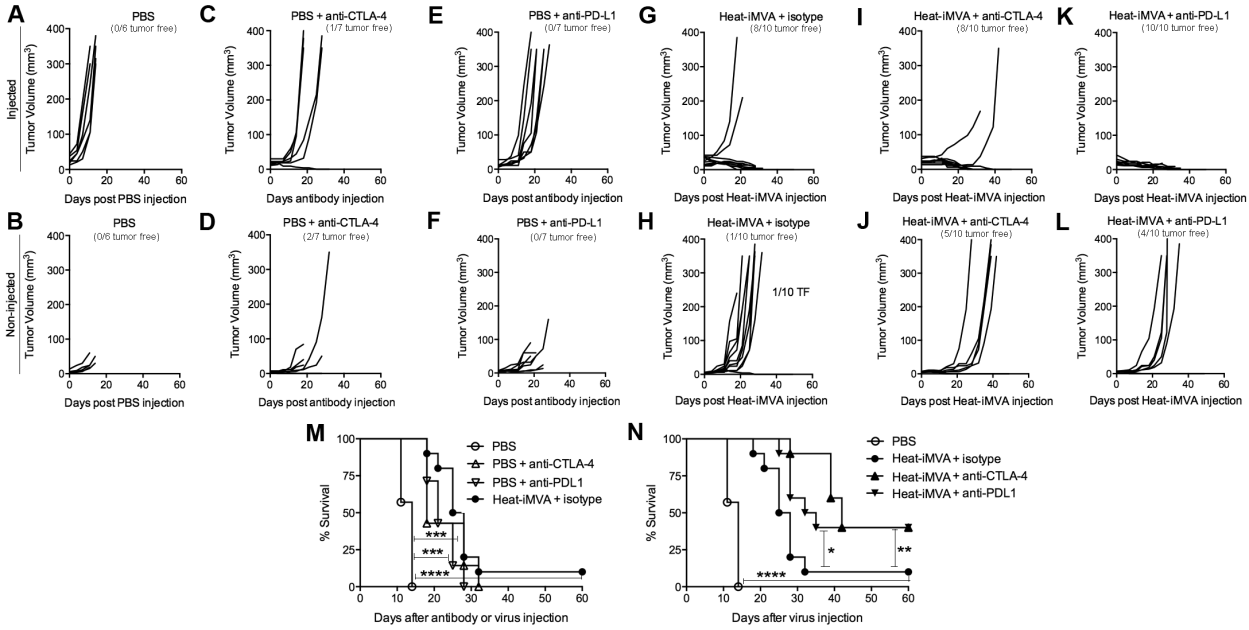
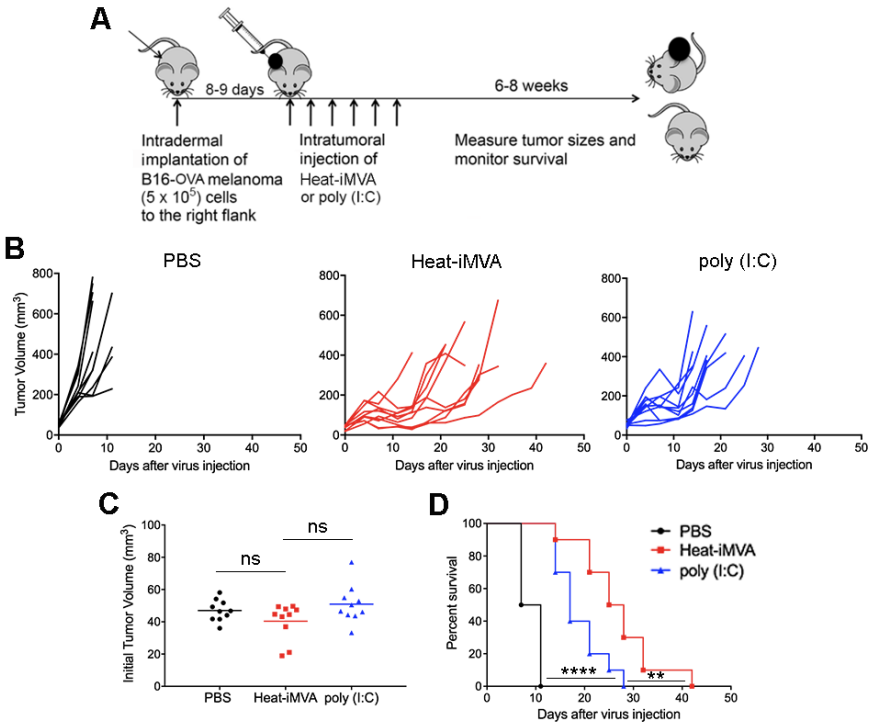


Fig. S9. **The combination of intratumoral injection of Heat-MVA with systemic delivery of anti-CTLA-4 or anti-PD-L1 antibodies significantly increases the overall response and cure rates in a MC38 bilateral tumor implantation model.** (**A-L**) Volumes of injected (**A**) and non-injected (**B**) tumor volume over days after PBS injection, after intratumoral injection of PBS and intraperitoneal delivery of anti-CTLA-4 antibody (**C**, **D**), after intratumoral injection of PBS and intraperitoneal delivery of anti-anti-PD-L1 antibody (**E**, **F**), after intratumoral injection of Heat-MVA and intraperitoneal delivery of isotype antibody control (**G**, **H**), after intratumoral injection of Heat-MVA and intraperitoneal delivery of anti-CTLA-4 antibody (**I**, **J**), after intratumoral injection of Heat-MVA and intraperitoneal delivery of anti-PD-L1 antibody (**K**, **L**). (**M**) Kaplan-Meier survival curve of tumor-bearing mice treated with PBS (n=6), anti-CTLA4 antibody (n=7), or anti-PD-L1 antibody (n=7; ***P< 0.001, Mantel-Cox test). (**N**) Kaplan-Meier survival curve of tumor-bearing mice treated with PBS (n=6), Heat-MVA + isotype control (n=10), Heat-MVA + anti-CTLA4 antibody (n=10), or Heat-MVA + anti-PD-L1 antibody (n=10; *P < 0.05; **P < 0.01; ****P < 0.0001; Mantel-Cox test). A representative experiment is shown, repeated once.



97 Fig. S10. **Intratumoral injection of Heat-iMVA is more effective than poly (I:C) in treating**
98 **large established tumors.** (**A**) Schematic diagram of a unilateral tumor implantation model with
99 large established B16-OVA model. B16-OVA melanoma cells (5 x 10^5 cells) were implanted
100 intradermally to the right flanks of C57B/6 mice. 8-9 days after tumor implantation, mice were
101 intratumorally injected with Heat-iMVA, poly (I:C), or PBS mock control twice weekly. Tumor
102 sizes were measured and the survival of mice was monitored. (**B**) Volumes of injected tumors
103 over days after injections with PBS, Heat-iMVA, or poly (I:C). (**C**) Initial tumor volumes on the
104 day of first injection. (**D**) Kaplan-Meier survival curve of tumor-bearing mice treated with PBS,
105 Heat-MVA, or poly (I:C) intratumorally (n=10; **P < 0.01; ****P < 0.0001; Mantel-Cox test).

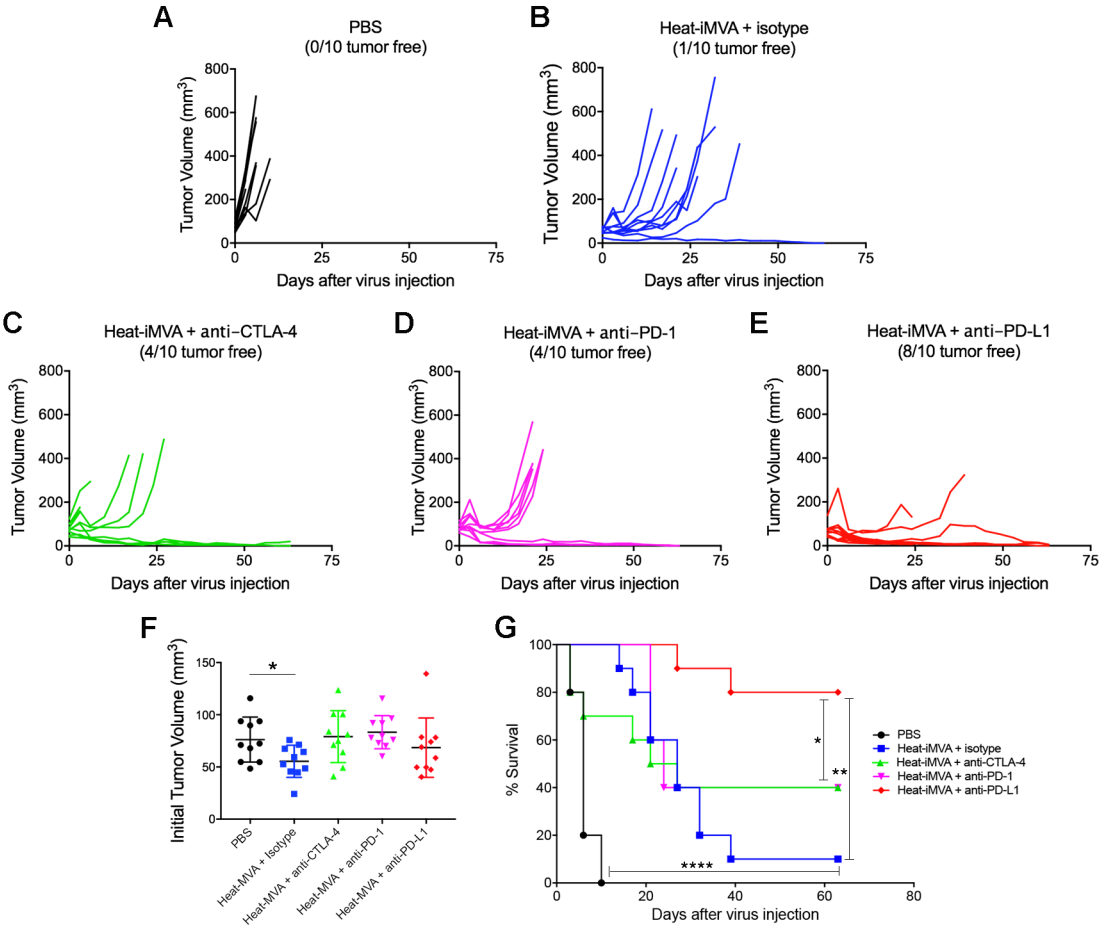


Fig. S11. **The combination of intratumoral injection of Heat-MVA with systemic delivery of immune checkpoint antibodies has synergistic effect in curing large established B16-F10 melanomas.** (**A**) Schematic diagram of a unilateral tumor implantation model with large established B16-F10 melanoma model. B16-F10 melanoma cells (5 x 10^5 cells) were implanted intradermally to the right flanks of C57B/6 mice. 8-9 days after tumor implantation, mice were intratumorally injected with 2 x 10^7 pfu of Heat-MVA twice weekly in the presence or absence of intraperitoneal delivery of anti-CTLA-4, anti-PD-1, or anti-PD-L1 antibodies. Tumor sizes were measured and the survival of mice was monitored. (**B**) Volumes of injected tumors over days after injections with PBS, or with Heat-iMVA in the presence of isotype control, or anti-CTLA-4, or anti-PD-1, or anti-PD-L1. (**C**) Initial tumor volumes prior to first treatment (n= 10; $*P < 0.05$; *t* test). (**D**) Kaplan-Meier survival curve of tumor-bearing mice treated with PBS, or Heat-iMVA in the presence of isotype control, or anti-CTLA-4, or anti-PD-1, or anti-PD-L1 antibodies (n=10; $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$; Mantel-Cox test).

mIFNA4 forward	5’-CCTGTGTGATGCAGGAACC-3’
mIFNA4 reverse	5’-TCACCTCCCAGGCACAGA-3’
mIFNB forward	5’-TGGAGATGACGGAGAAGATG-3’
mIFNB reverse	5’-TTGGATGGCAAAGGCAGT-3’
mIL6 forward	5’-AGGCATAACGCACTAGGTTT-3’
mIL6 reverse	5’-AGCTGGAGTCACAGAAGGAG-3’
mCCL4 forward	5’-GCCCTCTCTCTCCTCTTGCT-3’
mCCL4 reverse	5’-CTGGTCTCATAGTAATCCATC-3’
mCCL5 forward	5’-GCCCACGTCAAGGAGTATTTCTA-3’
mCCL5 reverse	5’-ACACACTTGGCGGTTCCTTC-3’
mCXCL10 forward	5’-GTCAGGTTGCCTCTGTCTCA-3’
mCXCL10 reverse	5’-TCAGGGAAGAGTCTGGAAAG-3’
mGAPDH forward	5’-ATCAAGAAGGTGGTGAAGCA-3’
mGAPDH reverse	5’-AGACAACCTGGTCCTCAGTGT-3’
hIFNB forward	5’-GCACTGGCTGGAATGAGACT-3’
hIFNB reverse	5’-CCTTGGCCTTCAGGTAATG-3’
hTNF forward	5’-AATAGGCTGTTCCCATGTAGC-3’
hTNF reverse	5’-AGAGGCTCAGCAATGAGTGA-3’
hIL6 forward	5’-AATTCGGTACATCCTCGACGG-3’
hIL6 reverse	5’-TTGGAAGGTTCAGGTTGTTTTCT-3’
hCCL4 forward	5’- AAAACCTCTTTGCCACCAATACC-3’
hCCL4 reverse	5’- GAGAGCAGAAGGCAGCTACTAG-3’
hCXCL9 forward	5’- AAACCCAGATTCAGCAGATG-3’
hCXCL9 reverse	5’- TCTTTTGACGAGAACGTTGAGA-3’
hCXCL10 forward	5’-ATTTGCTGCCTTATCTTTCTG-3’
hCXCL10 reverse	5’-TCTCACCCTTCTTTTTCATTGTAG-3’
hGAPDH forward	5’-ATCAAGAAGGTGGTGAAGCA-3’
hGAPDH reverse	5’-GTCGCTGTTGAAGTCAGAGGA-3’

Table S1. **Primer sequences for quantitative real-time PCR for the expression of type I IFN, proinflammatory cytokine and chemokine genes.**