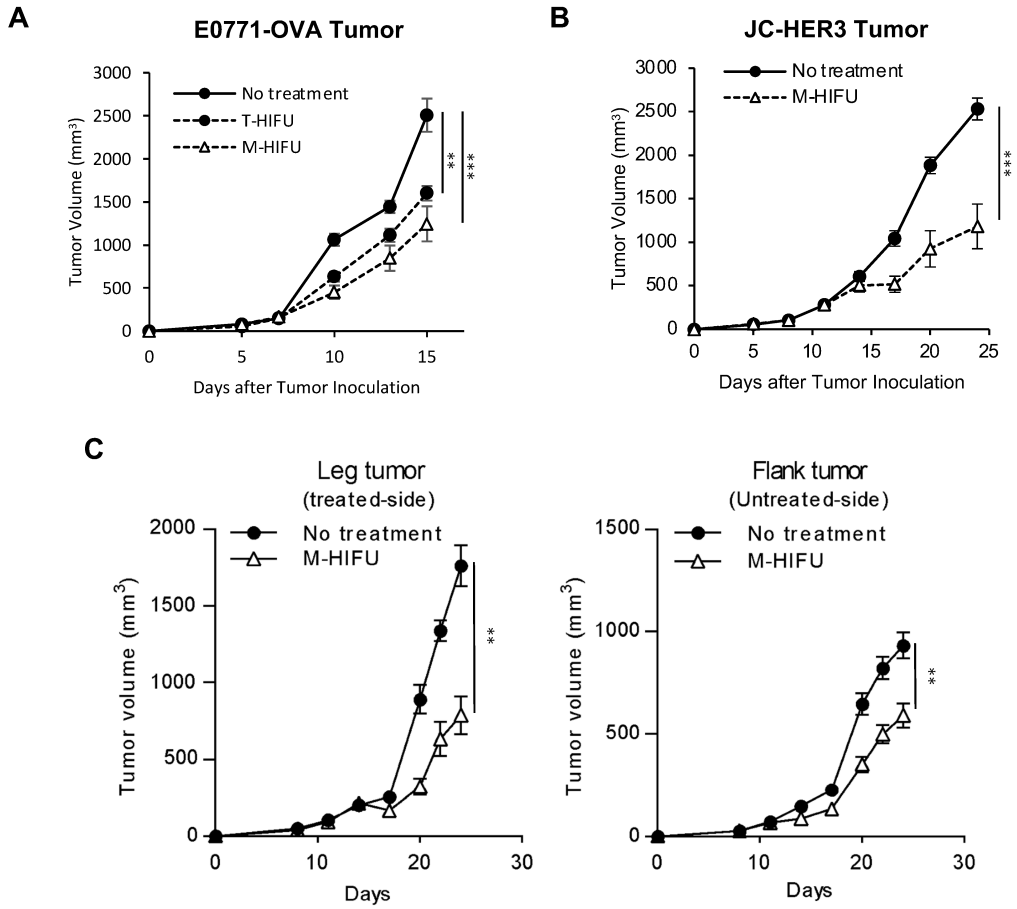
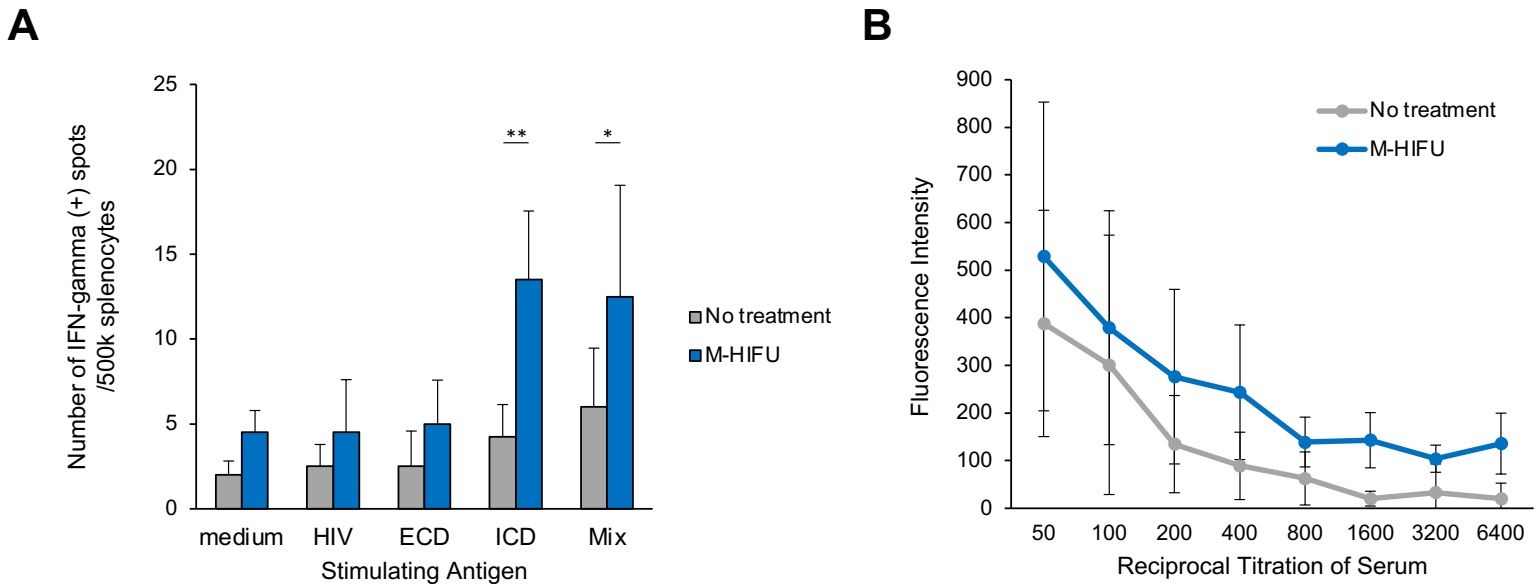
Supplementary Figure 1



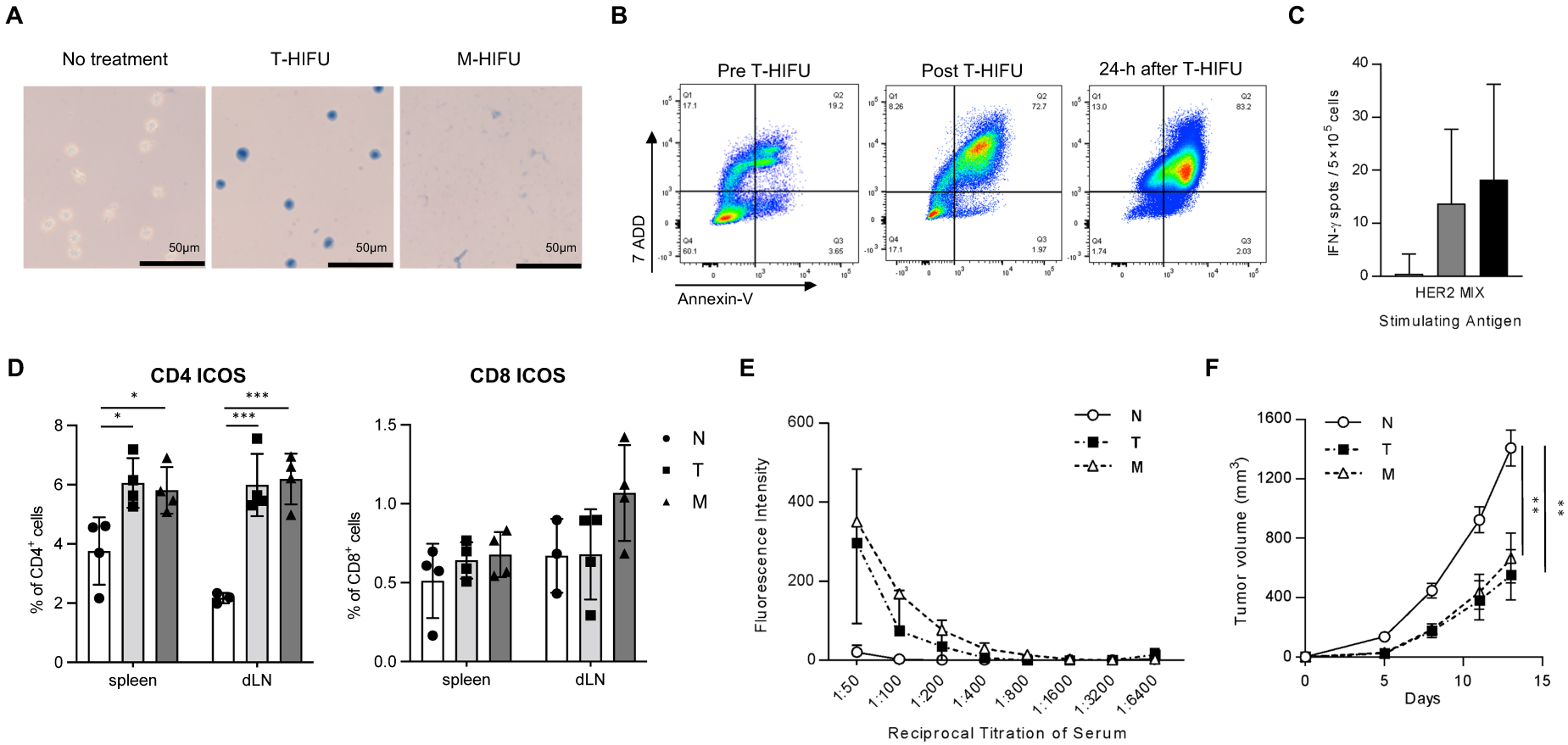
Supplementary Figure 1. Stronger tumor growth suppression and abscopal effect induced by M-HIFU against murine breast cancers. **(A)** E0771-OVA tumors grown in the leg of C57BL/6 mice were treated with M-HIFU or T-HIFU 7 days after tumor cell implantation. Tumor sizes were monitored every 2-3 days. n=5 mice (no treatment and T-HIFU) or 6 (M-HIFU). 16 mice in total. **(B)** JC-HER3 cells were subcutaneously injected into the left leg (1×10^6 cells) of HER3 transgenic mice. Established leg tumors at 5-7 mm in a diameter were treated with M-HIFU treatment on day 7. Growth curves of leg tumors in M-HIFU treated and untreated groups are shown. Bar represents mean ± SE. n=9 mice per each group. 18 mice in total. P values: $^{**}p < 0.005$, $^{***}p < 0.001$. **(C)** JC-HER3 cells were injected into both the left leg (1×10^6) and the right flank (5×10^5) of HER3 transgenic mice on day 0. Established leg tumors were treated with M-HIFU on day 11. The growth curves of M-HIFU-treated leg tumors and and untreated flank tumors were compared and shown. Bar represents mean ± SE. n=9 mice per each group. 18 mice in total. P value: $^{**}p < 0.01$.

Supplementary Figure 2



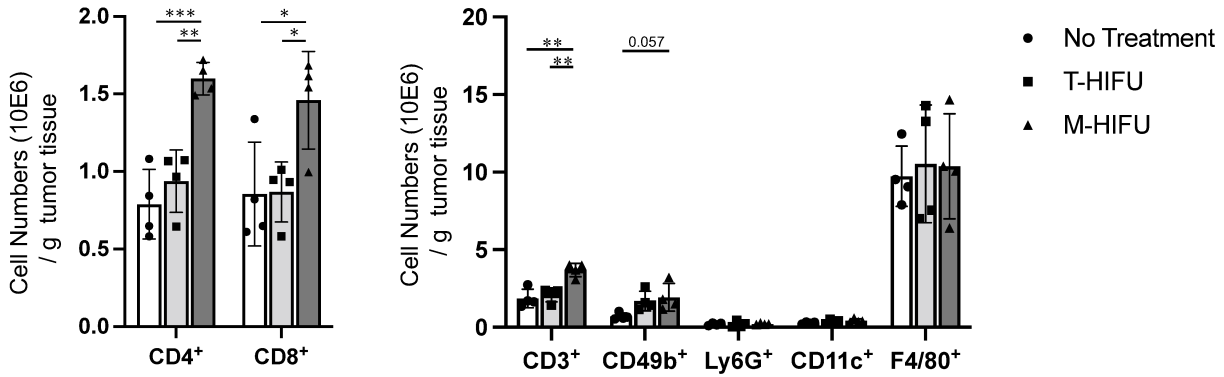
Supplementary Figure 2. HER3 antigen-specific immune responses induced in M-HIFU treated JC-HER3 tumor-bearing mice.
Twenty-four days after JC-HER3 cell inoculation to female HER3 transgenic mice (13 days after M-HIFU treatment), mice were euthanized, and spleen and blood were collected for the assays. **A)** IFN-gamma production by splenocytes was assessed by ELISpot assay. Average values of spot numbers for medium, HIV, HER3 peptide of ECD, ICD and mix (ECD+ICD) are shown. n=5 mice for each group. Bars represents SD. **B)** Anti-HER3 antibody in the serum of mice were evaluated with Cell-based ELISA, in which 4T1-HER3 cells (HER3-positive) and parental 4T1 cells (HER3-negative) were used for labeling with serial dilution of mouse serum. n=5 mice for each group. Bar represents mean SD.

Supplementary Figure 3



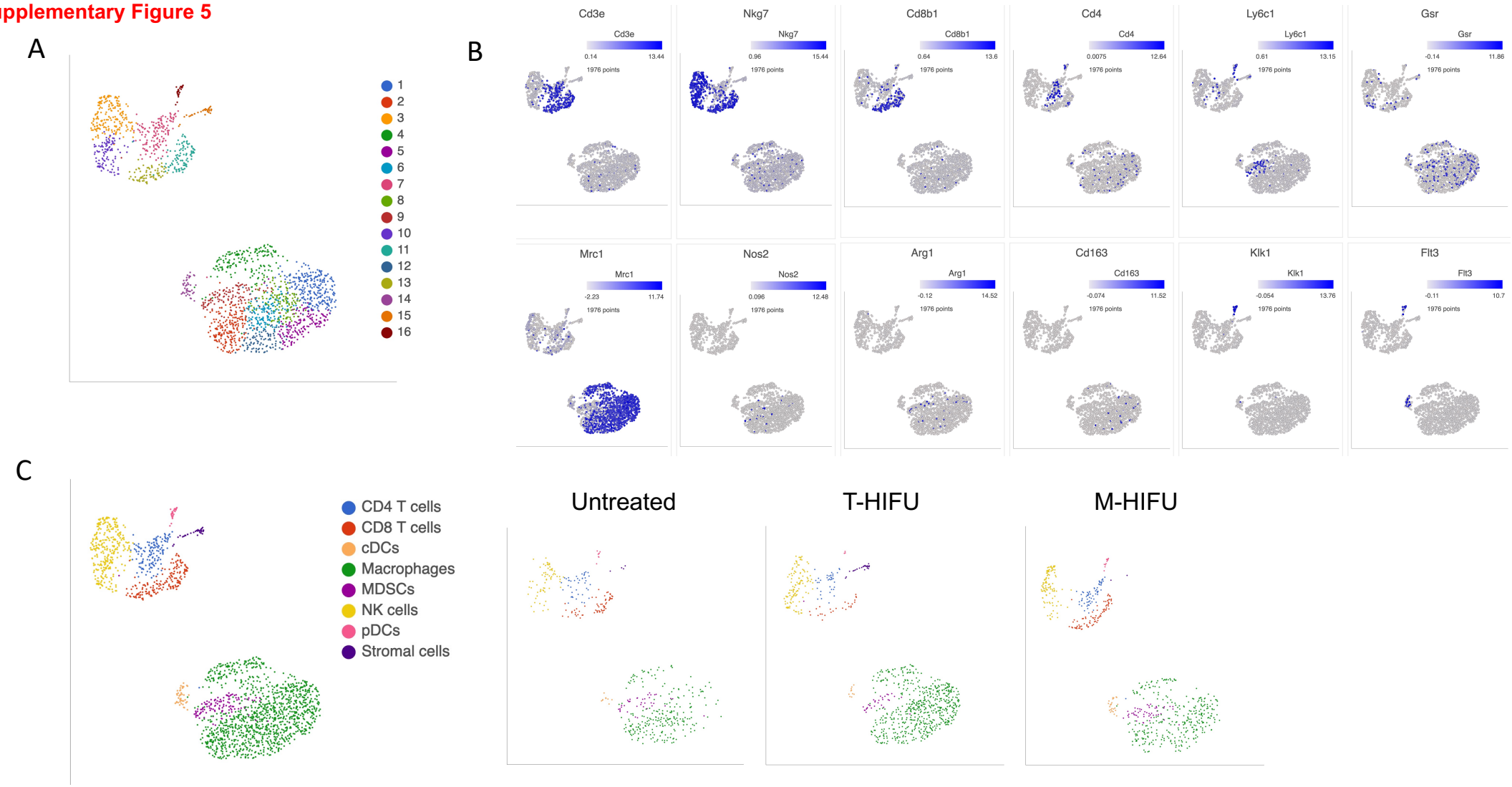
Supplementary Figure 3. Cell death and immunogenicity of HIFU treated MM3MG-HER2 breast cancer cells. (A) Cell suspensions of MM3MG-HER2 cells were treated with M- HIFU (Duty cycle 2%, PRF 2Hz, Power 200W, Time 20sec) or T-HIFU (Duty cycle 50%, PRF 1Hz, Power 50W, Time 10sec) *in vitro.* After *in vitro* HIFU, cells were stained with trypan blue. The experiment was performed in triplicate, showing consistent results. **(B)** The proportion of Annexin V+/7 ADD- or Annexin V+/7 ADD+ cells at pre-, post T-HIFU and twenty-four hours after T-HIFU were analyzed with flow cytometry. **(C-F)** On day -14 and -7, mice were vaccinated with 1×10^6 MM3MG-HER2 cells treated with M- HIFU or T-HIFU *in vitro*. Spleen and draining lymph nodes (both-side inguinal lymph nodes) were collected on day 0. **(C)** HER2 antigen-specific cellular response was analyzed by IFN-g ELISpot assay. Average values of spot numbers for HER2 peptide mix (ECD+ICD) are shown. n = 4 per group. **(D)** The percentages of $ICOS^+$ cells within the $CD4^+$ or $CD8^+$ cell population in spleen and draining lymph nodes were analyzed by flow cytometry and shown. n = 4 per group. **(E)** The levels of anti-HER2 antibody in the serum of the vaccinated mice were evaluated with cell-based ELISA. n = 3 per group. **(F)** As described in (C), mice were vaccinated by intradermal injection of *in vitro* HIFU-treated MM3MG-HER2 cells into the back on days -14 and -7. 1×10^6 MM3MG-HER2 cells were subcutaneously injected to the left legs of mice on day 0. Tumor growth in each group is shown. n = 10 mice per group. 30 mice in total. For C, D, and E, error bars represent SD. For F, error bars represent SE. *: *P* value < 0.05, **: *P* value < 0.01, ***: *P* value < 0.001.

Supplementary Figure 4



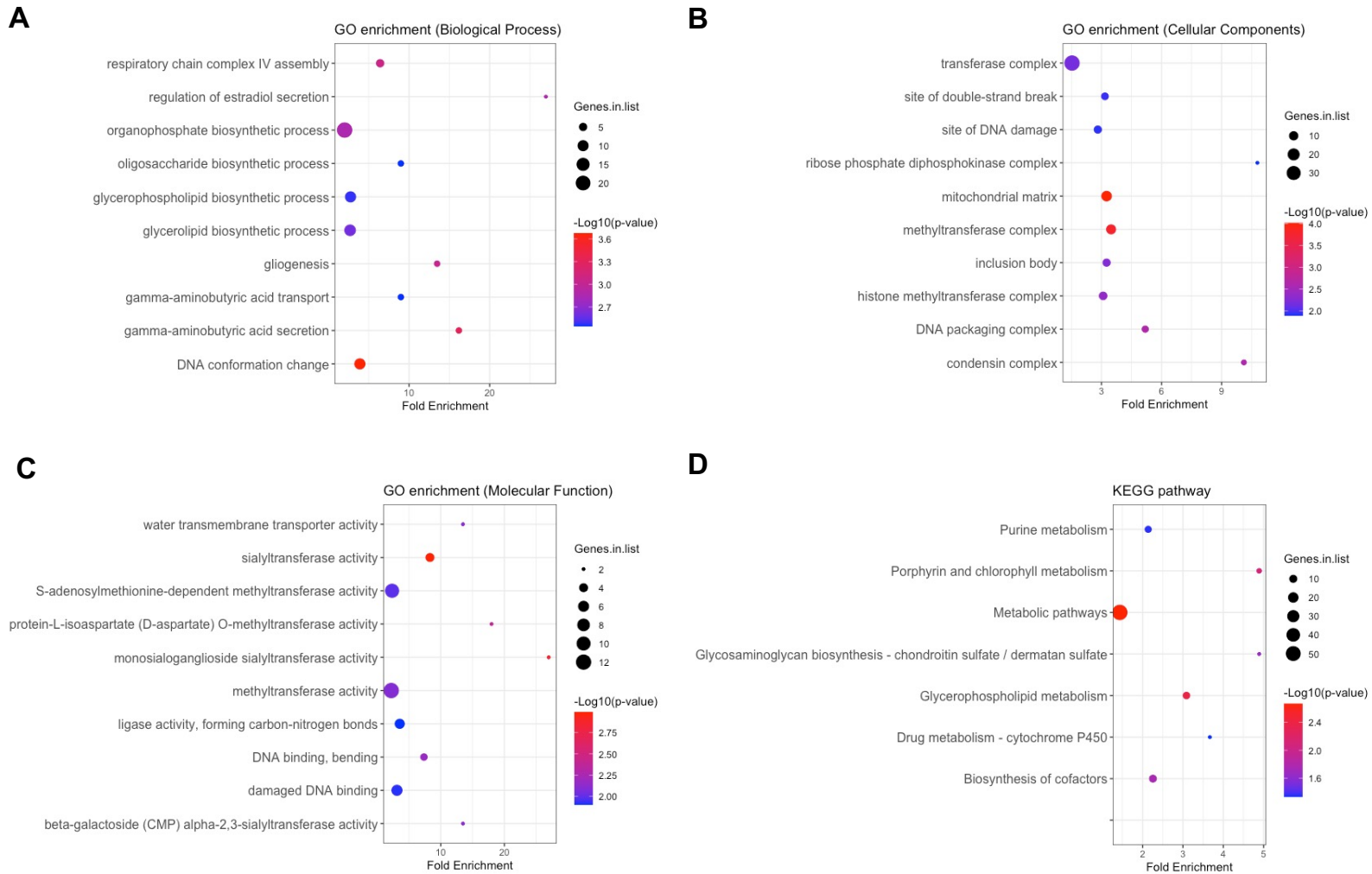
Supplementary Figure 4. Enhanced intratumoral infiltration of activated CD4+ and CD8+ cells by M-HIFU. 1×10^6 MM3MG-HER2 cells were injected into legs of BALB/c mice. Established leg tumors were treated with M-HIFU or T-HIFU on day 7 after tumor inoculation. Seven days after HIFU treatments of MM3MG-HER2 tumors in mice, tumors were collected and digested for flow cytometry analysis. The cell numbers of $CD4^+$, $CD8^+$, $CD49b^+$, Ly6G+, CD11c+ and F4/80+ cells in 1 g of tumor tissue were analyzed for each HIFU treatment group. The following formula was used. Numbers of infiltrating cells per 1 g of tumor tissue = [Cell count by trypan blue dye exclusion method using hemocytometer] x [percentages of CD45+ cells /100] x [percentages of cell types in CD45+ cells / 100] x [1 / weight of collected tumor (g)]. n = 4 per group and bar represents SD. One way ANOVA test was performed for statistical analysis. *: *P* value < 0.05, **: *P* value < 0.01, ***: *P* value < 0.001.

Supplementary Figure 5



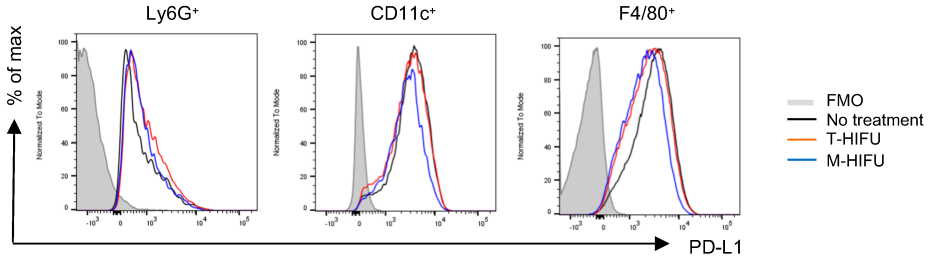
Supplementary Figure 5. Graph-based clustering of tumor-infiltrating leukocytes and Cluster Annotation. Established MM3MG-HER2 leg tumors in BALB/c mice were treated with T- or M-HIFU. Eight days after HIFU treatment, tumors were collected, and alive CD45+ Tumor-infiltrating leukocytes (TILs) were isolated from tumor digests by flow-based sorting. scRNA-sequencing was performed using Illumina system. Sequencing was performed as described in Materials and Methods. **(A)** Graph-based clustering is shown. **(B)** Feature plots are shown for cluster annotation. **(C)** Cluster Annotation was made and shown for TILs from untreated, M-HIFU and T-HIFU treated tumors.

Supplementary Figure 6



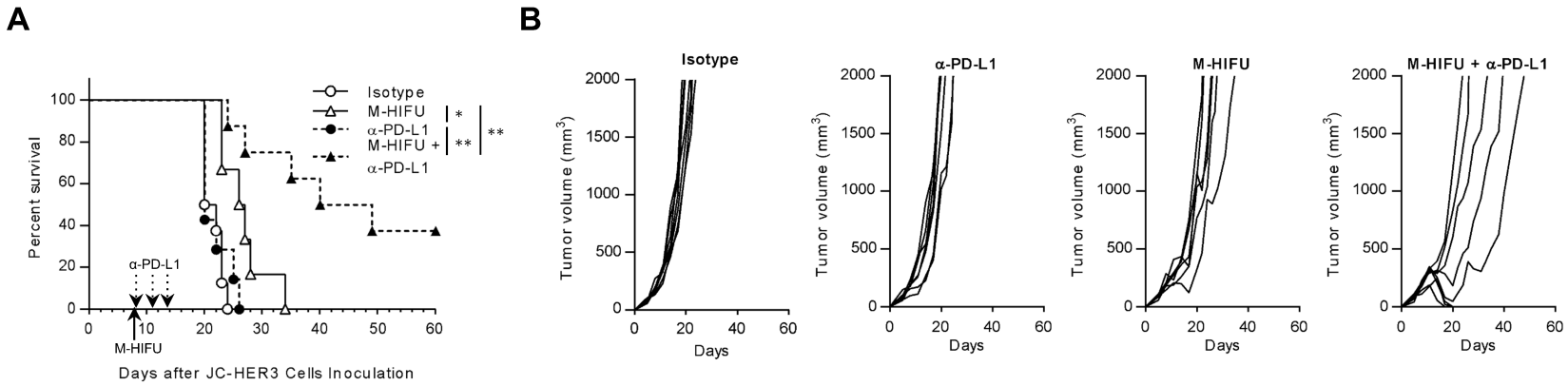
Supplementary Figure 6. Gene Ontology enrichment analysis and KEGG pathway analysis of the differentially expressed genes in tumor-infiltrating macrophages from T-HIFU-treated tumors. A-C) Differentially expressed genes that were upregulated in macrophages from T-HIFU treated tumors compared to macrophages from control untreated tumors were evaluated for their biological functions by GO enrichment analysis. Top 10 GO terms in biological process category (**A**), cellular components category **(B)** and molecular function category **(C)**, ranked by enrichment scores are shown. **D**) Differentially expressed genes were analyzed by KEGG pathway analysis. Top 10 Biological Process terms (**A-C**) or 7 KEGG pathways (**D**) that were significantly upregulated in T-HIFU treated tumors are shown. Only 7 KEGG pathways were significantly different.

Supplementary Figure 7



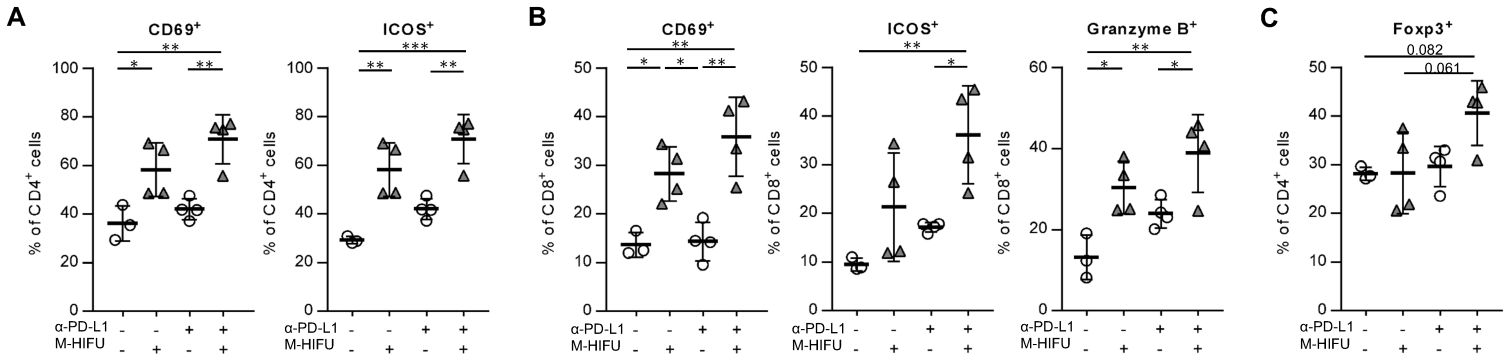
Supplementary Figure 7. Expression of immune checkpoint molecules on tumor associated immune cells in earlier phase after HIFU treatment. 1×10^6 MM3MG-HER2 cells were injected into legs of BALB/c mice. Established MM3MG-HER2 leg tumors were treated with T- or M-HIFU or left untreated. Three days after HIFU, tumors were collected and digested tumors were analyzed by flow cytometry. Ly6G+, CD11c+ or F4/80+ cells in alive CD45+ leukocytes were analyzed for their expression level of PD-L1. Representative cases are shown. Blue: M-HIFU, red: T-HIFU, black: no treatment, grey filled: isotype control.

Supplementary Figure 8



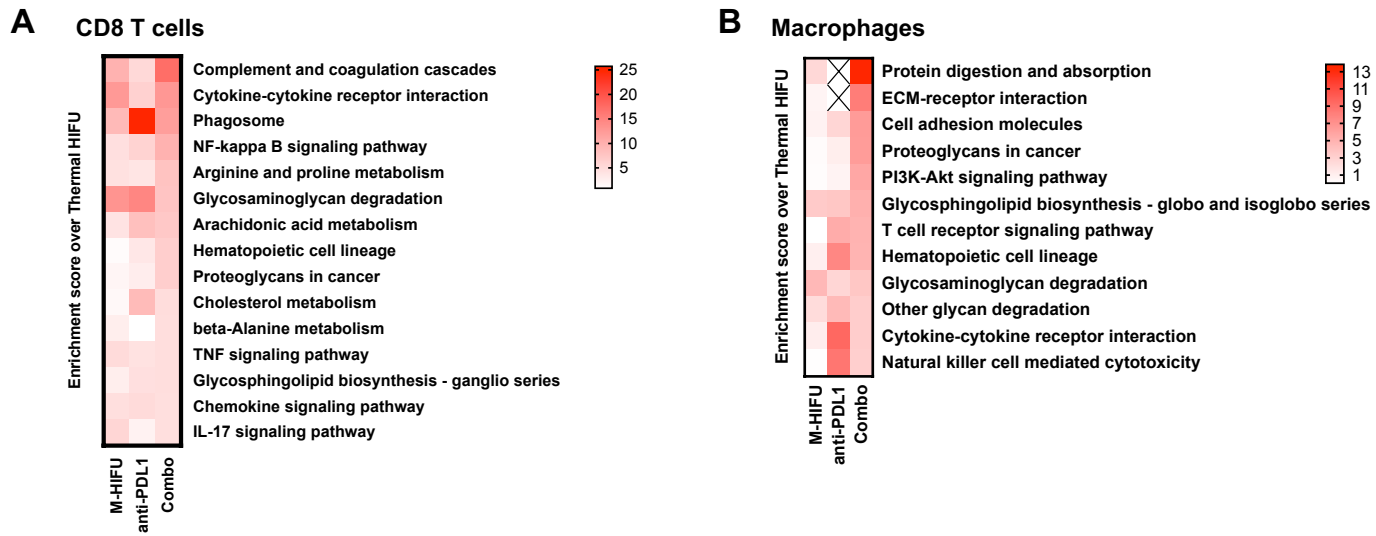
Supplementary Figure 8. Antitumor efficacy of combined M-HIFU and anti-PD-L1 antibody against JC-HER3 breast cancer model. **(A-B)** 1×10^6 JC-HER3 cells were injected into legs of BALB/c mice. Established leg tumors were treated with M-HIFU on day 8 after tumor inoculation. Anti-PD-L1 antibody (200 µg /100 µL) or Isotype IgG (200 µg /100 µL) were injected intraperitoneally on day 8, 11 and 15. **(A)** Survival curves are shown and log-rank test was performed. **(B)** Individual tumor growth curves are shown. n=6 mice (M-HIFU), 7 (anti-PD-L1) or 8 (Isotype, Combination). 29 mice in total. Error bar represents mean ± SD. ∗: *P* value < 0.05, ∗∗: *P* value < 0.01.

Supplementary Figure 9



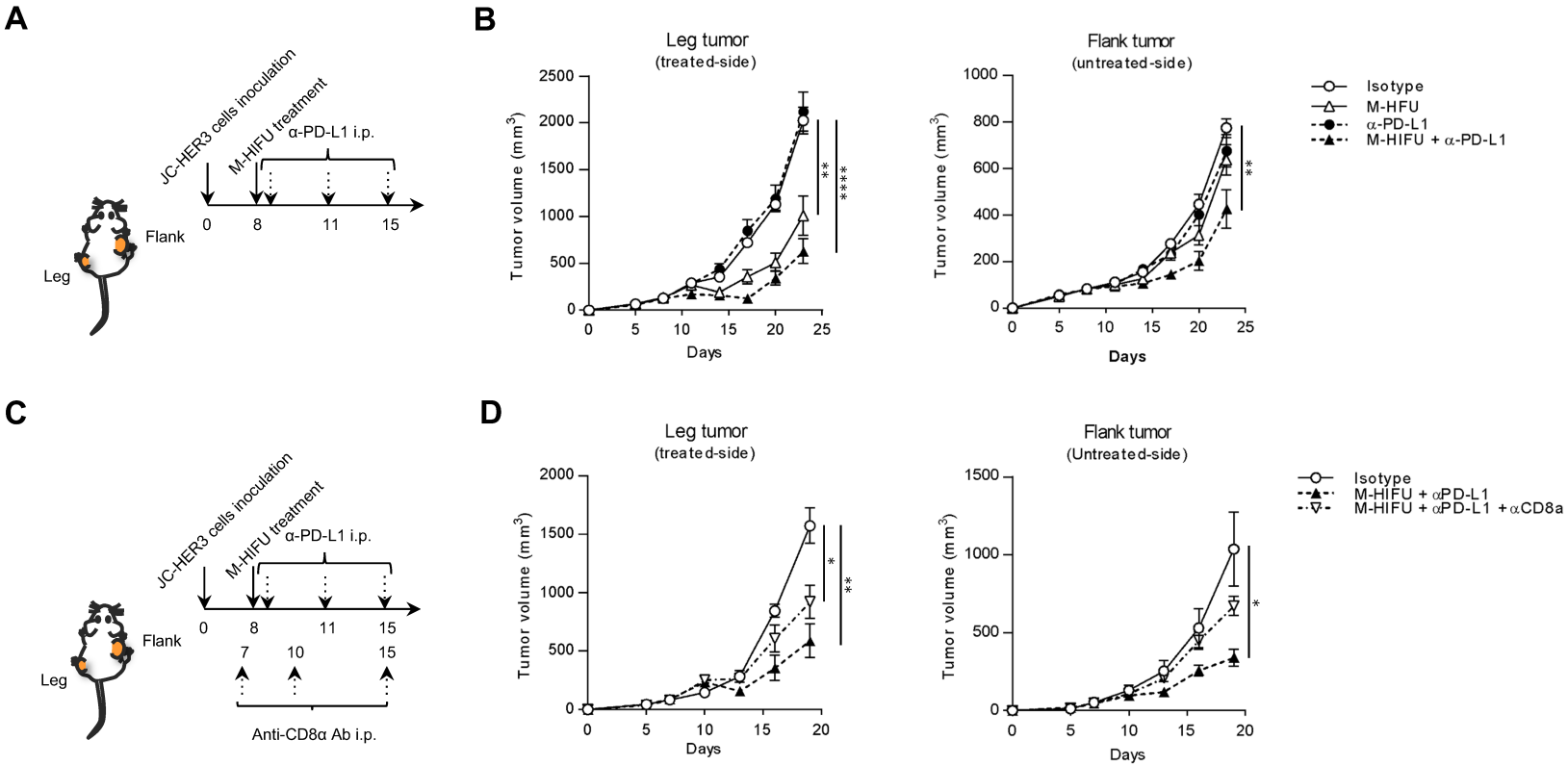
Supplementary Figure 9. Flow Cytometry Analysis of TILs in MM3MG-HER2 tumors treated with combined M-HIFU and anti-PD-L1 antibody. Established MM3MG-HER2 leg tumors were treated with M-HIFU followed by intraperitoneal injection of anti-PD-L1 antibody or Isotype IgG 5 and 8 days after M-HIFU treatment, and tumors were collected 9 days after M-HIFU. Tumors were minced and enzymatically digested to collect single cell suspension of tumor-infiltrating leukocytes. Alive singlet CD45+ leukocytes were analyzed for the following; **(A) t**he expression of CD69 and ICOS by $CD4^+$, **(B)** the expression of CD69, ICOS and Granzyme B by $CD8^+$ cells, and (**C**) the expression of $Foxp3^+$ by $CD4^+$ cells. N=4 per each group. Error bars represent SD. ∗: *P* value < 0.05, ∗∗: *P* value < 0.01, ∗∗∗: *P* value < 0.001.

Supplementary Figure 10



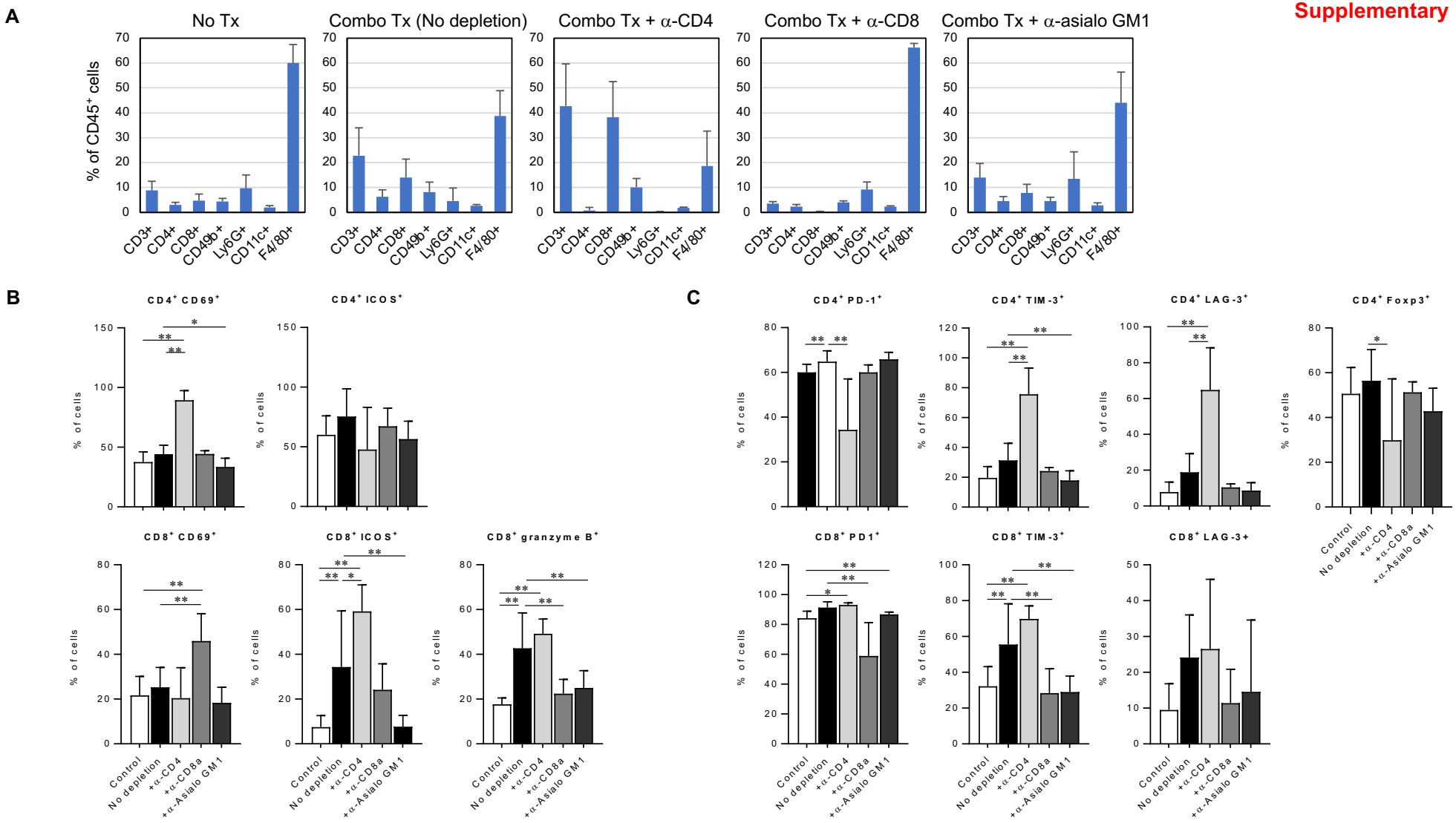
Supplementary Figure 10. KEGG pathway analysis of the differentially expressed genes in tumor-infiltrating CD8 T cells and macrophages. Differentially expressed genes upregulated in treatment groups compared to no treatment control were analyzed by KEGG pathway analysis using Partek Flow software. Top 15 KEGG pathways for CD8 T cells **(A)** and top 12 pathways for macrophages **(B)** that showed the highest enrichment scores in the combination treatment are shown.

Supplementary Figure 11



Supplementary Figure 11. Enhanced abscopal effect induced by combined M-HIFU and anti-PD-L1 antibody. **(A)** JC-HER3 cells were injected into both the left leg (1×10^6) and the right flank (5×10^5) of HER3 transgenic mice on day 0. Established leg tumors were treated with M-HIFU on day 8. Anti-PD-L1 antibody (200 µg /100 µL) or Isotype IgG (200 µg /100 µL) were injected intraperitoneally on day 8, 11, 15 and 18. **(B)** Tumor growth curves of leg tumors and flank tumors are shown for each treatment group. n=7 mice per each group. 28 mice in total. **(C)** The mice were treated as described in Figure 4E legend in the presence of depleting antibody for $CD8a^+$ cells on day 7, 10 and 15. **(D)** Tumor growth curves of both leg and flank tumors are shown. n=5 mice per each group. 15 mice in total. Error bar represents mean ± SE; ∗: *P* value < 0.05, ∗∗: *P* value < 0.01, ∗∗∗: *P* value < 0.001, ∗∗∗∗: *P* value < 0.0001.

Supplementary Figure 12



Supplementary Figure 12. Activation and exhaustion status of T cells infiltrating into remote tumors in mice administered with depletion antibodies. MM3MG-HER2 cells were injected into both the left leg (1×10^6) and the right flank (5×10^5) of BALB/c mice on day 0. Established leg tumors were treated with M-HIFU on day 7. Anti-PD-L1 antibody (100 µg /100 µL) or Isotype IgG (100 µg /100 µL) were injected intraperitoneally on day 10, 13 and 16. The mice were also treated with depleting antibody for $CD4^+$, $CD8a^+$ and NK cells on day 6, 9 and 14. Mice were euthanized on day19 after tumor inoculation, and remote untreated-tumors were collected. **A)** Immune cell profile was analyzed for each group. **B)** CD69 and ICOS on residual intratumoral $CD4^+$ cells and CD69, ICOS and granzyme B on residual intratumoral $CD8^+$ cells were analyzed with flow cytometry. **C)** immune checkpoint molecules on residual intratumoral T lymphocytes and Treg population were analyzed with flow cytometry. N=3 mice for CD 4 depletion group, n=5 mice for other groups. Bar represents SD.