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Supplemental Information

Eliciting anti-cancer immunity by genetically engineered multifunctional exosomes

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Table S1. List of primer sequences used for molecular cloning. Restriction enzyme sites EcoRI and NotI are underlined and italicized.

DNA fragment for cloning	Primer sequence
PD-1	Forward: 5'-CAGTGTGCTG*GAATTC*GGCTTGGGGATATCCACC-3'
	Reverse: 5'-CCGGACTACCACCGCCTCCGCTAGCGAGGGGCCAAGAGCAGTGTCCATCC-3'
CD9-OX40L	Forward: 5'-CCCTCGCTAGCGGAGGCGGTGGTAGTCCGGTCAAAGGAGGCACCAAGTGCATCAAATACC-3'
	Reverse: 5'-GATCTCGA*GCGGCCGC*CTTAATGGTGGTGGTGATGGTGAAGG-3'
PD-1-CD9-OX40L	Forward: 5'-CAGTGTGCTG*GAATTC*GGCTTGGGGATATCCACC-3'
	Reverse:5'-GATCTCGA*GCGGCCGC*CTTAATGGTGGTGGTGATGGTGAAGG-3'

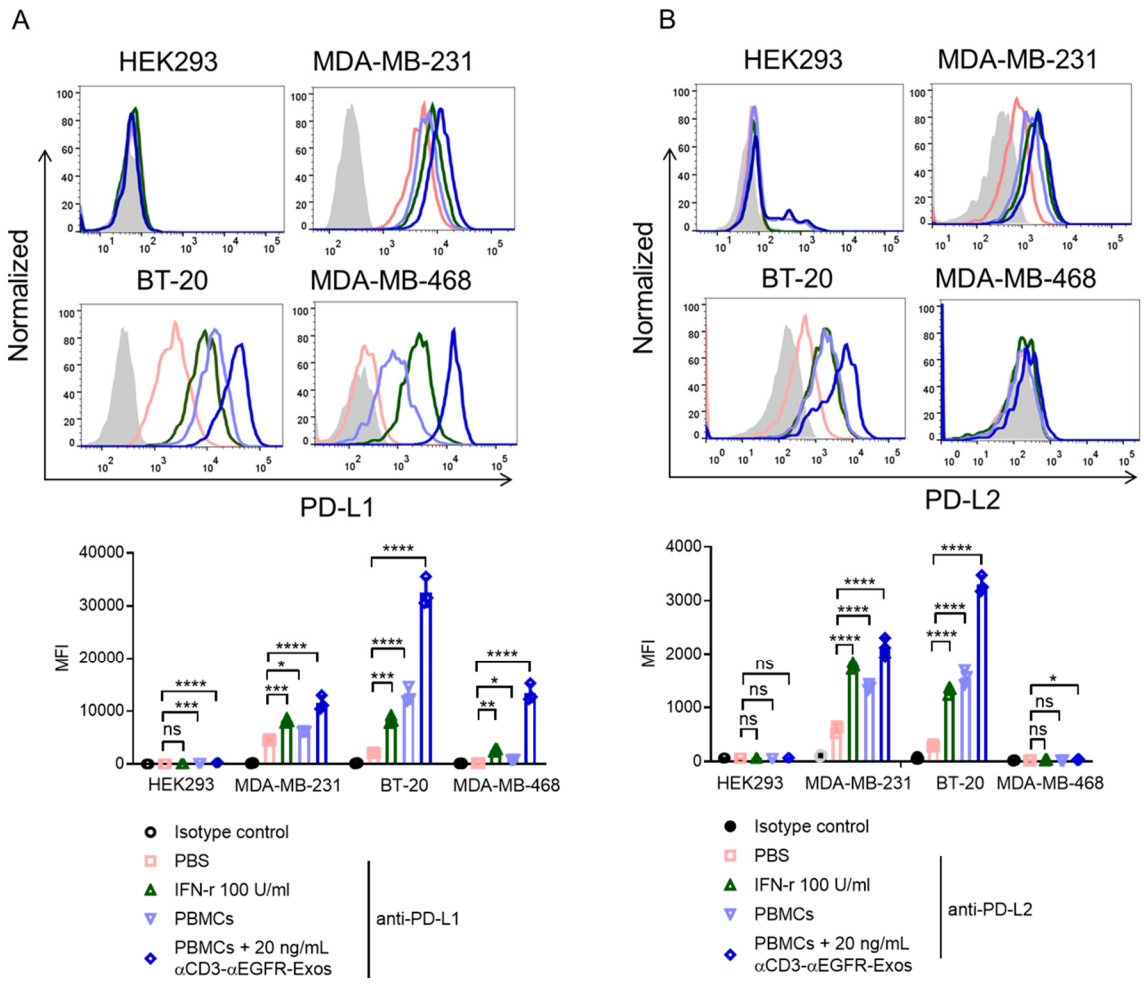

Figure S1. Flow cytometric analysis of expression levels of PD-L1 and PD-L2 at varied conditions. (A) and (B) Surface expression levels of PD-L1 (A) and PD-L2 (B) for HEK293 and three TNBC cell lines without and with stimulations. HEK293, MDA-MB-231, BT-20, and MDA-MB-468 cells were treated with 100 U/mL IFN-γ or human PBMCs (PBMC:TNBC/HEK293=2:1) in the absence or presence of 20 ng/mL αCD3-αEGFR-Exos for 48 hours at 37℃. Non-treated and treated cells were then analyzed for PD-L1 and PD-L2 expression by flow cytometry. Lower panels: quantitative representations of mean fluorescence intensities (MFIs) of PD-L1 (A) or PD-L2 (B) for each cell line. Data are shown as mean ± SD of triplicates. ns = not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ (ordinary one-way ANOVA test).

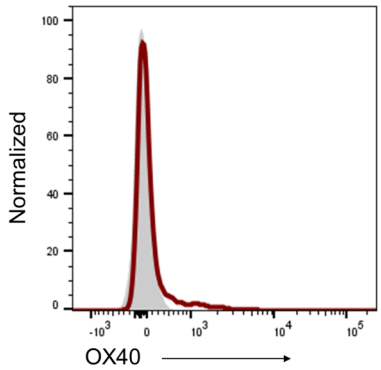

Figure S2. Flow cytometric analysis of OX40 expression on non-activated T cells.

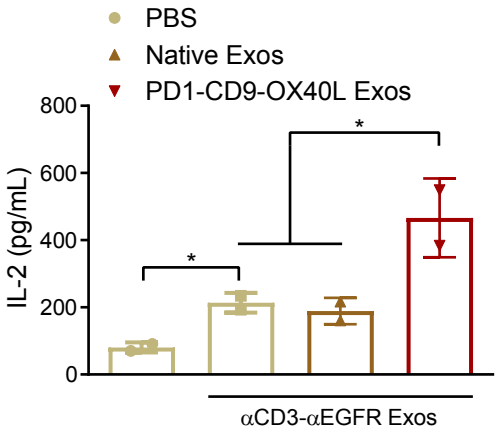

Figure S3. Enhancing T-cell activation by PD-1-OX40L-Exos. Human PBMCs were mixed with BT-20 cells at a ratio of 2:1 and incubated without or with αCD3-αEGFR-Exos (20 ng mL^{-1}) in the absence or presence of 10 μg mL^{-1} PD-1-OX40L-Exos or native exosomes for 48 hours. The levels of secreted IL-2 were measured by ELISA. Data are shown as mean ± SD of duplicates. * $P < 0.05$ (two-tailed unpaired *t* test).

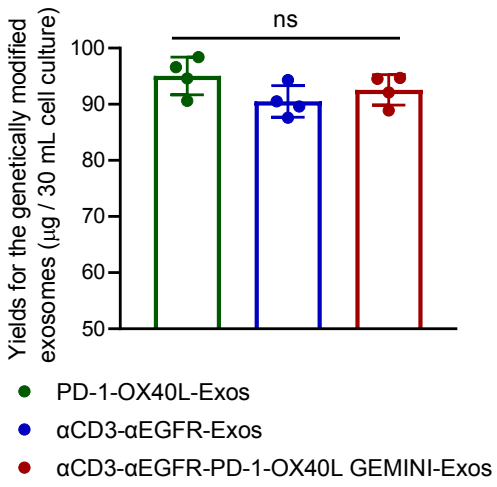

Figure S4. Yields of the genetically modified exosomes. Data are shown as mean ± SD (n=4). ns = not significant, P > 0.05 (ordinary one-way ANOVA test).

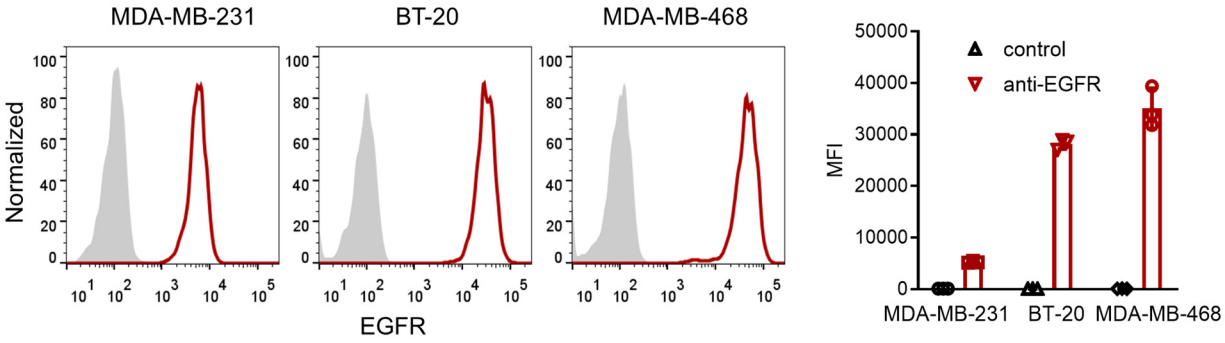

Figure S5. Flow cytometric analysis of EGFR expression for three TNBC cell lines. Right panel: quantitative representations of MFIs of EGFR for each cell line. Data are shown as mean ± SD of triplicates.

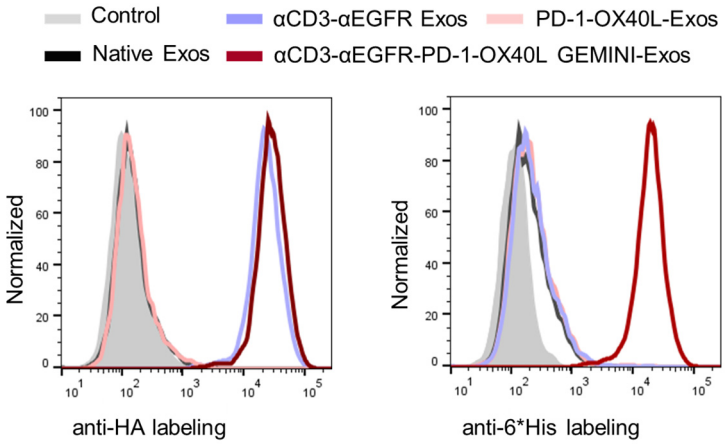

Figure S6. Flow cytometry of the binding of αCD3-αEGFR-PD-1-OX40L GEMINI-Exos to MDA-MB-468 cells (PD-L1$^-$ PD-L2$^-$ OX40$^-$ CD3$^-$ EGFR$^+$) as detected by the anti-HA or anti-6×His antibody.

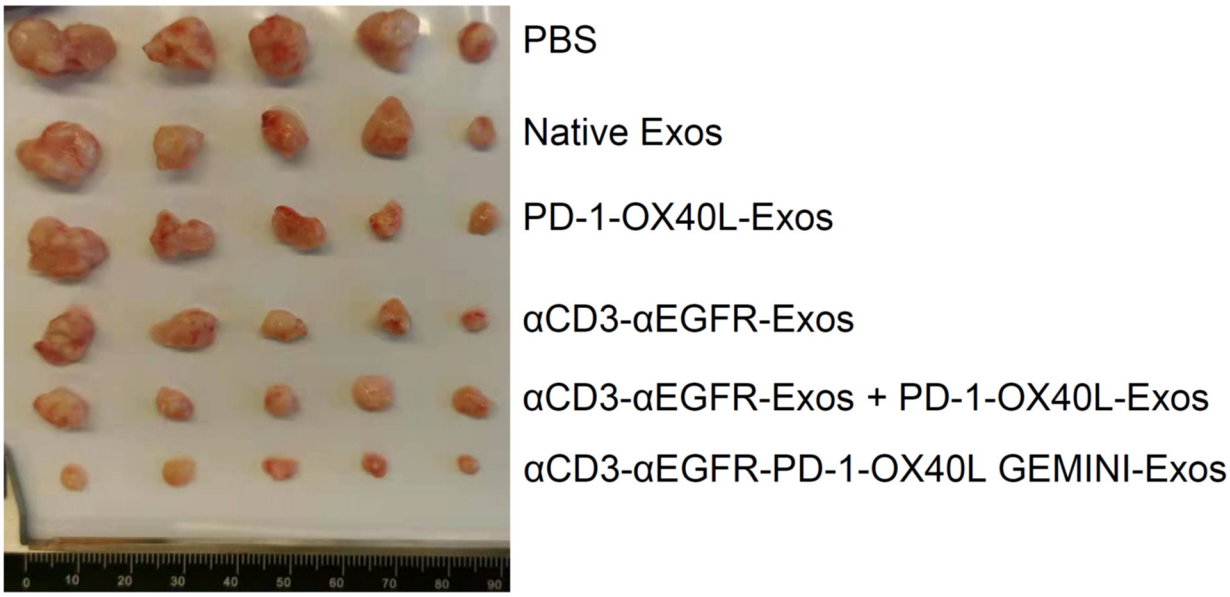

Figure S7. Photographs of xenografted mouse tumors at the endpoint.

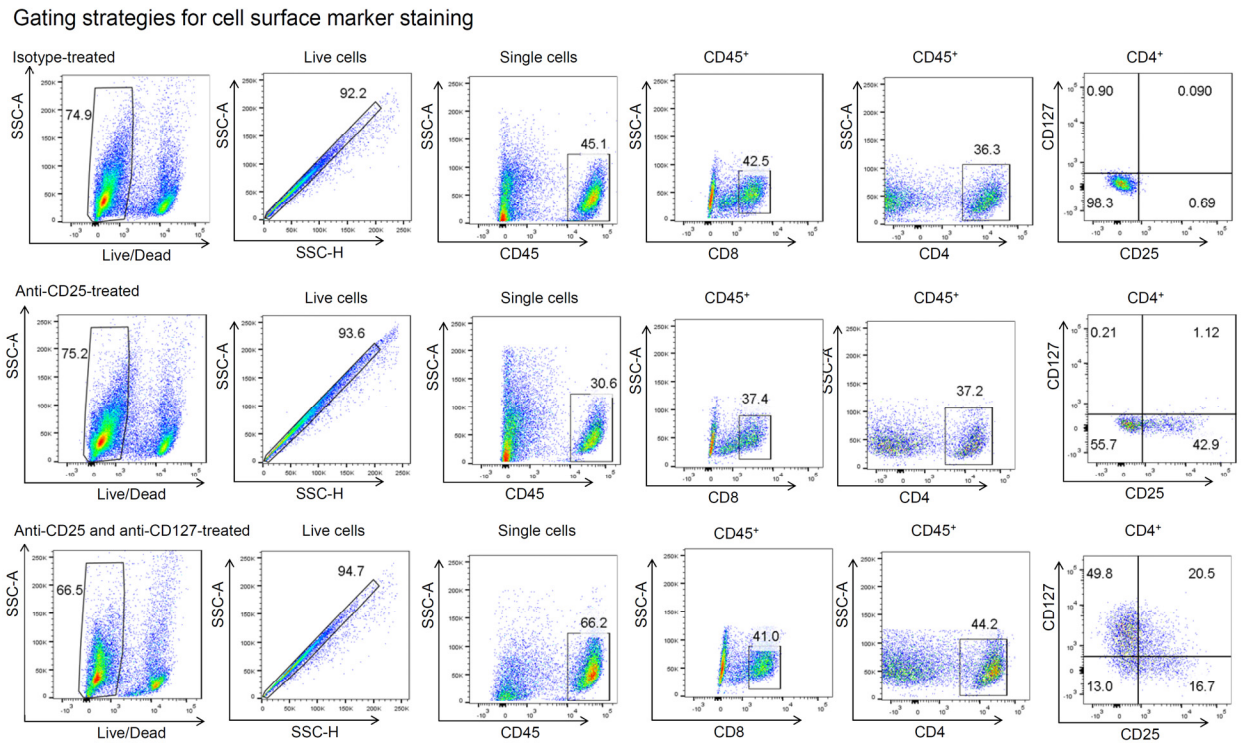

Figure S8. Gating strategy for $CD4^+$ $CD25^+$ $CD127^-$ Tregs.

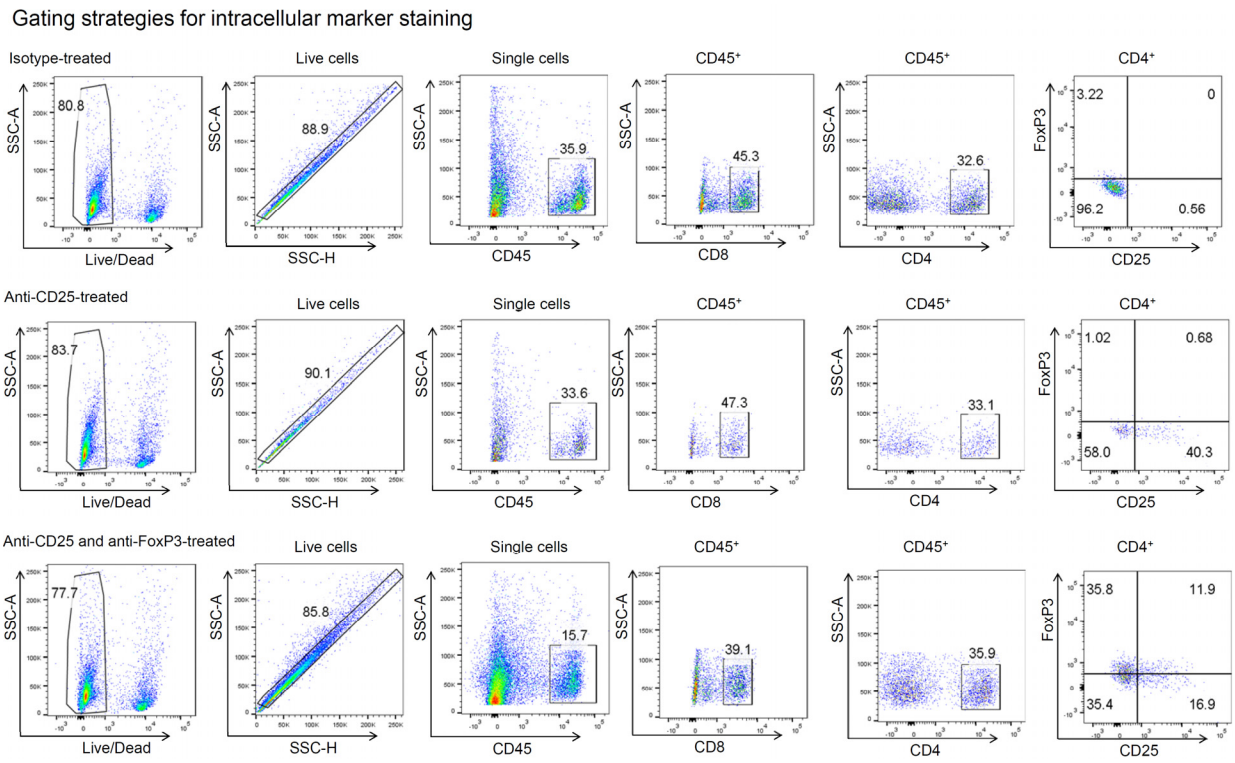

Figure S9. Gating strategy for $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs.

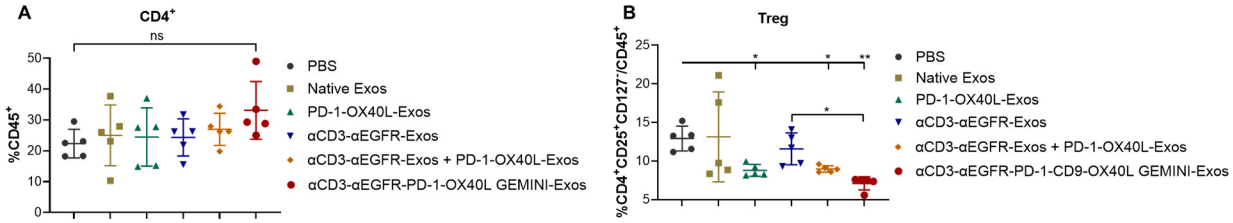

Figure S10. Immune phenotyping of tumor infiltrating lymphocytes. (A) Percentages of $CD4^+$ T cells in $CD45^+$ cells in tumors. (B) Percentages of $CD4^+$ $CD25^+$ $CD127^-$ Tregs in $CD45^+$ cells in tumors. At the end of the *in vivo* efficacy study, tumors were harvested and disaggregated into single-cell suspensions. After immunostainings, cells were analyzed by flow cytometry for the expression of CD45, CD4, CD8, CD25, and CD127. Data are shown as mean ± SD (n=5). ns = not significant, * $P < 0.05$, and ** $P < 0.01$ (ordinary one-way ANOVA test).

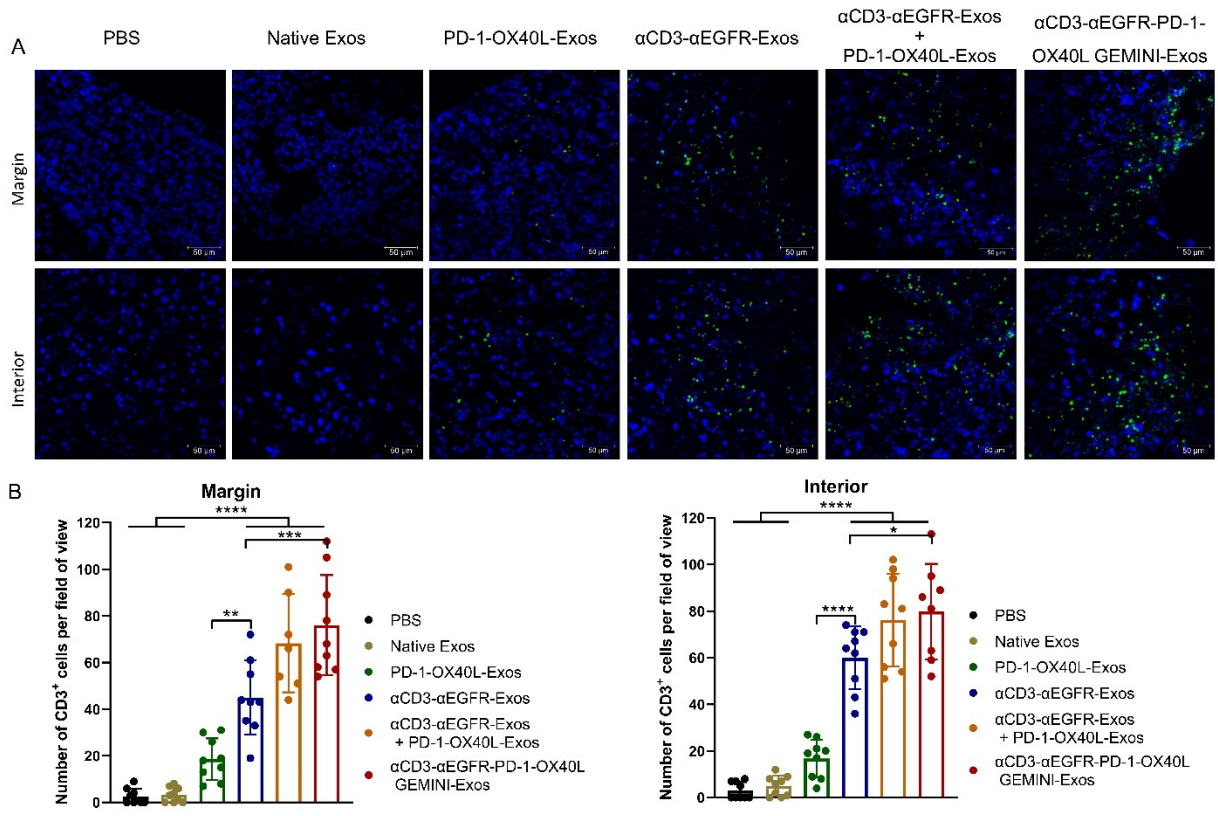

Figure S11. Immunohistofluorescence analysis of tumor-infiltrating T lymphocytes. (A) Representative immunohistofluorescence images of the margin and interior of frozen tumor sections from PBS- and exosomes-treated mice. Green: $CD3^+$ cells stained with the anti-CD3 antibody. Blue: nuclei stained with DAPI. Scale bars: 50 µm. (B) Quantitative representation of the number of $CD3^+$ cells from each field of view along the margin and interior of each tumor from PBS- and exosomes-treated groups (up to three fields of view per region and three mice per group). Data are shown as mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $p < 0.0001$ (ordinary one-way ANOVA test).

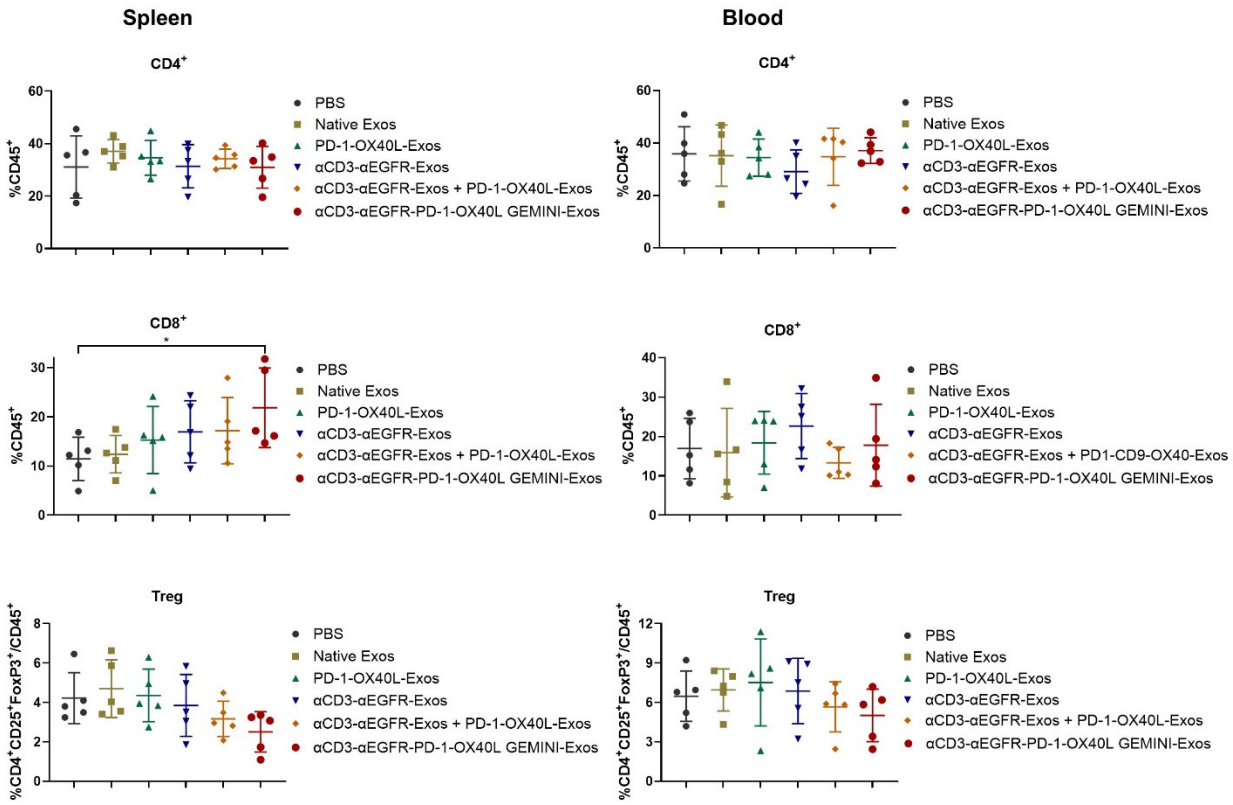

Figure S12. Immune phenotyping of lymphocytes in spleen and blood. At the end of the *in vivo* efficacy study, blood and spleen were harvested and spleen samples were disaggregated into single-cell suspensions. After immunostainings, cells were analyzed by flow cytometry for the expression of CD45, CD4, CD8, CD25, and FoxP3. Percentages of $CD4^+$ T cells (top), $CD8^+$ T cells (middle), and $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs (bottom) in $CD45^+$ cells in spleen (left) and blood (right) were determined. Data are shown as mean ± SD (n=5). * $P < 0.05$ (ordinary one-way ANOVA test).