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Supplementary Information for

TLR1/2 ligand enhances anti-tumor efficacy of CTLA-4 blockade by increasing intratumoral Treg depletion

Naveen Sharma, Jean Vacher, James P. Allison

Email: nsharma1@mdanderson.org (N.S.), JAllison@mdanderson.org (J.P.A)

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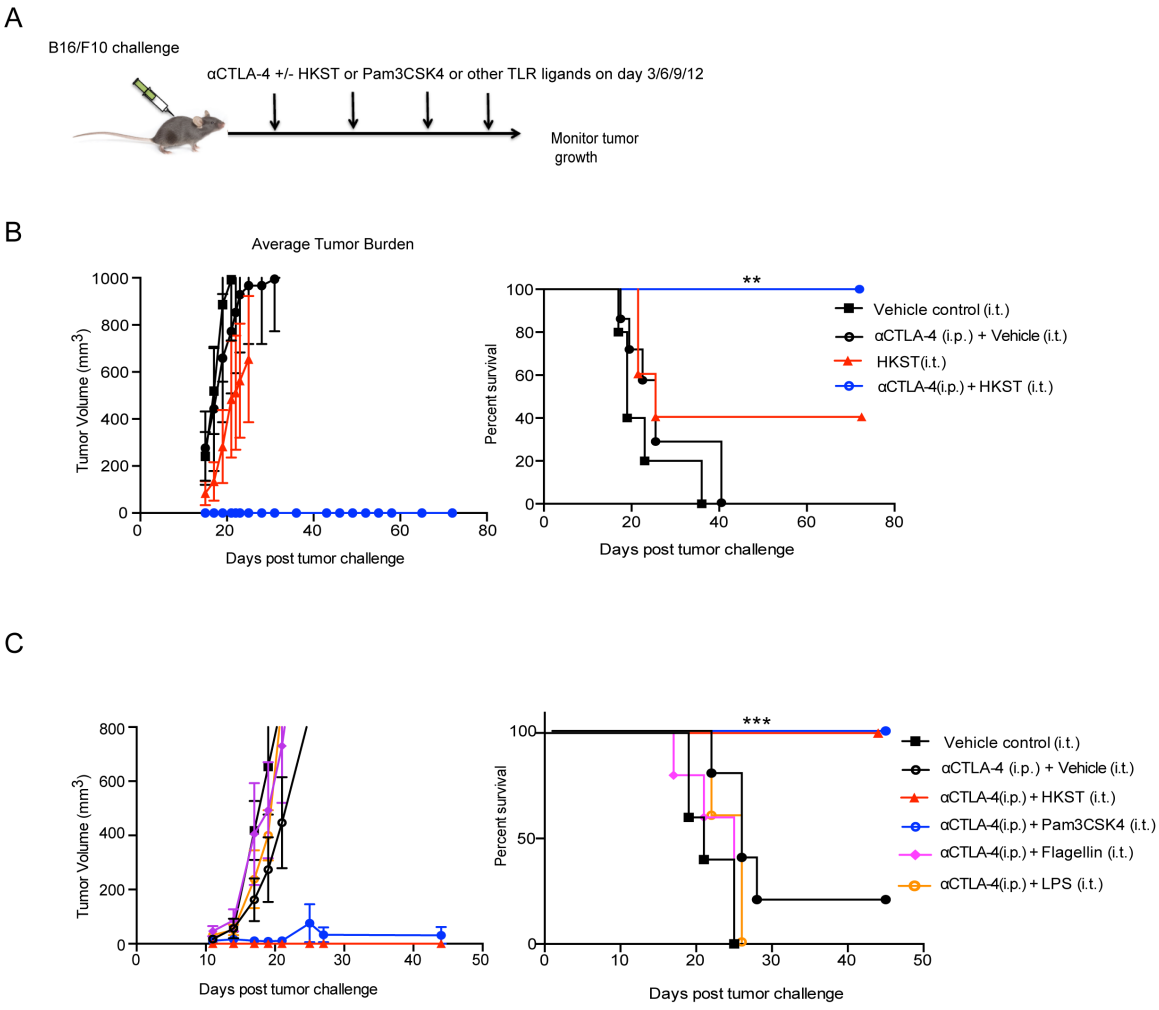

Fig. S1. HKST and Pam3CSK4 Cooperate with Anti–CTLA-4 Antibody to Promote Tumor Rejection and Survival. (A) Treatment schedule of Pam3CSK4, HKST and other TLR ligands (intratumoral) and anti–CTLA-4 antibody (intraperitoneal) in B16/F10 tumor challenged mice. (B) Average tumor growth and survival of mice treated with combination of anti-CTLA4 antibody and HKST. (C) Average tumor growth and survival of mice treated with combination of anti-CTLA4 antibody and HKST or other TLR ligands. Mice were challenged with $3x10^5$ B16/F10 cells and were given 4 doses of treatments as indicated every third day starting at day 3 after tumor challenge. Data are representative of 2 independent experiments with 5-8 mice per group. i.p., intraperitonial treatment, i.t., intratumoral treatment. Error bars represent the mean ± SEM. **p<0.01, **p<0.001 (Mantel-Cox test). Survival curve significance is calculated between anti-CTLA-4 plus vehicle and anti-CTLA-4 plus Pam3CSK4.

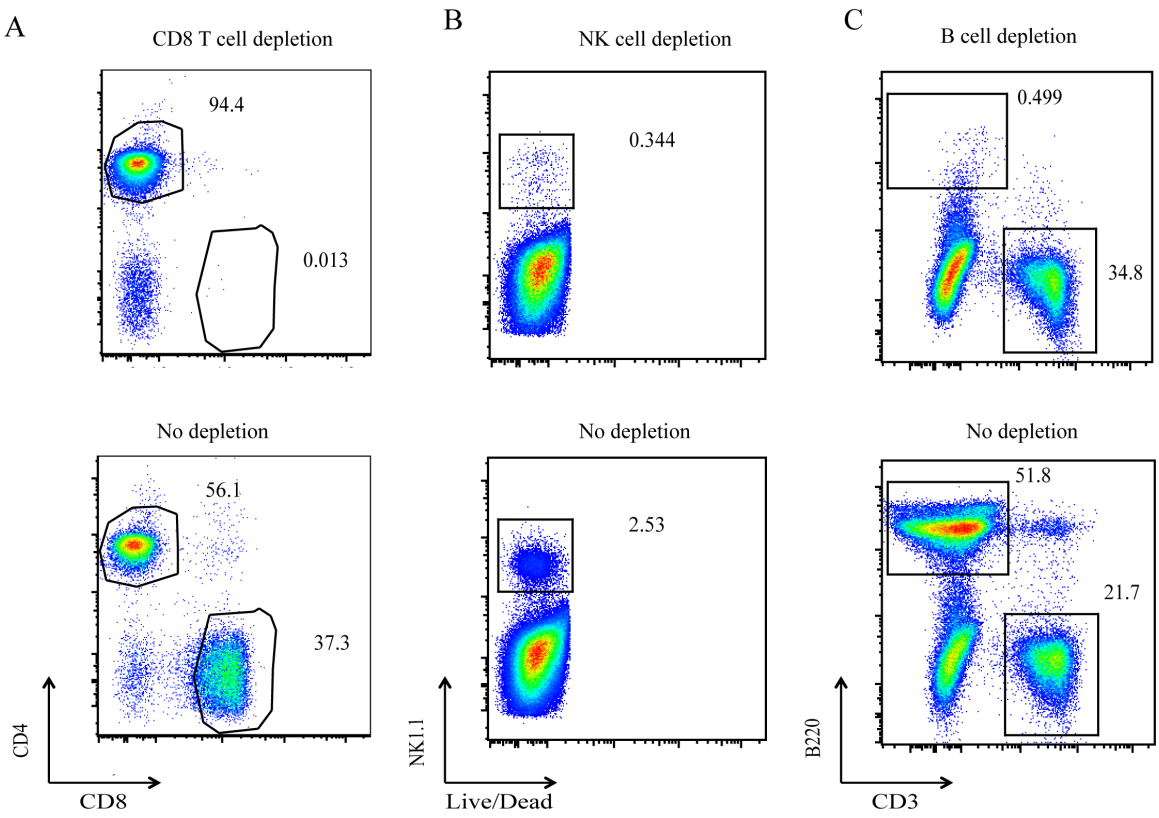

Fig. S2. *In vivo* Depletion Efficacy of Antibodies. Representative flow dot plots of peripheral blood from mice treated with (Top) different depleting antibodies for: (A) CD8 T cells, (B) NK cells and (C) B cells and (Bottom) compared with untreated control. Peripheral blood was collected 2 days after the last dose of antibody treatment. Data are representative of 2 independent experiments.

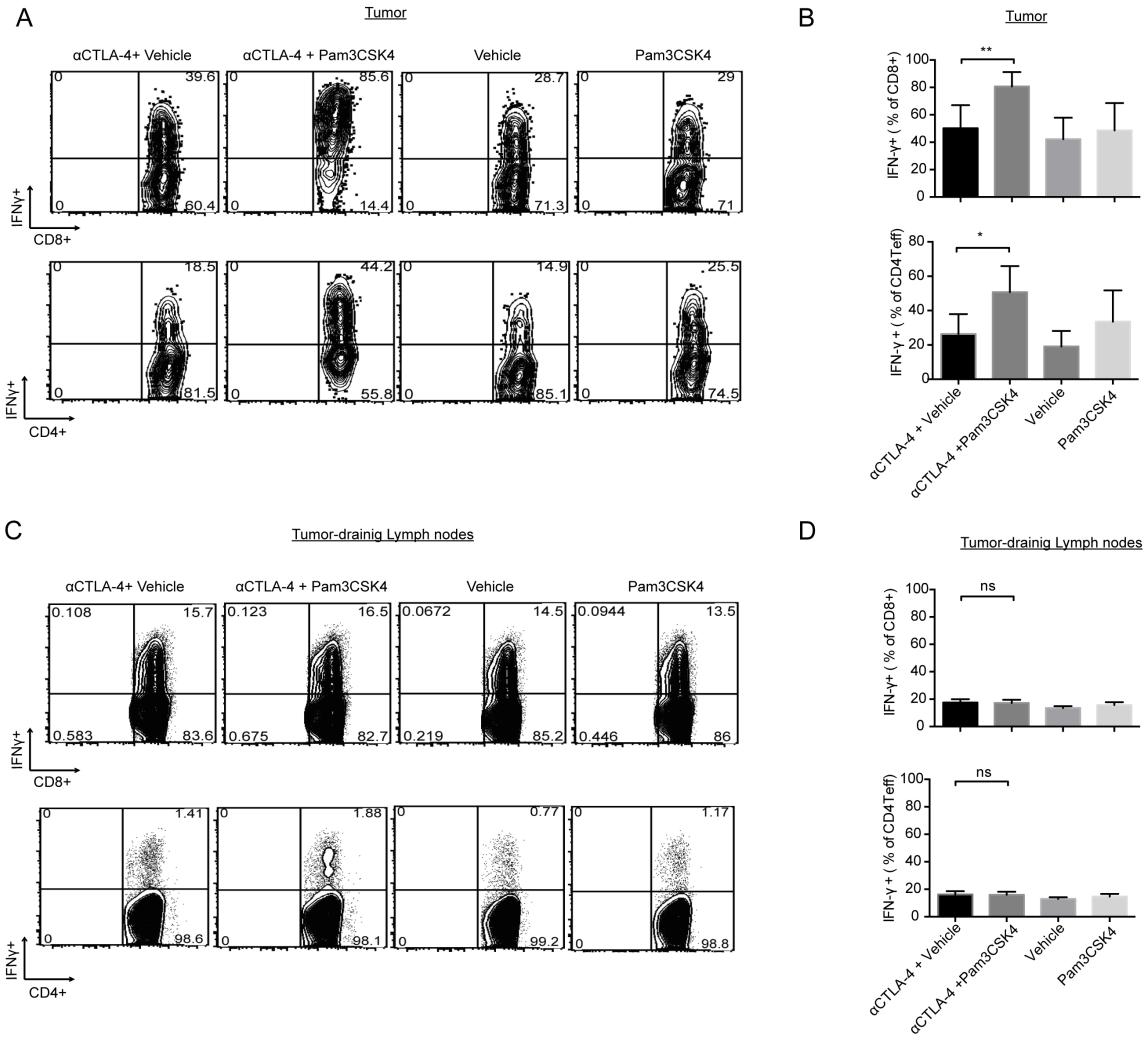

Fig. S3. Enhanced IFN-γ Production from TILs from Mice Treated by Combination of Pam3CSK4 plus CTLA-4 blockade. Mice were challenged with B16/F10 cells and were given indicated treatments, tumors were harvested, activated *ex vivo* with BD leukocyte activation cocktail and stained with indicated antibodies as described in Materials and Methods. (A) Representative flow cytometry plots of IFN-γ in CD8 T cells and CD4 Teff cells from TILs. The numbers in the quadrants are relative frequencies, and plots are representative. (B) Bar graphs show the frequencies of IFN-γ producers among tumor-infiltrating CD8 T cells and CD4 Teff cells. (C) Representative flow cytometry plots of IFN-γ in CD8 T cells and CD4 Teff cells from draining lymph nodes. The numbers in the quadrants are relative frequencies, and plots are representative. (D) Bar graphs show the frequencies of IFN-γ producers among tumor-infiltrating CD8 T cells and CD4 Teff in draining lymph nodes. Data are representative of 3 or 4 experiments with 5 animals per group. Error bars represent the mean ± SD. ns, not significant, *p<0.05, **p<0.01 (Student's t-test).

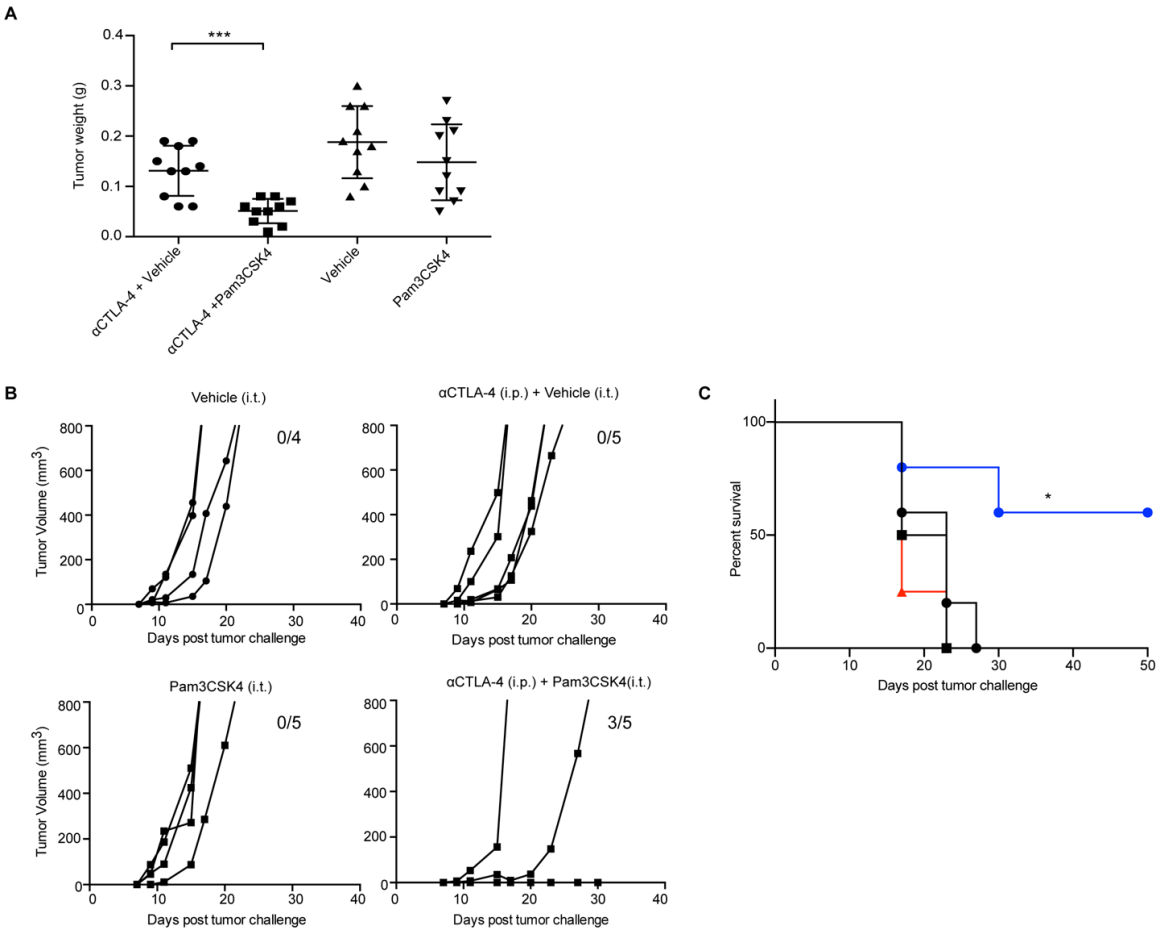

Fig. S4. Decreased Tumor burden in Mice given Delayed Treatment of Pam3CSK4 Plus Anti-CTLA-4 antibody. (A) Mice were challenged with $6x10^5$ B16/F10 cells and were given 2 doses of treatments as indicated on day 9 and 12 after tumor challenge. Tumors were harvested at day 14 after tumor challenge and cumulative tumor weights of the indicated treatment groups in 2 of 3 independent experiments. (B) Individual tumor growth and (C) Survival of mice in each treatment group. Mice were challenged with $3x10^5$ B16/F10 cells and were given 4 doses of treatments as indicated every third day starting at day 9 after tumor challenge. Data are representative of 1 of 2 experiments with 4-5 animals per group. *$p<0.05$***$p<0.001$ (Student t-test).

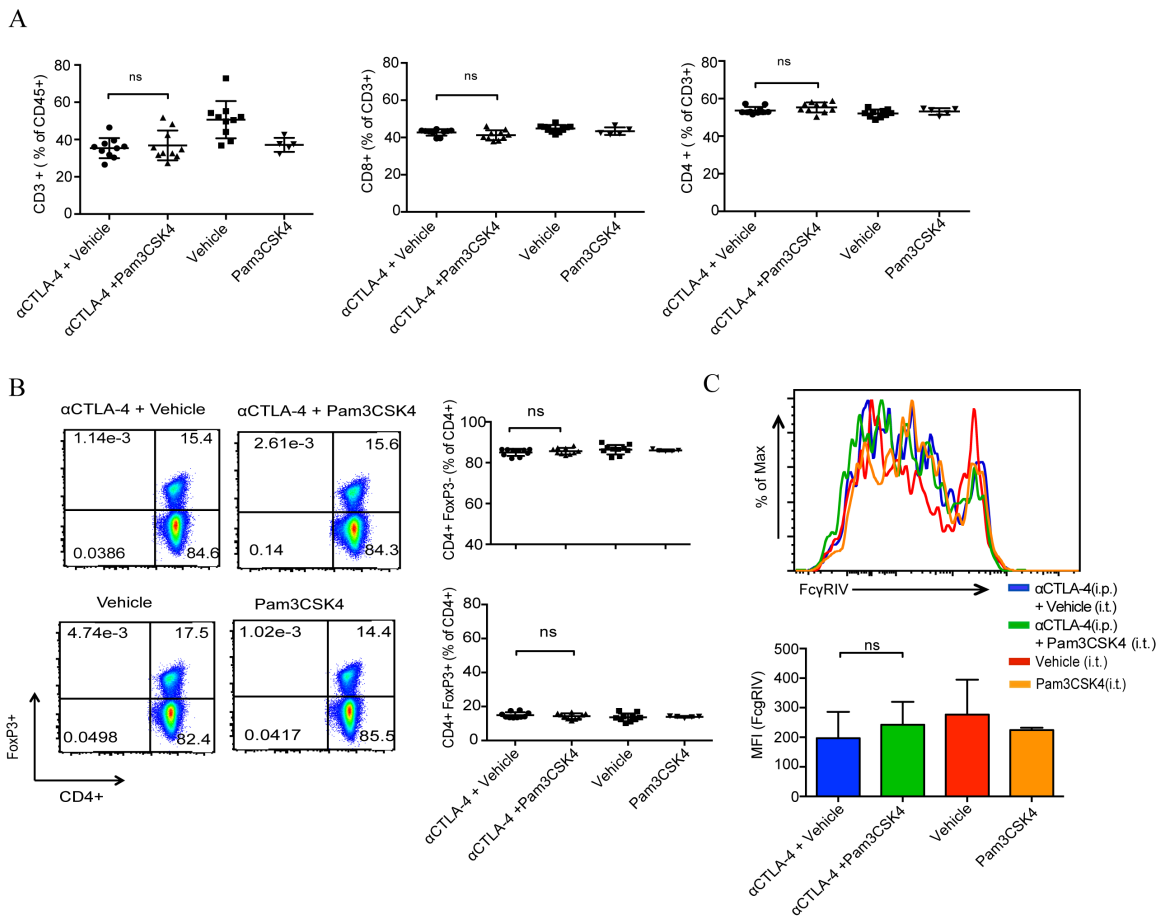

Fig. S5. Frequencies of Different T-Cell Subsets and FcγRIV expression on macrophages in Draining Lymph Nodes Do Not Differ Between Mice Treated with Combination and Mice Treated with Anti–CTLA-4 Antibody Alone. Mice were challenged with $6x10^5$ B16/F10 cells and were given 2 doses of treatments as indicated on day 9 and 12 after tumor challenge. Cells from draining lymph nodes were harvested at day 14 after tumor challenge and stained with indicated antibodies as described in Materials and Methods. (A) Cumulative frequencies of CD3, CD8, and CD4 T cells from 2 of 3 or 4 independent experiments were calculated as the percentages of CD45+ cells or CD3+ cells. (B) Representative dot plot showing the relative frequencies of CD4+ Foxp3+ Tregs and CD4+Foxp3- Teff cells as percentages of CD4+ T cells and cumulative frequencies of CD4+ Foxp3+ Tregs and CD4+ FoxP3- Teffs from 2 of 3 or 4 independent experiments were calculated as the percentages of CD4+ T cells. (C) Representative flow histogram plot (Top) and mean fluorescence intensity bar graph (Bottom) of FcγRIV expression on CD11b+ GR1- F-4-80+ dLN resident macrophages from B16/F10 challenged mice and given treatments as indicated. Data are representative of 3 or 4 independent experiments with 4 or 5 mice per group. Error bars represent the mean ± SD. ns, not significant (Student t-test).

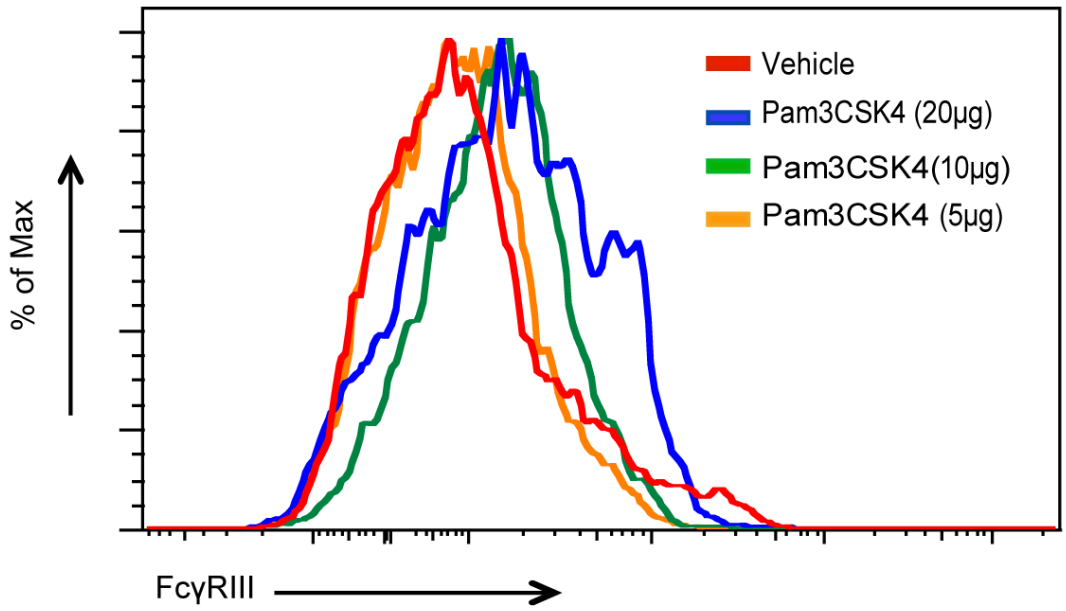

Fig. S6. Pam3CSK4 enhances FcγRIII expression on CD11b+ cells. PBMCs from healthy individuals were stimulated *ex vivo* with TLR1/2 ligand Pam3CSK4 at different doses for 24 hours and then stained with LIVE/DEAD fixable blue, anti-CD11b and anti-FcγRIII (CD16). Gated live CD11b+ cells were analyzed for FcγRIII expression. Data is representative flow cytometry plot from 2 experiments.

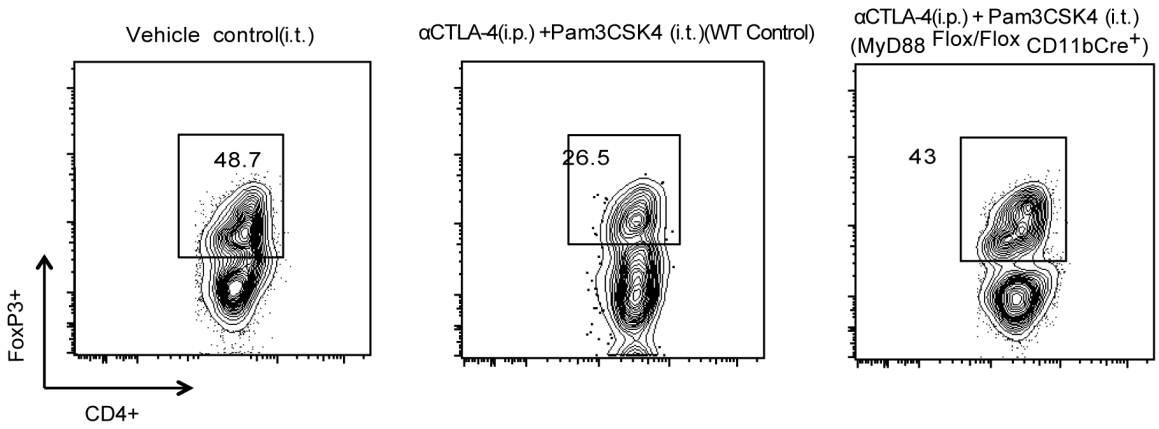

Fig. S7. Combination Treatment Did Not Deplete Intratumoral Tregs in MyD88$^{Flox/Flox}$ CD11bCre^{+} mice. Tumors were harvested once it reached 1000 mm^3 at the end of survival experiment, cells were isolated and stained with indicated antibodies as described in materials and method. Representative flow cytometry plots showing CD4+FoxP3+ (Treg) relative frequencies from indicated group of mice. Data are representative of two experiments with 2-4 mice per group.

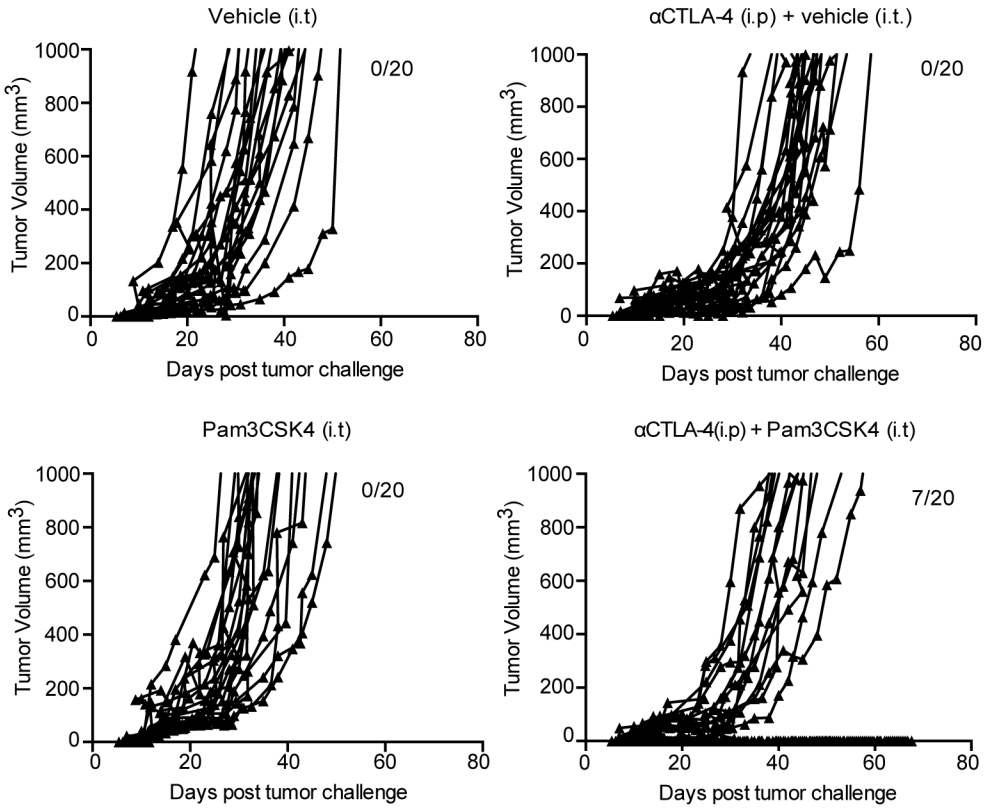

Fig. S8. Individual Tumor Growth Curves of Various Treatment Groups in Mouse Pancreatic Tumor model. Mice were challenged with $1x10^5$ mT5 cells and were given indicated treatments. Numbers on the upper right side represent tumor-free mice. Data are cumulative of 3 independent experiments with 5-7 mice per group.

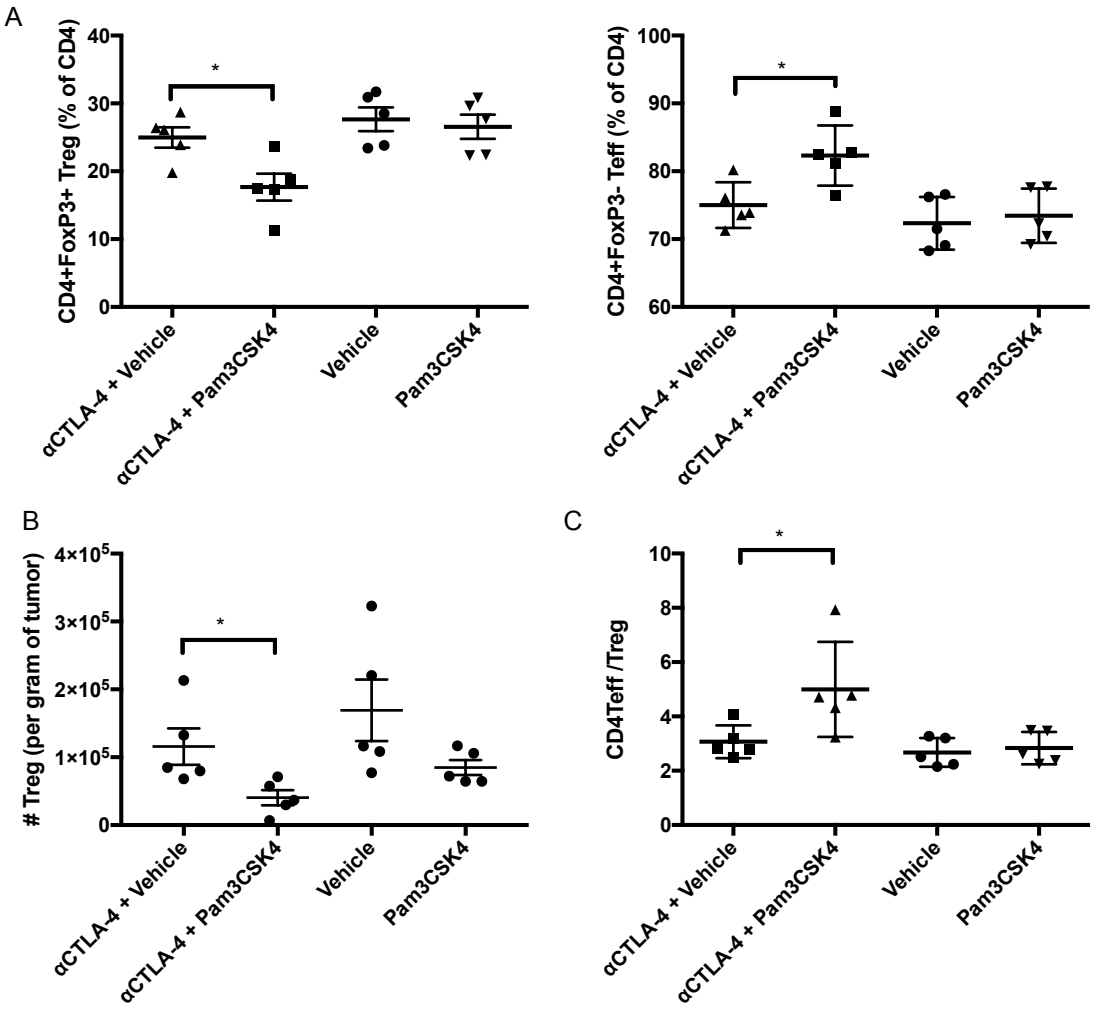

Fig. S9. Combination Treatment Depletes Intratumoral Tregs in mT5 Tumor Model And Increases CD4Teff/Treg Ratio. Mice were challenged with mT5 cells and were given indicated treatments, cells from tumors were harvested and stained with indicated antibodies as described in Materials and Methods. (A) Frequencies of CD4+FoxP3+ Treg and CD4+FoxP3- Teff cells as percentages of CD4+ cells, (B) Treg density as the absolute numbers of the cells per gram of tumor and (C) Intratumoral CD4Teff/ Treg ratios. Data are representative of 2 experiments with 4-5 mice per group. Error bars represent the mean ± SD.*$p<0.05$ (Student's t-test).

Table S1: Top 20 genes differentially upregulated with combination treatment of Pam3CSK4 plus 9H10 antibody with 9H10 antibody treatment as a baseline

Probe	Accession no.	Log2 fold change	P-value
Marco	NM_010766.2	3.06	0.000366
Emr1	NM_010130.1	2.72	1.89E-05
Itgal	NM_008400.2	2.47	0.000126
Ebi3	NM_015766.2	2.43	1.47E-05
Irak3	NM_028679.3	2.33	3.55E-05
Tlr1	NM_030682.1	2.29	1.22E-05
Itgam	NM_001082960.1	2.22	1.75E-05
Spn	NM_001037810.1	2.16	0.000248
Fcgr4	NM_144559.1	2.01	0.000818
Tnfrsf1b	NM_011610.3	1.97	3.61E-05
Fcer1g	NM_010185.4	1.94	4.33E-05
Ccl3	NM_011337.1	1.92	0.000266
Tyrobp	NM_011662.2	1.85	2.03E-05
Lilrb3	NM_011095.2	1.83	5.00E-05
Pou2f2	NM_001163554.1	1.83	0.00033
Syk	NM_011518.2	1.77	0.000124
Il16	NM_010551.3	1.76	5.38E-05
Itgb2	NM_008404.4	1.73	0.000167
Tagap	NM_145968.2	1.72	0.00199