Supplementary Materials: The RXR Agonist MSU42011 Is Effective for the Treatment of Preclinical HER2+ Breast Cancer and Kras-Driven Lung Cancer

Ana S. Leal, Jessica A. Moerland, Di Zhang, Sarah Carapellucci, Beth Lockwood, Teresa Krieger-Burke, Bilal Aleiwi, Edmund Ellsworth, Karen T. Liby

Supplementary Text

Supplemental methods

CD4-FOXP3 T cell treatment

From a single cell suspension of splenocytes from a wild type mouse, 10^7 cells were incubated in 100 µL of Mojo Buffer (Biolegend) with 10 µL of pre-diluted Biotin-antibody cocktail mix for 15 minutes, followed by 10 µL of pre-diluted streptavidin nanobeads for an additional 15 minutes on ice (Biolegend MojoSortTM CD4 T cell isolation kit). The mixture was then placed on the column in the magnetic separator and washed with 3 mL of Mojo Buffer. Isolated CD4 T cells were plated in a 24 well plate at 106 ml/well in RPMI media supplemented with 10% FBS, anti-mouse CD28 (clone 37.51, 3 µg/mL, Biolegend), recombinant IL-2 (5 ng/mL, Biolegend), and recombinant human TGFβ (5 ng/mL, R&D). Plates were coated with anti-mouse CD3ε (clone 145-2C11, 3µg/mL, Biolegend) over night at 4oC. After 24 hours, LG268 or bexarotene were added at 100 nM and CD4 T cells were cultured for an additional 4 days. On day 5 cells were collected and RNA was extracted using triZOL(Thermo Fisher Scientific) following the manufacturer's instructions. Two micrograms of RNA were reverse transcribed, and 1 µl of complementary DNA from this reaction was added to 12.5 µl of Bio-Rad iQ SYBRGREEN Supermix (Hercules, CA), 1 µl of validated RT2 quantitative PCR (qPCR) FOXP3 (F: 5'-CTC GTC TGA AGG CAG AGT CA -3'; R: 5'-TGG CAG AGA GGT ATT GAG GG -3') or RLP13A (F: 5'-GTT GCC TTC ACA GCG TA-3'; R: 5'-AGA TGG CGG AGG TGC AG-3) primers and DNAse-free water for real-time qPCR. All expression data were normalized using RLP13A as the housekeeping control.

CD11b and CD3 isolation

Tumors from MMTV-neu female mice were minced separately and incubated in digestion media consisting of collagenase (300 U/ml, Sigma), dispase (1 U/ml, Worthington), and DNAse (2 U/ml, Calbiochem) for 30 minutes at 37°C with stirring. Cells were then passed through a 40 µm cell strainer (BD Falcon). From a single cell suspension 107 cells were incubated in 100 µL of Mojo Buffer (Biolegend) with 10 µL of pre-diluted Biotin-antibody cocktail mix for 15 minutes, followed by 10 µL of pre-diluted streptavidin nanobeads for an additional 15 minutes on ice (CD11b cell isolation kit, Miltenyi). The mixture was then placed on the column in the magnetic separator and washed with 3 mL of Mojo Buffer. CD11b positive cells were then eluted from the column. Cells were lysed in RIPA buffer (1 M Tris-Cl, pH 7.4, 0.5 M EDTA, 5 M NaCl, 1% triton-X, 25 mM deoxycholic acid, 0.1% SDS) containing protease inhibitors (PMSF, aprotinin and leupeptin) for whole cell lysates.

CD3 T cells were isolated from the tumors using the same approach as described above for CD4.

RT-qPCR analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA concentrations were determined by NanoDrop, and 2 μg RNA was used to synthesize cDNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Primers were ordered from IDT. iQ SYBR Green Supermix (Bio-Rad, Berkeley, CA) and the QuantStudio 7 Flex Real-Time PCR system were used to detect gene expression. The delta-delta Ct method was used to assess relative gene expression. Values were normalized to the reference gene GAPDH and expressed as fold change compared to saline control samples.
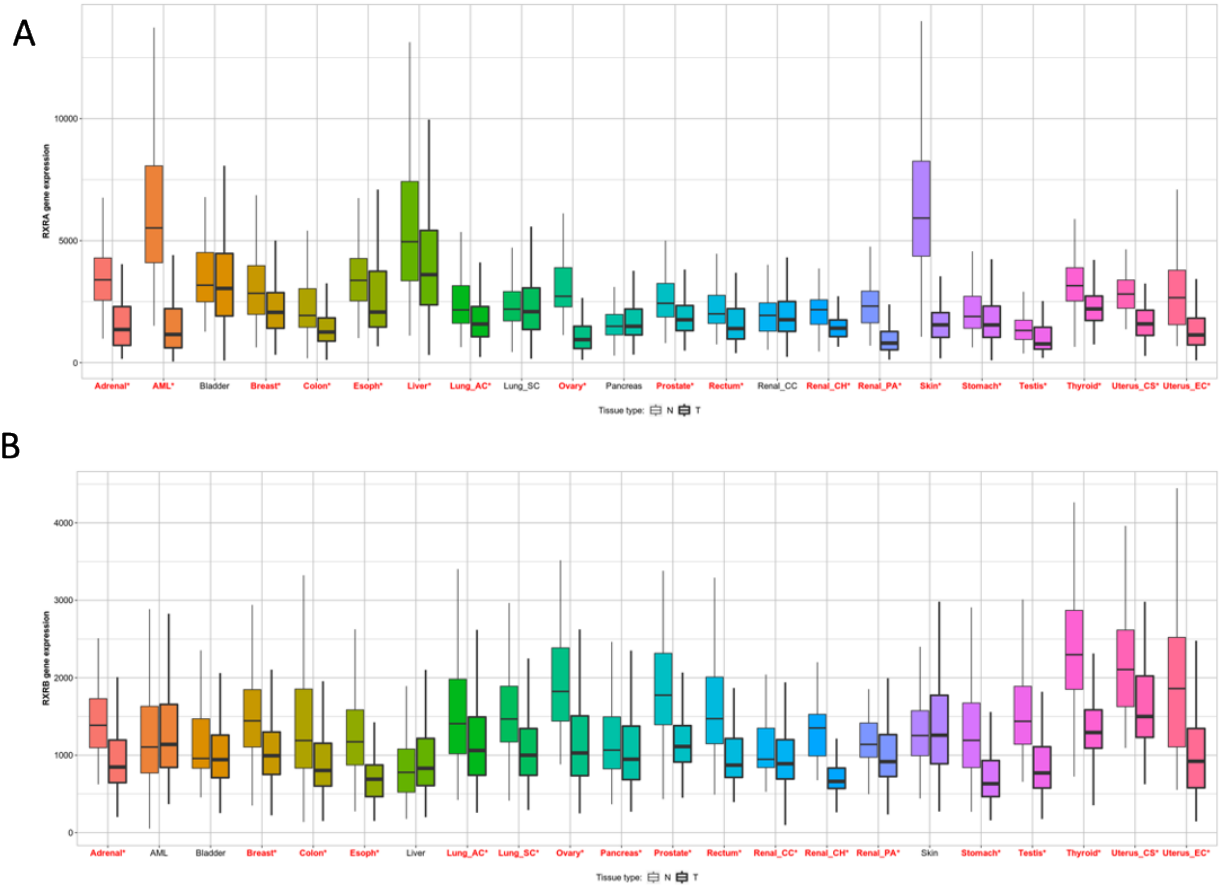
Figure S1. Expression of RXRα (A) and RXRβ (B) in tumors vs. normal tissue. Data analyzed/accessed through KMPLOT.
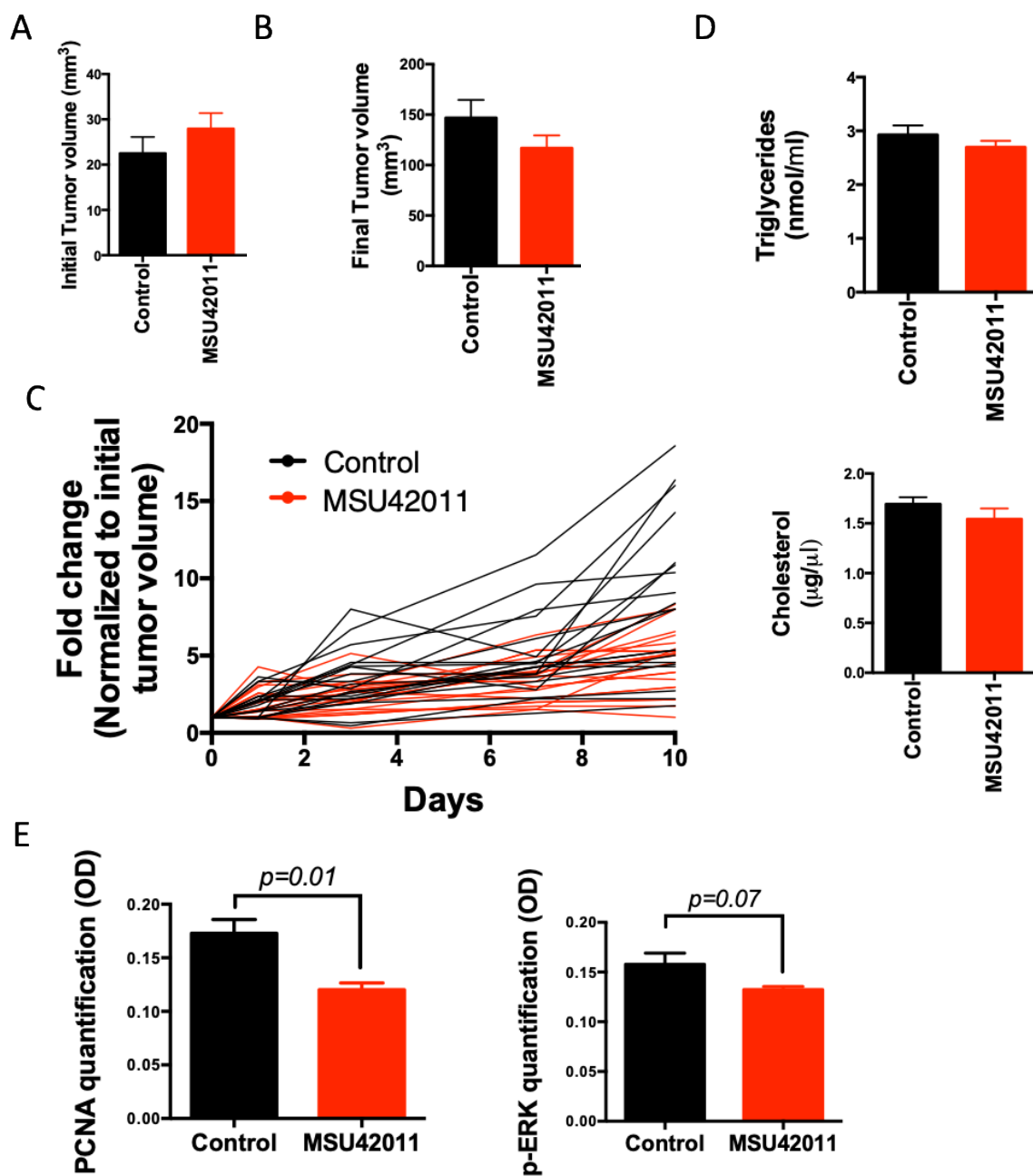
Figure S2. Tumor volume in MMTV-Neu mice treated with MSU42011. (A) Initial and (B) final tumor volumes. (C) individual fold changes of tumors over 10 days of treatment. (D) Plasma triglycerides and cholesterol levels in MMTV-Neu after 10-day treatment with MSU42011. (E) Quantification of PCNA and p-ERK intensity staining by optical density (OD), *n* = 4/group.
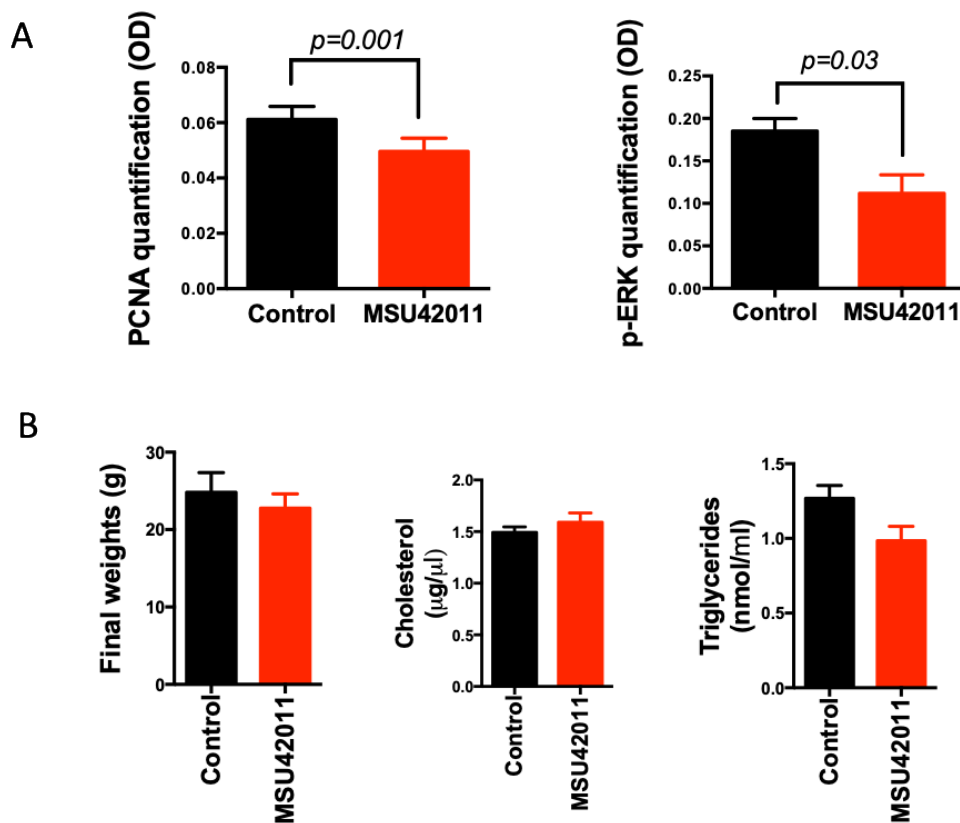
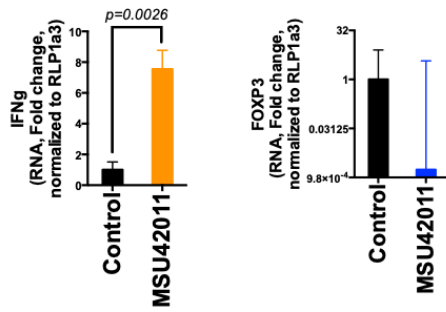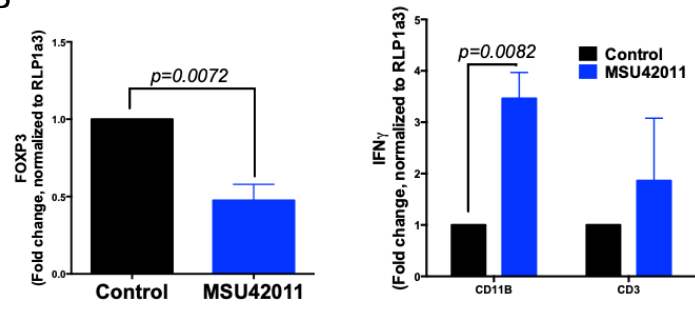**Figure S3.** (A) Quantification of PCNA (n = 3) and p-ERK (n = 4) intensity staining by optical density (OD). (B) Final total body weight, plasma triglycerides and cholesterol levels in A/J mice after 12 weeks of treatment with control or MSU42011 (100 mg/Kg).
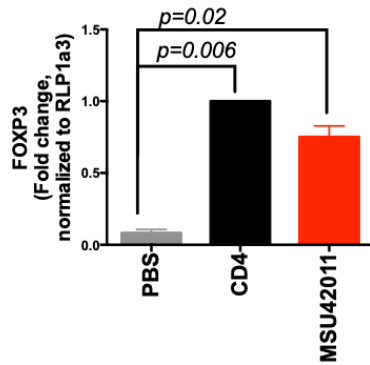
Figure S4: Treatment with MSU42011 reduced FOXP3 and increased IFNγ. (A) RT-PCR analysis of whole tumor lysates from MMTV-Neu mice treated with control or MSU42011 diet for 10 days. FOXP3 and IFNγ were evaluated by qPCR and RLP13a was used as control, n = 4/group. (B) Magnetically sorted CD3 and CD11b positive cells were collected from MMTV-Neu tumors treated for 10 days with MSU42011 or control diet (n = 3 tumors per group). FOXP3 levels were determined in the CD3 population by RT-PCR, which was also used to determine the levels of IFNγ mRNA in both CD3 and CD11b positive populations. (C) In vitro treatment of CD4 T cells isolated from the spleen of mice treated with MSU42011. CD4 T cells were isolated by magnetic bead separation and treated with anti-CD3, CD28, IL-2 and TGFβ for 24 hours alone, to induce a FOXP3 phenotype; MSU42011 was then added, and treatment maintained for 4 additional days. Cells were collected and RT-PCR was used to determine the levels of FOXP3.
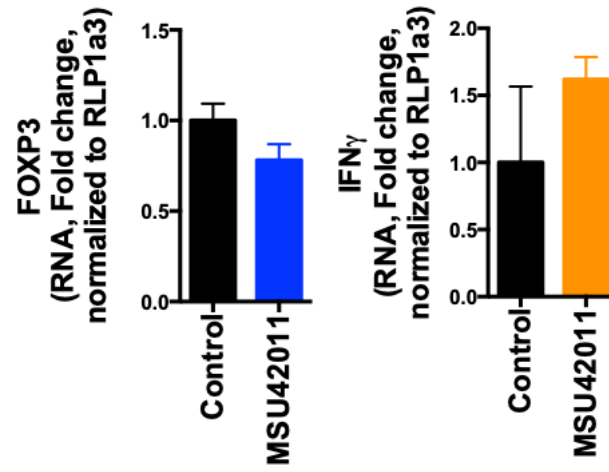
Figure S5. Evaluation of FOXP3 and increase of IFNγ mRNA in lungs of A/J mice treated with MSU42011. RT-PCR analysis of whole lung lysates of A/J mice treated with control or MSU42011. FOXP3 and IFNγ were determined by qPCR and RLP13a was used as control, *n* = 5/group.
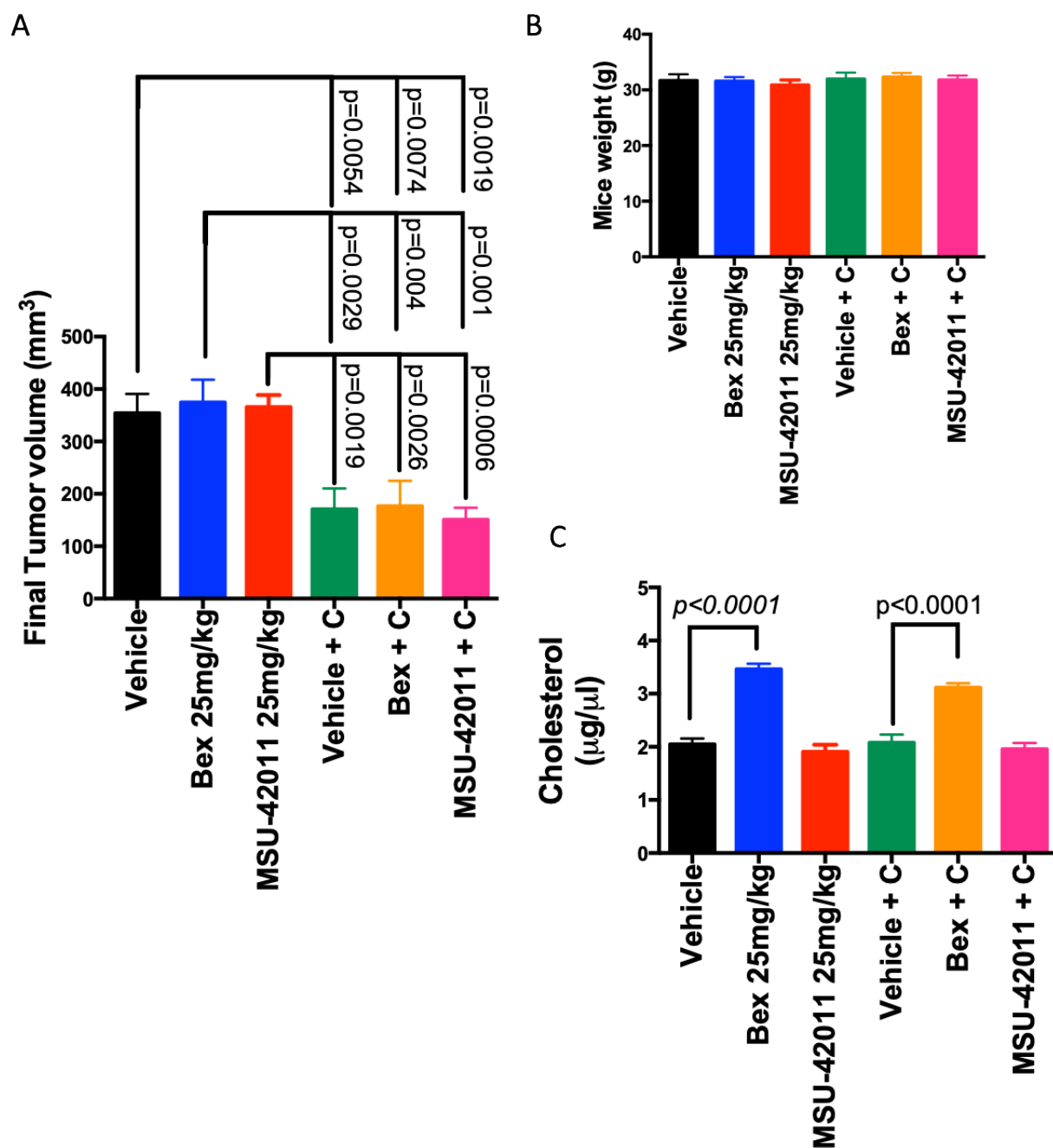
Figure S6. Nude mice were injected s.c. with human A549 lung cancer cells. Once a tumor reached 4 mm in diameter, mice were treated as shown and described in Fig 2. (A) Final tumor volumes, (B) average mouse weight, and (C) cholesterol levels in the plasma at the time of necropsy.
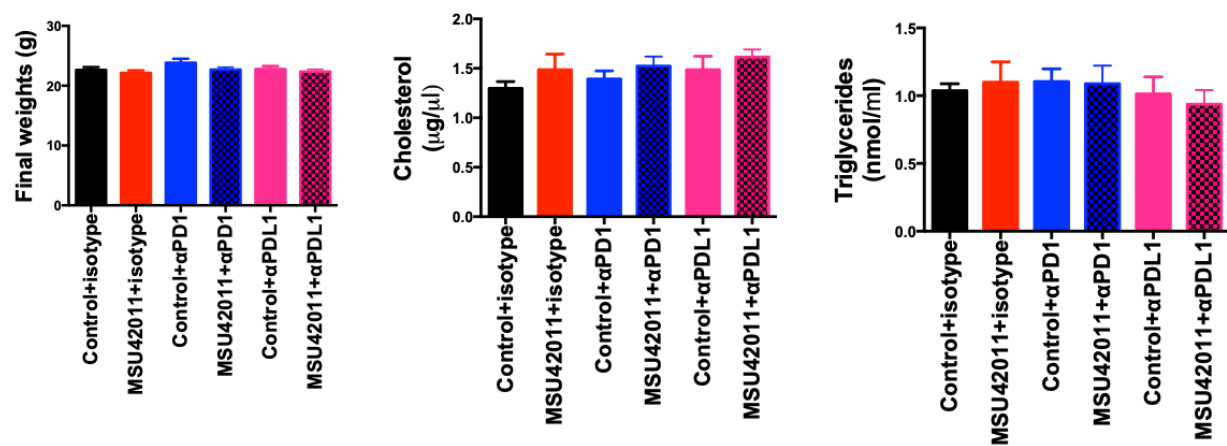
Figure S7. Final total body weight, levels of cholesterol and triglycerides in A/J mice treated with control, MSU42011, anti-PD1 or anti-PDL1 antibodies, or the combinations as described in Fig. 7. .
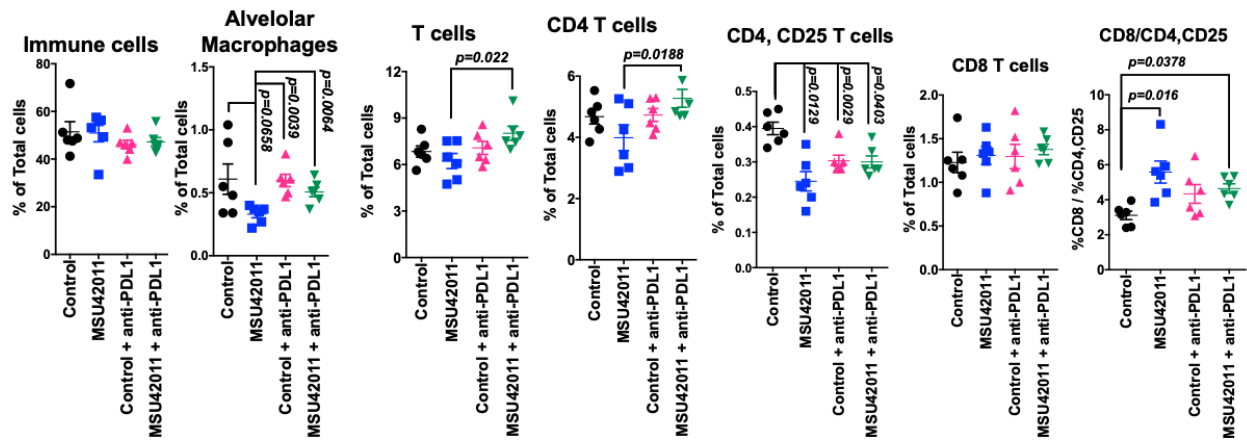
Figure S8. Flow cytometry analysis of selected groups of A/J mice treated as described in Fig.7.