ERK Inhibition Improves Anti-PD-L1 Immune Checkpoint Blockade in Preclinical Pancreatic Ductal Adenocarcinoma

SUPPLEMENTARY INFORMATION

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Running title: Imaging PD-L1 in PDAC

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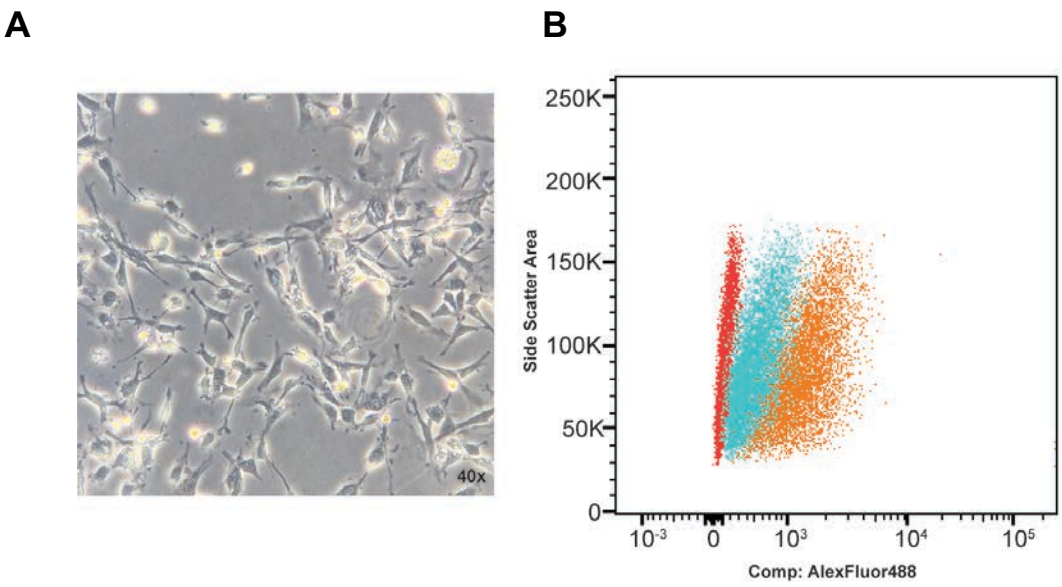
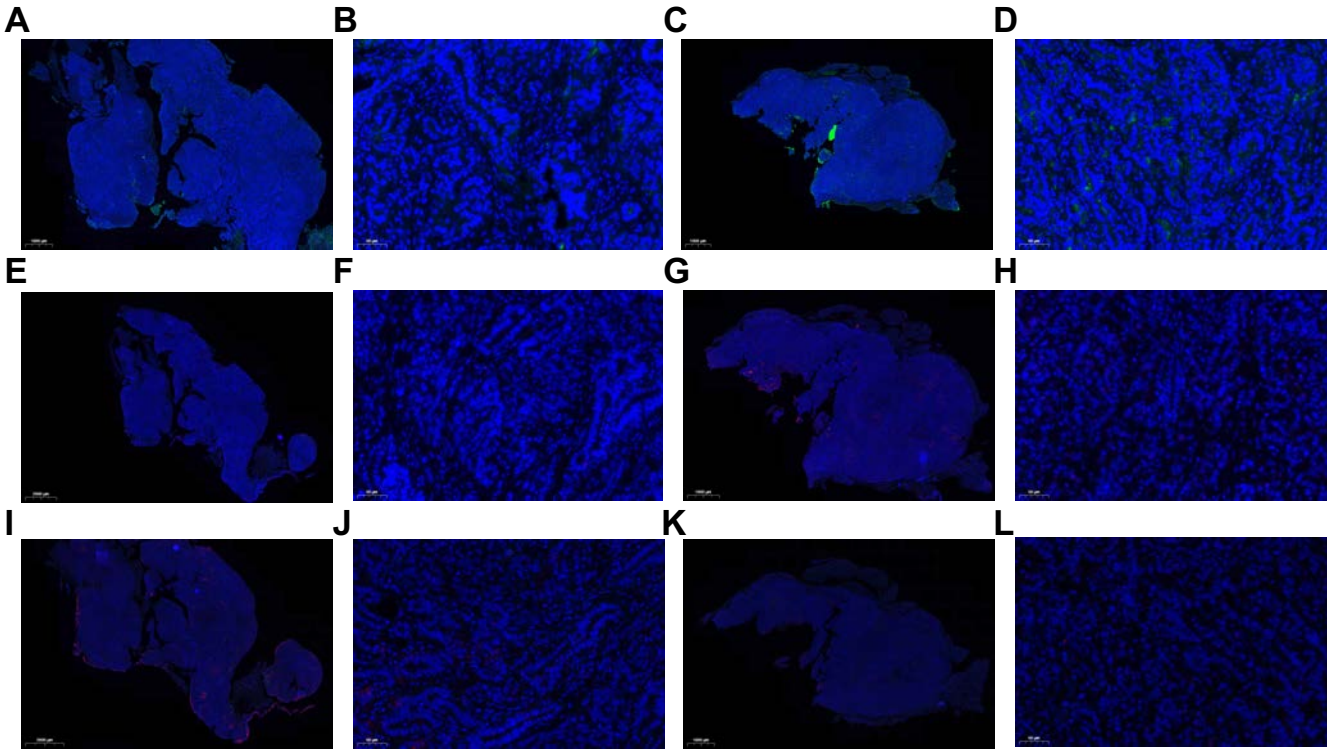
MATERIALS AND METHODS: PROTOCOLS

Immunofluorescence. The tissue sections were blocked first for 30 min in Background Blocking reagent (Innovex, catalog#: NB306). A rabbit polyclonal c-myc (Abcam cat# ab32072) antibody was used in 2 μg/mL concentration. The incubation with the primary antibody was done for 5 h, followed by 60 min incubation with biotinylated goat anti-rabbit IgG (Vector labs, cat#: PK6101) in 5.75 μg/mL. Blocker D, Streptavidin- HRP and TSA Alexa488 (Life Tech, cat# B40932) is used for 16 min. A goat anti-mouse PD-L1 antibody (goat polyclonal, R&D Systems, cat# AF1019) was used in 2 μg/ml concentration. The incubation with the primary antibody is done for 5 h followed by biotinylated rabbit anti-goat IgG {Vectastain ABC Kit (Rabbit IgG)} from Vector PK4005, 5.75 μg/mL. Blocker D, Streptavidin- HRP and TSA Alexa 594 (Life Tech, cat#B40957) is used for 16 min. All slides were counterstained in 5 μg/mL DAPI dihydrochloride (2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride), Sigma D9542, for 5 minutes at room temperature, mounted with anti-fade mounting medium Mowiol {Mowiol 4-88 (CALBIOCHEM code: 475904)} and coverslipped.

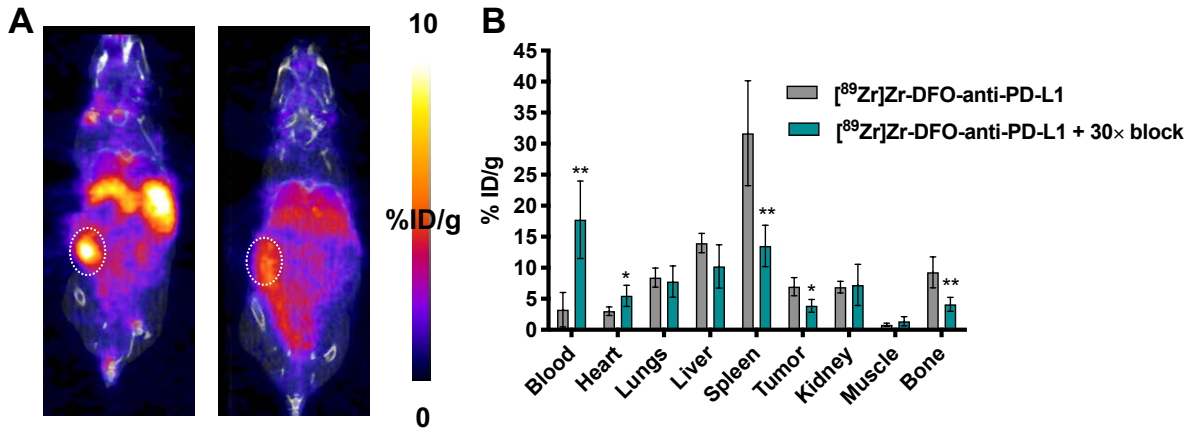
Immunohistochemistry. The chromogen was 3,3 diaminobenzidine tetrachloride (DAB) and sections were counterstained with hematoxylin. Whole-slide digital images were generated on a scanner (Pannoramic 250 Flash III, 3DHistech, 40x/0.95NA objective, Budapest, Hungary) at a resolution of 0.121 μm per pixel. Image analysis was performed with HALO software Area Quantification module v.1.0 (Indica Labs, Albuquerque, NM) and the Cytonuclear Quantification module 2.1.3. The region of interest was manually defined as viable tumor tissue, excluding necrotic tumor tissue and adjacent non-tumor tissues. ROI selection, area quantification algorithm optimization, OD threshold determination, and validation of the results were aided by an ACVP board-certified veterinary pathologist (AM).



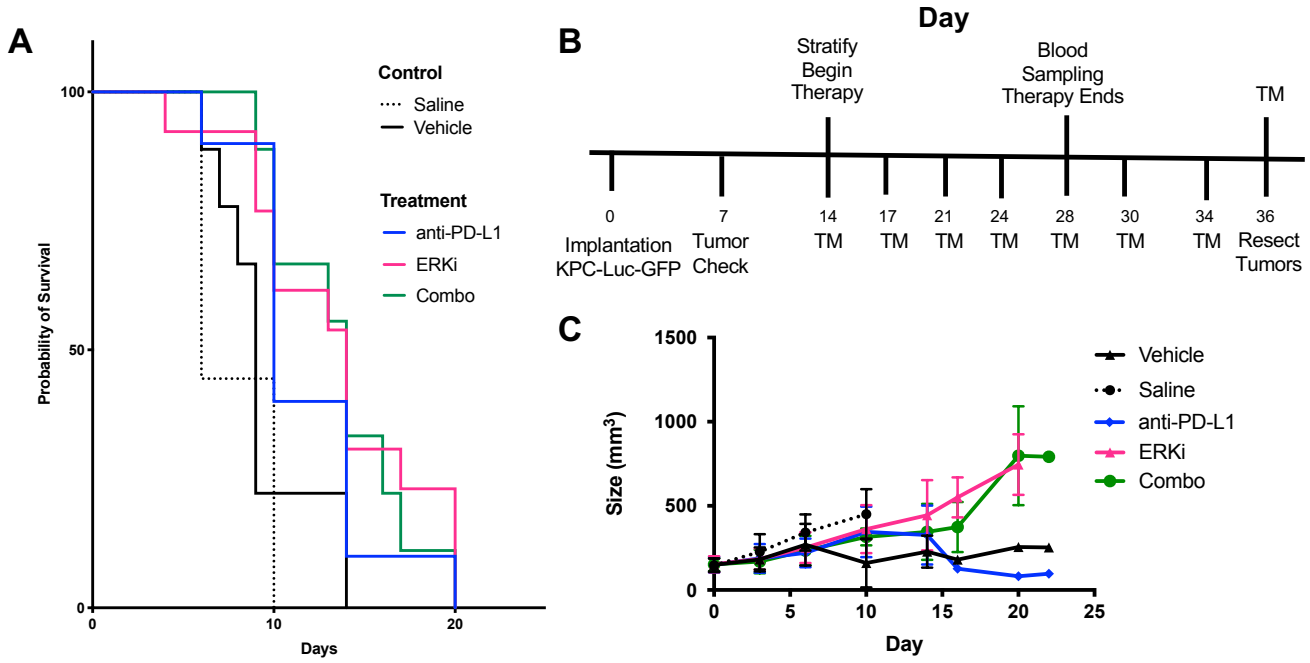
Supplementary Figure 1. Fibroblast isolation from orthotopic PDAC tumor microenvironment. **A**. Primary cancer-associated fibroblasts (CAFs) isolated from orthotopically implanted KPC tumors. **B**. Characterization of CAFs shows positive staining with alpha-smooth muscle actin (orange) via flow cytometry. Blue population represents the isotype control, and red cells are the negative population.



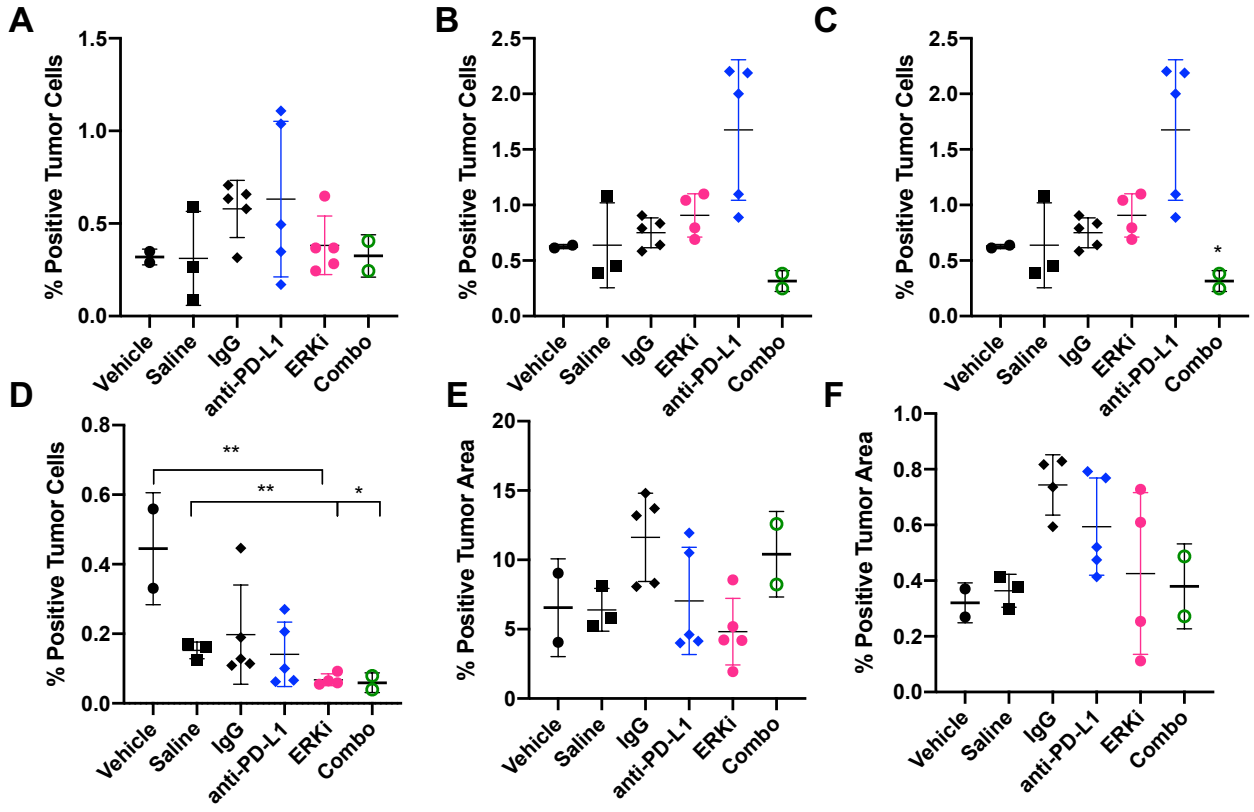
Supplementary Figure 2. Controls for immunofluorescence experiments to confirm no cross-reactivity or non-specific binding, and to identify regions to exclude via secondary entrapment. A. Goat anti-PD-L1 (single) with rabbit secondary (AlexaFluor488) at 1x and 20x (**B**). **C**. Goat IgG with rabbit secondary (AlexFluor 488) at 1× and 20× (**D**). **E**. Rabbit anti-c-MYC (single) with goat secondary (AlexaFluor594) at 1× and 20× (**F**). G. Rabbit IgG with goat secondary (AlexFluor 594) at 1× and 20× (**H**). I. Rabbit anti-c-MYC (single) with goat secondary (AlexaFluor594) at 1× and 20× (**J**). **K**. Rabbit IgG with goat secondary (AlexFluor 594) at 1× and 20× (**L**).



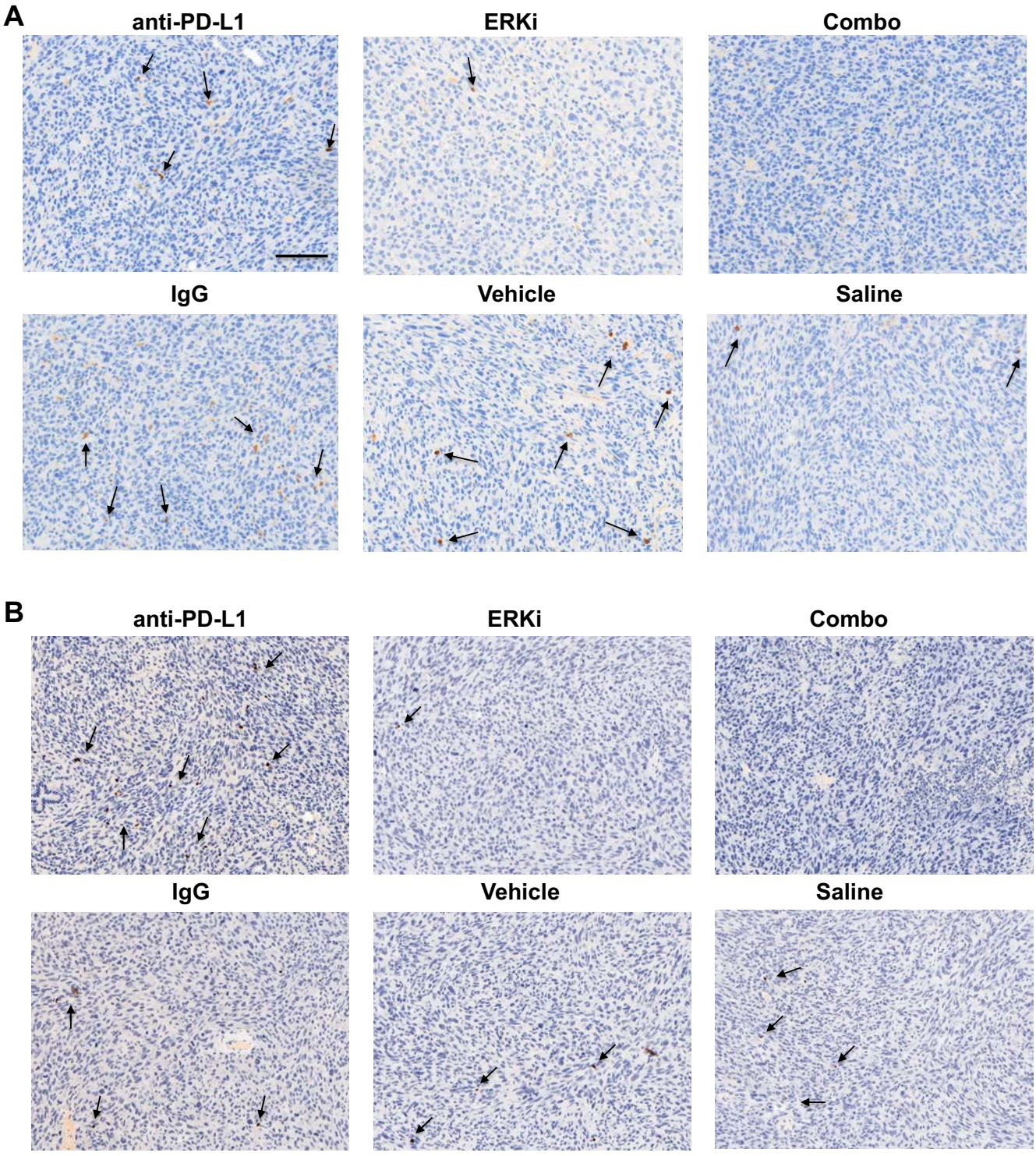
Supplementary Figure 3. Blocking study with [^{89}Zr]Zr-DFO-anti-PD-L1. Representative PET/CT of [^{89}Zr]Zr-DFO-anti-PD-L1 side-by-side with 30× blocking dose. There is a significant decrease in tumor uptake, along with a noticeable blocking effect in the spleen and bone, which can be attributed to non-target PD-L1 expression. The significant increase in the blood in the blocking cohort is also of note.



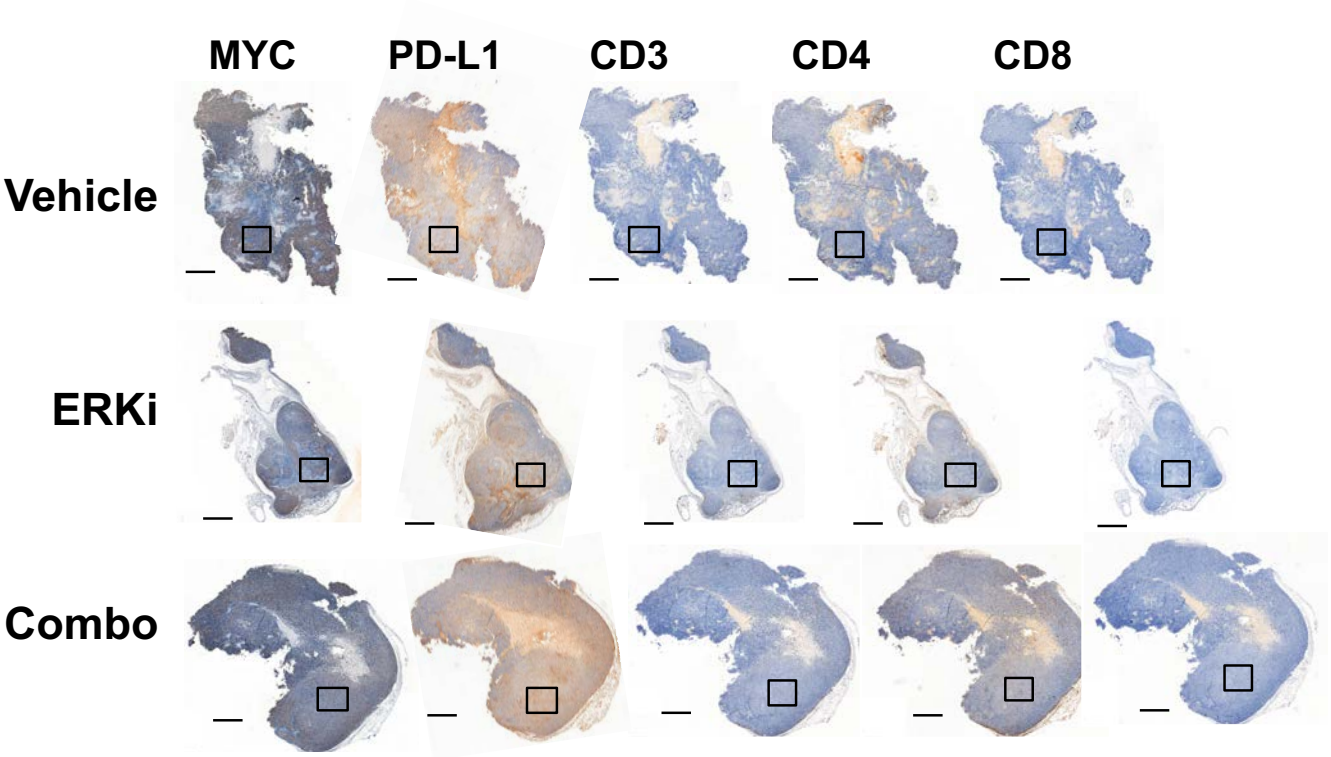
Supplementary Figure 4. Combination ERK inhibition and anti-PD-L1 does not improve survival in subcutaneous tumors. A. Survival curve of treatment arms over 20-day time period, drugging period was during the first two weeks. No significant survival observed between treatment and control groups. **B.** Timeline of tumor implantation and measurements. **C.** Tumor measurements until study endpoint. Abbreviations: TM: tumor measurements.



Supplementary Figure 5. Ex vivo analysis of endpoint tissues suggests immunosuppressive mechanism. A. Quantification of CD3 staining in % positive tumor cells using cytonuclear analysis. **B.** Quantification of CD4 staining in % positive tumor cells using cytonuclear analysis. The combination arm is significant compared to PD-L1, ERKi, and each control (Saline, Vehicle, and IgG). **C.** Quantification of CD8 staining in % positive tumor cells using cytonuclear analysis. **D.** Quantification of FoxP3 staining in % positive tumor cells using the cytonuclear analysis. Vehicle vs. ERKi is significant, Saline vs. ERKi and Saline vs. Combo also significant. **E.** Quantification of MYC staining using the % positive tumor area quantification module. **F.** Quantification of PD-L1 % positive tumor area quantification module. $P < 0.05$.



Supplementary Figure 6. **Immunohistochemical analysis of ex vivo tissues collected at endpoint indicate a potential immunosuppressive mechanism.** Representative images of serial sections from the same tumors stained for CD4 (**A**) and FoxP3 (**B**) with arrows indicating significant clusters of cells. There is no CD4 or FoxP3 staining in the combo arm in this view, along with trending decreases of these cells in each treatment arm compared to respective controls. Scale bar in top left panel indicates 50 μm and each subsequent slice is similar at 20×.



Supplementary Figure 7. **Immunohistochemical analysis of ex vivo tissues collected at single endpoint.** Representative images of serial sections from the same treated tumors stained for MYC, PD-L1, CD3, CD4, and CD8 at 1× magnification. Scale bars indicate 1 mm. Boxes indicate location of zoomed in (20× magnification, 50 μm scale bar) snapshots for Figure 6.

Supplementary Table 1. Immunohistochemistry details for TME analysis.

Antibody	Company	Cat No.	Primary Concentration
anti-CD3	abcam	ab135372	1 to 250
anti-CD8a	invitrogen	4SM15	1 to 1000
anti-CD4	invitrogen	4SM95	1 to 250
anti-MYC	abcam	ab32072	1 to 100
anti-PD-L1	R&D	AF1019	1 to 100
anti-FoxP3	invitrogen	14-5773-82	1 to 100