# Metformin Reduces PD-L1 on Tumor Cells and Enhances the Anti-tumor Immune Response Generated by Vaccine Immunotherapy

Luis E. Munoz[1], Lei Huang[1], Ramireddy Bommireddy[1], Richa Sharma[2], Lenore Monterroza[1], Rohini N. Guin[1], Sarah Samaranayake[1], Christopher D. Pack[3], Sampath Ramachandiran[3], Shaker JC. Reddy[3], Mala Shanmugam[2], and Periasamy Selvaraj[1*].

[1]Department of Pathology and Laboratory Medicine,

[2]Department of Hematology and Medical Oncology,

Emory University School of Medicine, Atlanta, GA USA.

[3]Metaclipse Therapeutics Corporation, Atlanta, GA USA.

***Address for Correspondence:**

Periasamy Selvaraj, PhD.

Emory University

Woodruff Memorial Building, Room 7309

101 Woodruff Circle

Atlanta, GA 30322

Phone: 404-727-5929

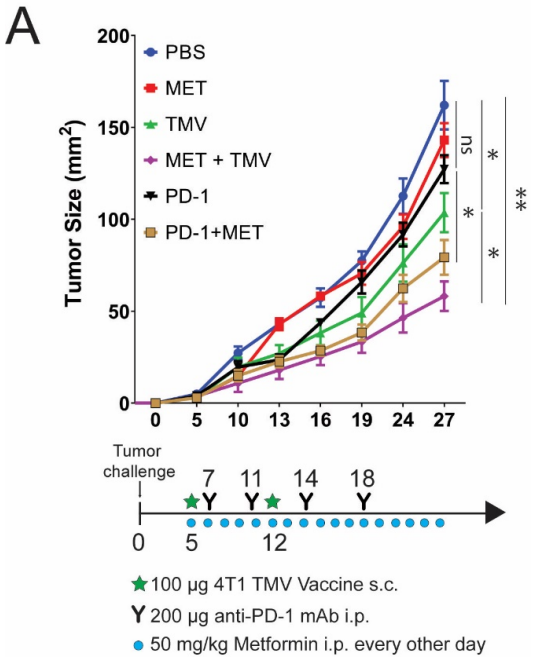
Fax: 404-727-5764

Email: pselvar@emory.edu

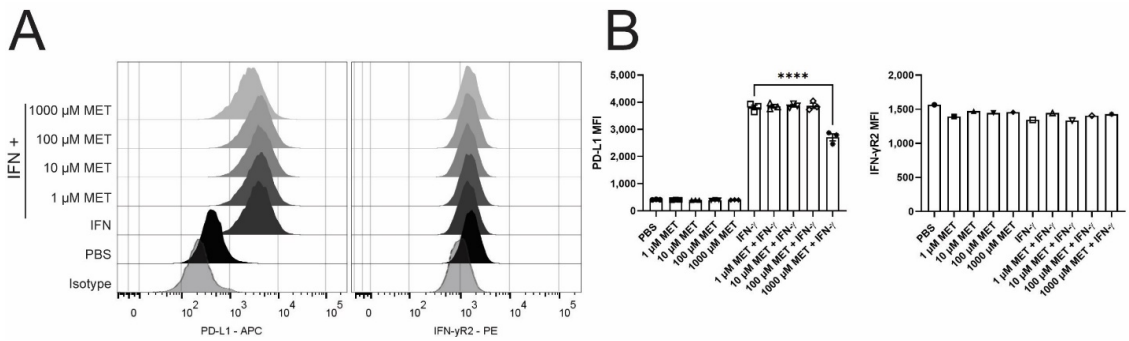


Munoz LE, *et al. J Immunother Cancer* 2022; 9:e002614. doi: 10.1136/jitc-2021-002614

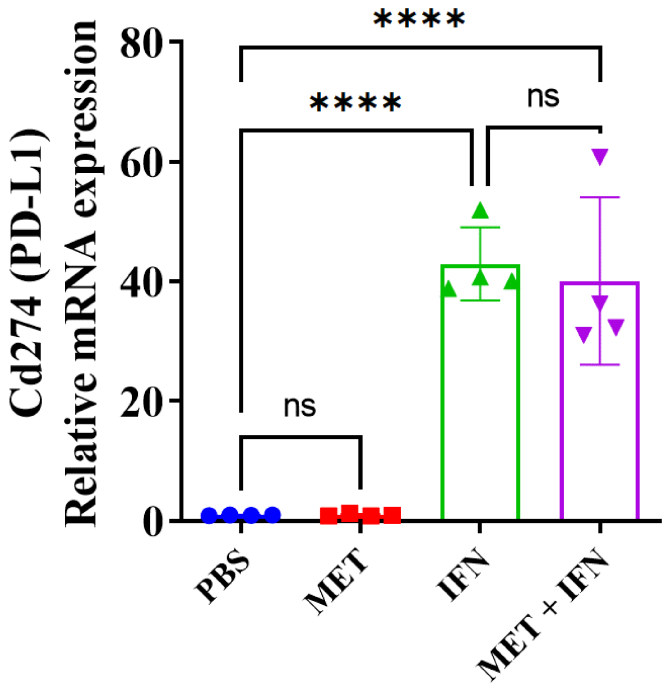
**SUPPLEMENTARY MATERIAL**



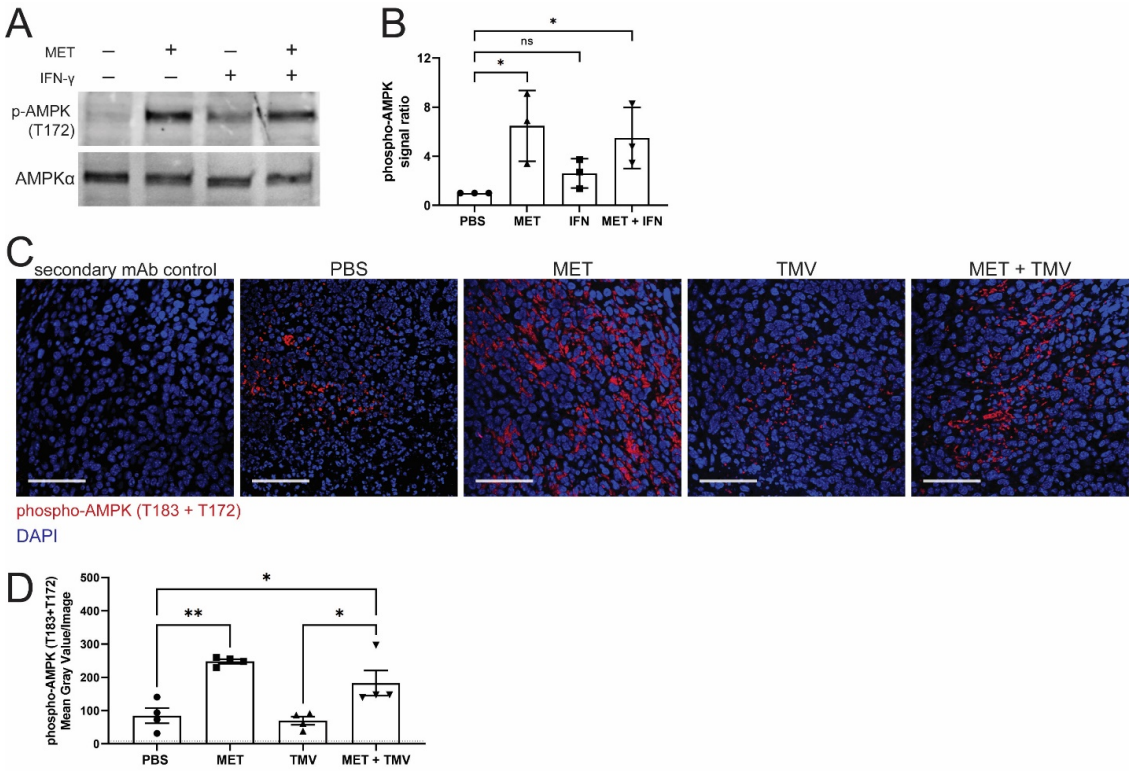

**Supplemental Figure S1. Metformin improves TMV vaccine and PD-1 blockade immunotherapy in anti-PD-1 mAb resistant 4T1 tumor model. A**, tumor growth in BALB/c mice inoculated with 4T1 cells, TMV vaccine, metformin, and with anti-PD-1 mAb (Clone RMP1-14) as indicated in the treatment outline (n=5 mice per group, mean ± SEM). * $p<0.05$, ** $p<0.01$, ns $p>0.05$. A and B analyzed by two-way ANOVA with Tukey's post test for multiple comparisons.




**Supplemental Figure S2. Effect of varying levels of Metformin on IFN-γ-induced PD-L1 expression.** 4T1 cells were cultured in media treated with PBS as a control, 20 ng/mL of IFN-γ, and metformin at concentrations ranging from 1 to 1000 µM and surface expression of PD-L1 (n=3) and IFN-γR2 (n=1) was determined by flow cytometry. **A**, representative histograms of PD-L1 and IFN-γR2 after treatment. **B**, MFI quantifications from A (mean ± SD). Analysis in B done by a one-way ANOVA with Tukey's post test for multiple comparisons. **** $p<0.001$.




**Supplemental Figure S3. PD-L1 mRNA induction by IFN-γ is not reduced by metformin.** RT-qPCR analysis of PD-L1 mRNA levels in 4T1 cells treated with IFN-γ and metformin or both. Data represent Log2 mRNA expression (mean ± SD) normalized to TBP expression from three independent biological repeats (n=3 repeats 5 samples per group). Analysis in done by a one-way ANOVA with Tukey's post test for multiple comparisons. **** $p<0.001$, ns $p>0.05$.




**Supplemental Figure S4. Metformin induces AMPK phosphorylation of 4T1 cancer cells *in vitro* and within 4T1 tumors *in vivo*.** **A**, western blot of phospho-AMPK (T172) and overall AMPK-α in 4T1 cells after IFN-γ and metformin treatment *in vitro*. **B**, quantification of phospho-AMPK signal from 3 independent blots normalized to PBS control (mean ± SD). **C**, FFPE sections from 4T1 tumors in Figure 1A were stained for DAPI and phospho-AMPK (T183+T172), representative images are shown, scale bar represents 100µm, secondary mAb control done on PBS group. **D**, quantification of the mean gray value of phospho-AMPK signal per image from C (n=4, mean ± SEM). Analysis in B done by one-way ANOVA with Holm's post test for multiple comparisons. Analysis in D done by one-way ANOVA with Tukey's post test for multiple comparisons.* $p<0.05$, ** $p<0.01$, ns $p>0.05$.