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

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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-yR2 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 \textit{in vitro} and within 4T1 tumors \textit{in vivo}. \textbf{A}, western blot of phospho-AMPK (T172) and overall AMPK-\(\alpha\) in 4T1 cells after IFN-\(\gamma\) and metformin treatment \textit{in vitro}. \textbf{B}, quantification of phospho-AMPK signal from 3 independent blots normalized to PBS control (mean ± SD). \textbf{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 100\(\mu\)m, secondary mAb control done on PBS group. \textbf{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 \(p>0.05\).