

Supplementary Materials for

Selective Effects of PD-1 on Akt and Ras Pathways Regulate Molecular Components of the Cell Cycle and Inhibit T Cell Proliferation

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Fig. S4. PD-1 signaling inhibits activation of Akt and phosphorylation of GSK-3.

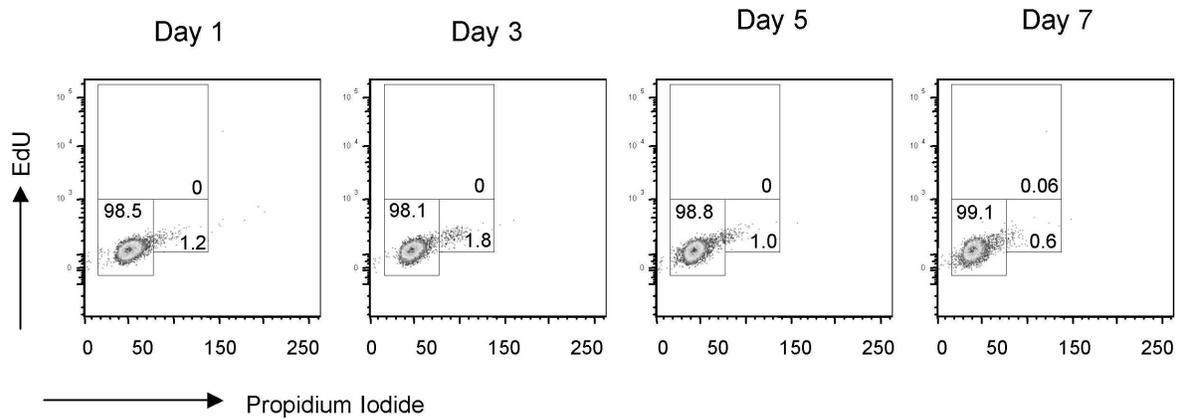


Fig. S1. Effects of PD-1 signaling on cell cycle progression. In parallel with cultures of CD4⁺ T cells with tosylactivated magnetic beads conjugated with activating antibodies, CD4⁺ T cells were cultured in media alone and were analyzed for cell cycle distribution at the indicated times. The percentages of cells in G0/G1 (lower left region), S-phase (upper region), and G2/M (lower right region) were identified by incubation with EdU followed by Alexa Fluor 647-labeled azide. The cells were also stained with propidium iodide and analyzed by flow cytometry. The data are representative of three experiments.

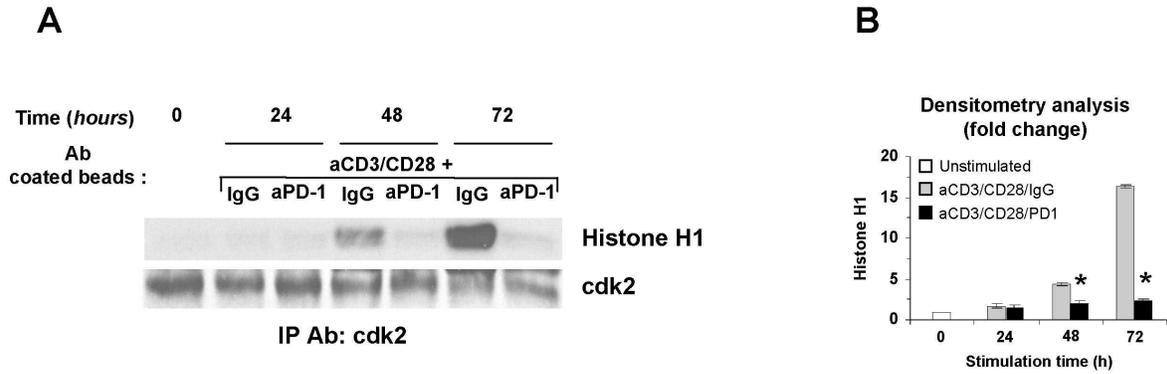


Fig. S2. PD-1 inhibits the kinase activity of Cdk2. **(A)** CD4⁺ T cells were activated through CD3 and CD28 with or without stimulation through PD-1 for the indicated times. Cell lysates were prepared, immunoprecipitations were performed with Cdk2-specific antibody conjugated to agarose beads, and kinase activity was examined by in vitro kinase reaction with histone H1 as the exogenous substrate (top panel). Western blotting analysis with a Cdk2-specific antibody confirmed the presence of comparable amounts of Cdk2 protein in the samples (bottom panel). **(B)** Bar graph shows densitometric analysis of changes in H1 phosphorylation normalized to Cdk2 abundance per sample and are expressed as the fold-change relative to mean values before stimulation (defined as 1). * $P < 0.05$; n = 3 experiments.

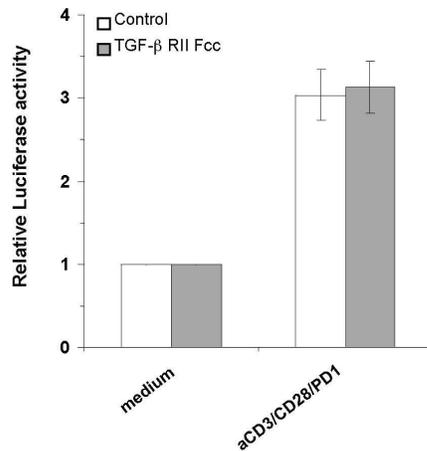
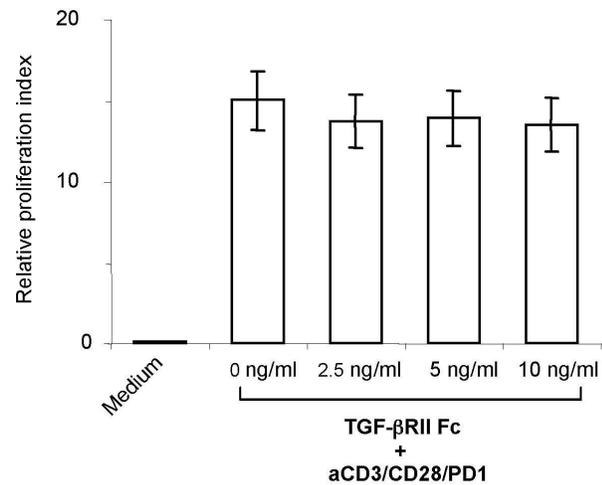
A**B**

Fig. S3. Neutralization of TGF- β does not reverse the effect of PD-1 signaling on the transcriptional activation of Smad3 or the antiproliferative function of PD-1. **(A)** Primary human CD4⁺ T cells were transfected with a Smad-dependent reporter plasmid and were subsequently cultured in medium alone or were stimulated for 24 hours with tosylactivated magnetic beads conjugated with mAbs against CD3, CD28, and PD-1 in medium alone or in the presence of the TGF β RII-Fc chimera (10 ng/ml), which neutralizes the effects of TGF- β . The transcriptional activity of Smad3 was then examined by luciferase assay. ($P > 0.05$, $n = 3$ experiments). **(B)** Primary human CD4⁺ T cells were cultured with tosylactivated magnetic beads conjugated with mAbs against CD3, CD28, and PD-1 in medium alone or in the presence of various concentrations of TGF β RII-Fc chimera, and cellular proliferation was examined. ($P > 0.05$, $n = 3$ experiments).

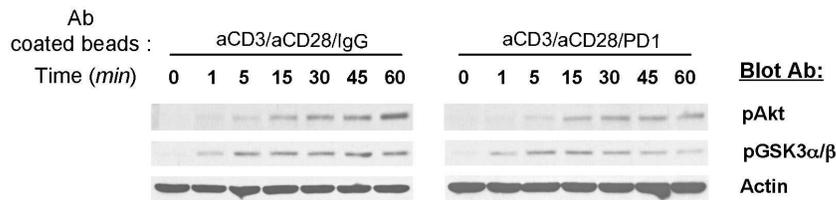
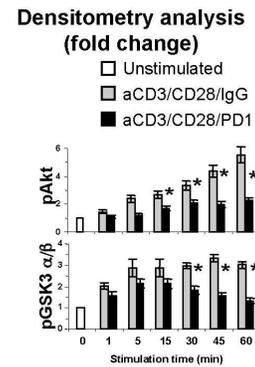
A**B**

Fig. S4. PD-1 signaling inhibits activation of Akt and phosphorylation of GSK-3. **(A)** CD4⁺ T cells isolated from PHA blasts were cultured activated through CD3 and CD28 with or without stimulation through PD-1 for the indicated times. Cell lysates were prepared, and the activation of Akt was examined by Western blotting with a phospho-specific antibody (pAkt). Phosphorylation of GSK-3 was examined by Western blotting with an antibody specific for pGSK-3 α/β (Ser^{21/9}). **(B)** Bar graphs show densitometric analysis of changes in the abundance of phosphorylated substrates normalized to that of β -actin to control for equal loading and expressed as the fold-change relative to mean values before stimulation (defined as 1). * $P < 0.05$; $n = 3$ experiments.