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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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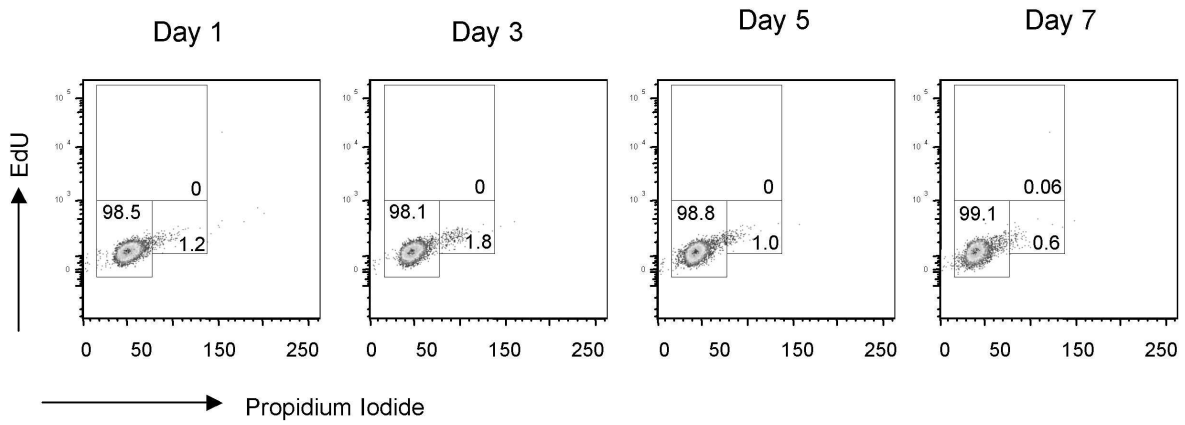

Fig. S1. Effects of PD-1 signaling on cell cycle progression. In parallel with cultures of $CD4^+$ T cells with tosylactivated magnetic beads conjugated with activating antibodies, $CD4^+$ T cells were cultured in media alone and were analyzed for cell cycle distribution at the indicated times. The percentages 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.

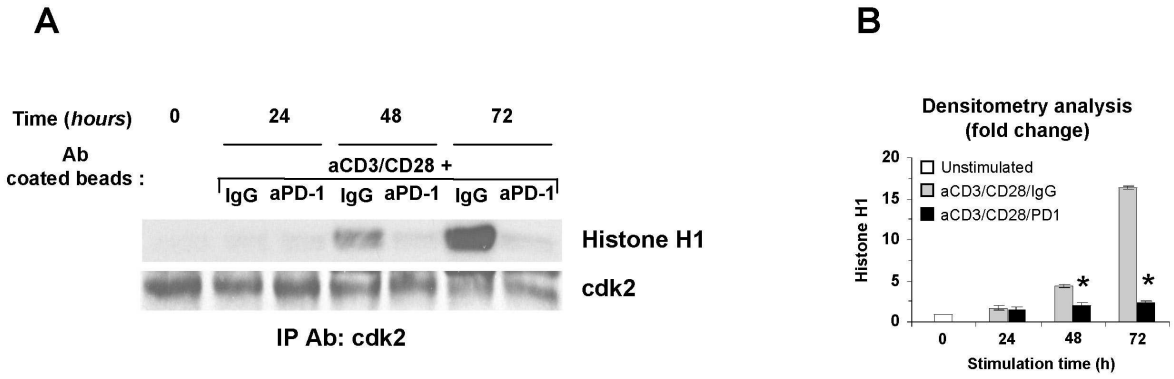

Fig. S2. PD-1 inhibits the kinase activity of Cdk2. (**A**) $CD4^+$ T cells were activated through CD3 and CD28 with or without stimulation through PD-1 for the indicated times. Cell lysates were prepared, immunoprecipitations were performed with Cdk2-specific antibody conjugated to agarose beads, and 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). *$\underline{P}$ < 0.05; n = 3 experiments.

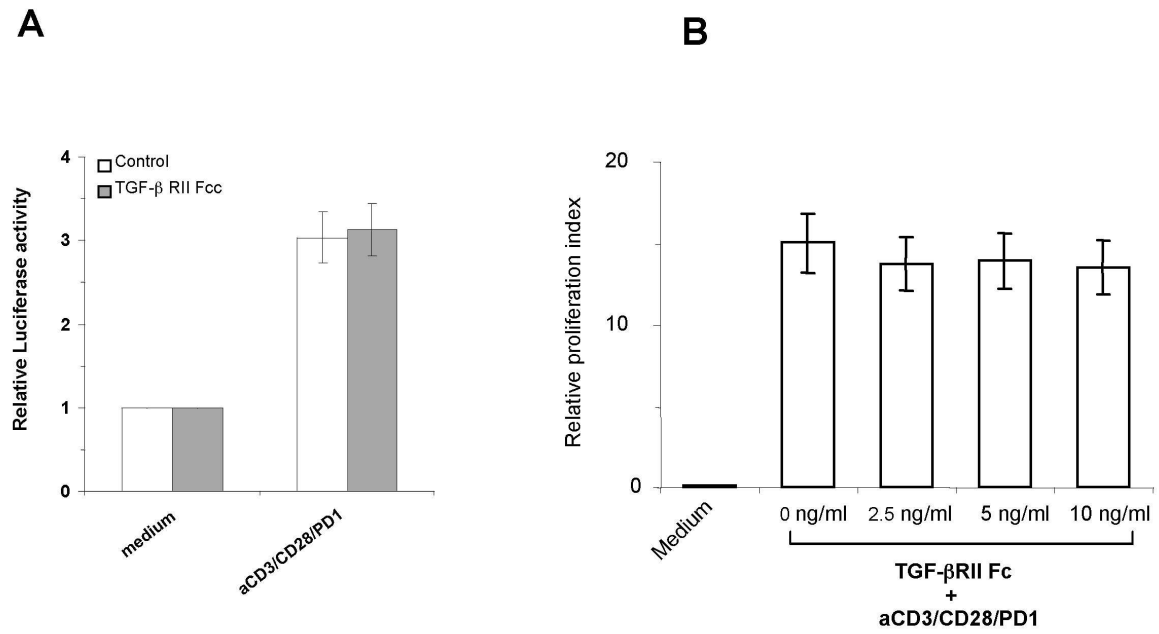

Fig. S3. Neutralization of TGF-β does not reverse the effect of PD-1 signaling on the transcriptional activation of Smad3 or the antiproliferative function of PD-1. (**A**) Primary human $CD4^+$ T cells were transfected with a Smad-dependent reporter plasmid and were subsequently cultured in medium alone 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 TGFβRII-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 TGFβRII-Fc chimera, and cellular proliferation was examined. ($P > 0.05$, n = 3 experiments).

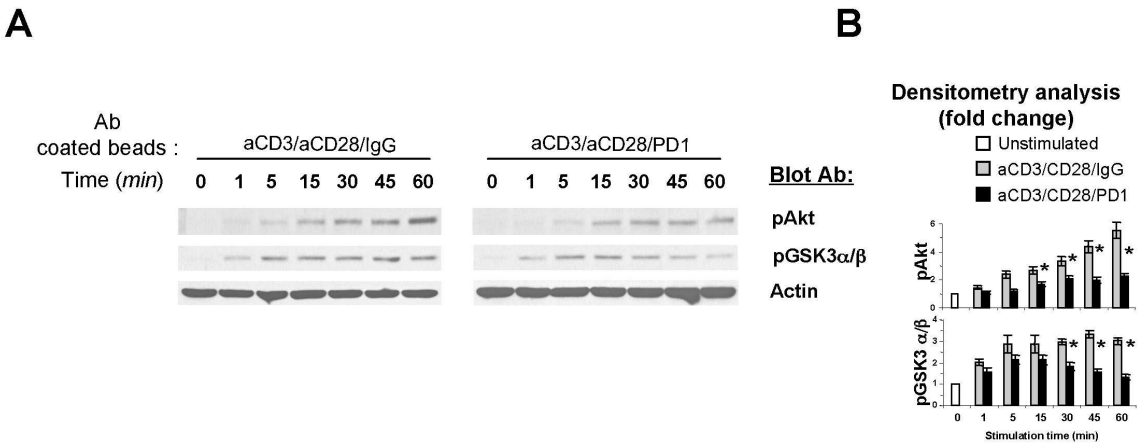

Fig. S4. PD-1 signaling inhibits activation of Akt and phosphorylation of GSK-3. (**A**) $CD4^+$ T cells isolated from PHA blasts were cultured activated through CD3 and CD28 with or without stimulation through PD-1 for the indicated times. Cell lysates were prepared, and the activation of Akt was examined by Western blotting with a phospho-specific antibody (pAkt). Phosphorylation of GSK-3 was examined by Western blotting with an antibody specific for pGSK-3α/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.