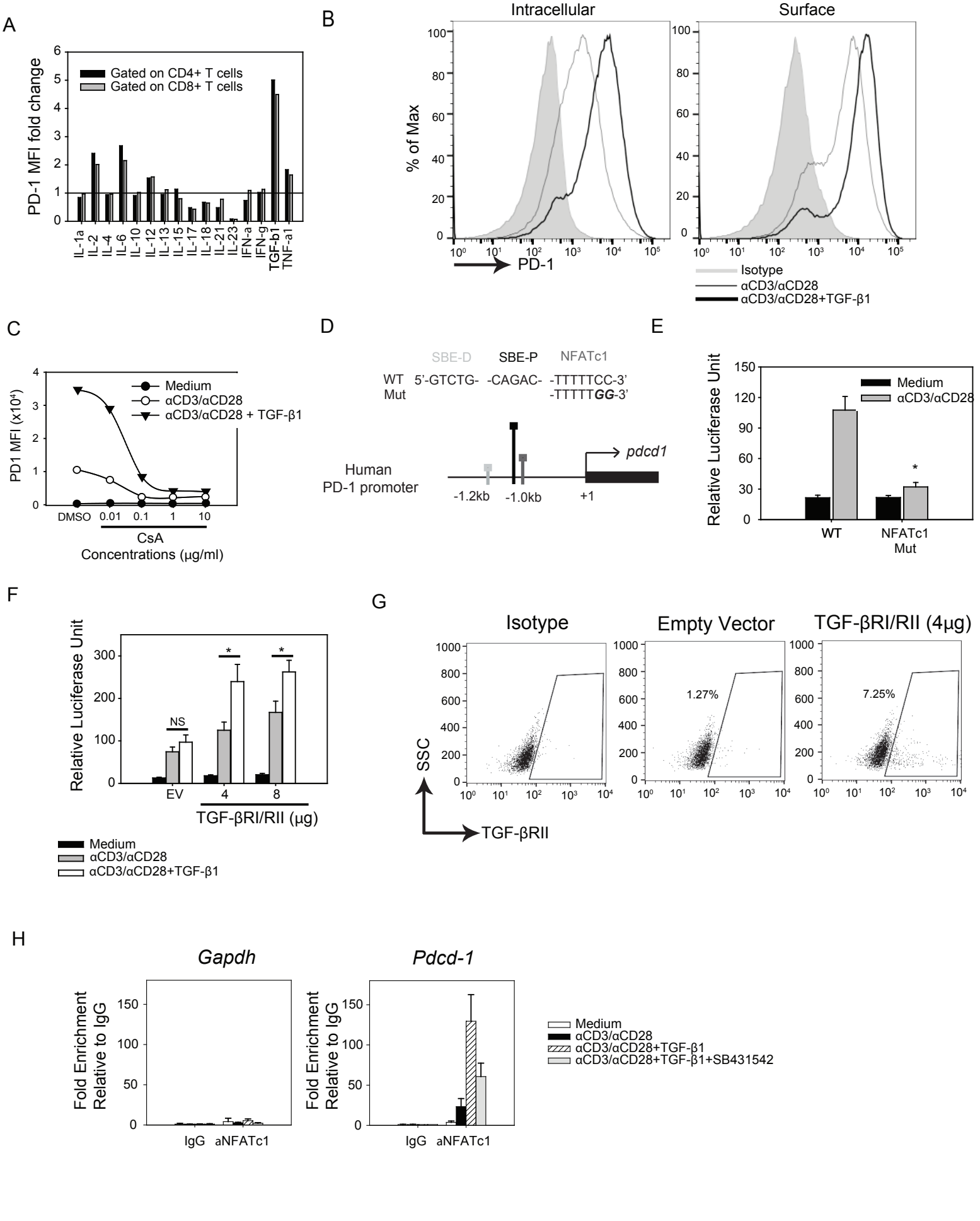
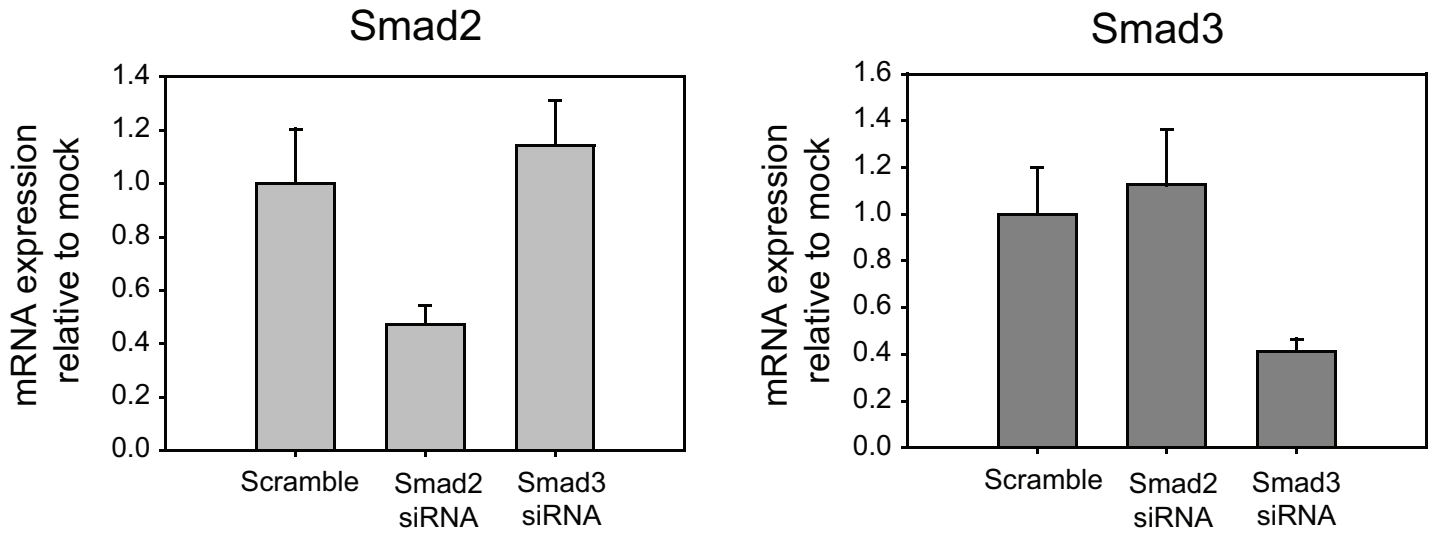
Supplemental Figure 1

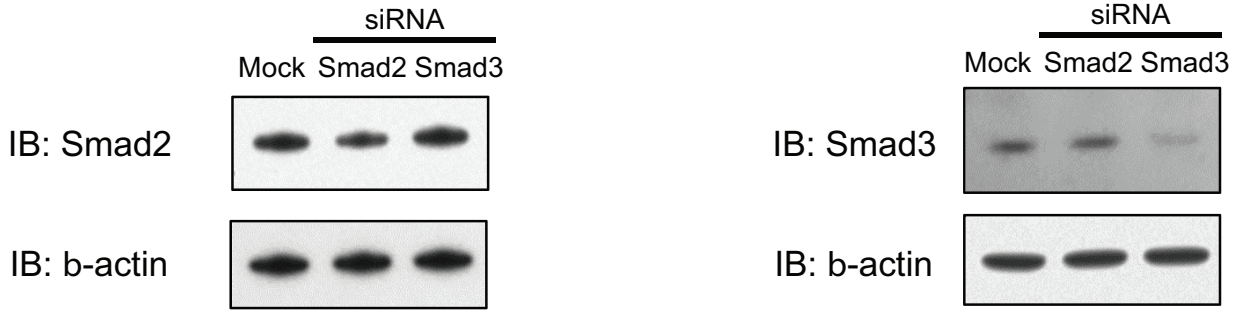


Supplemental Figure 2

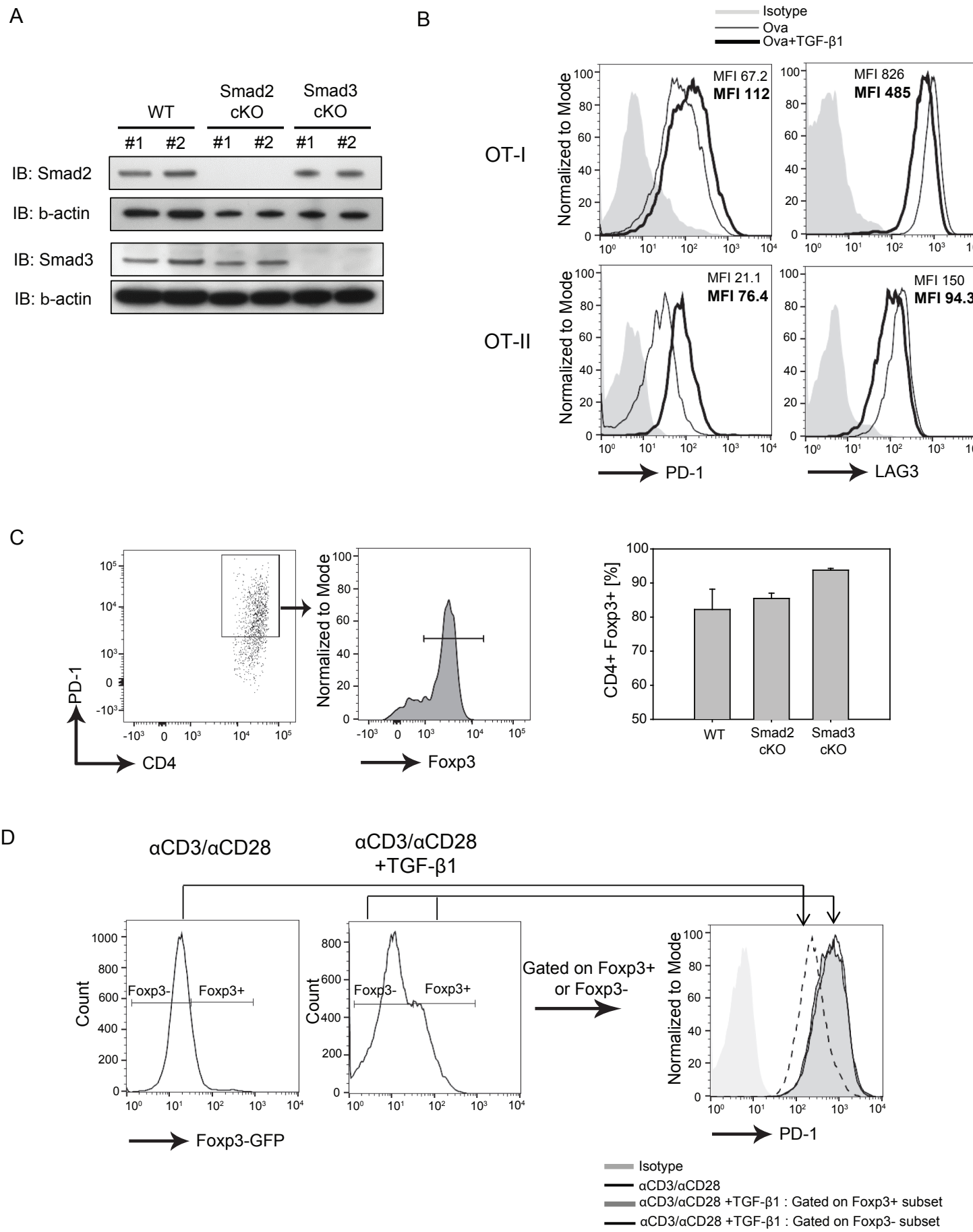
A



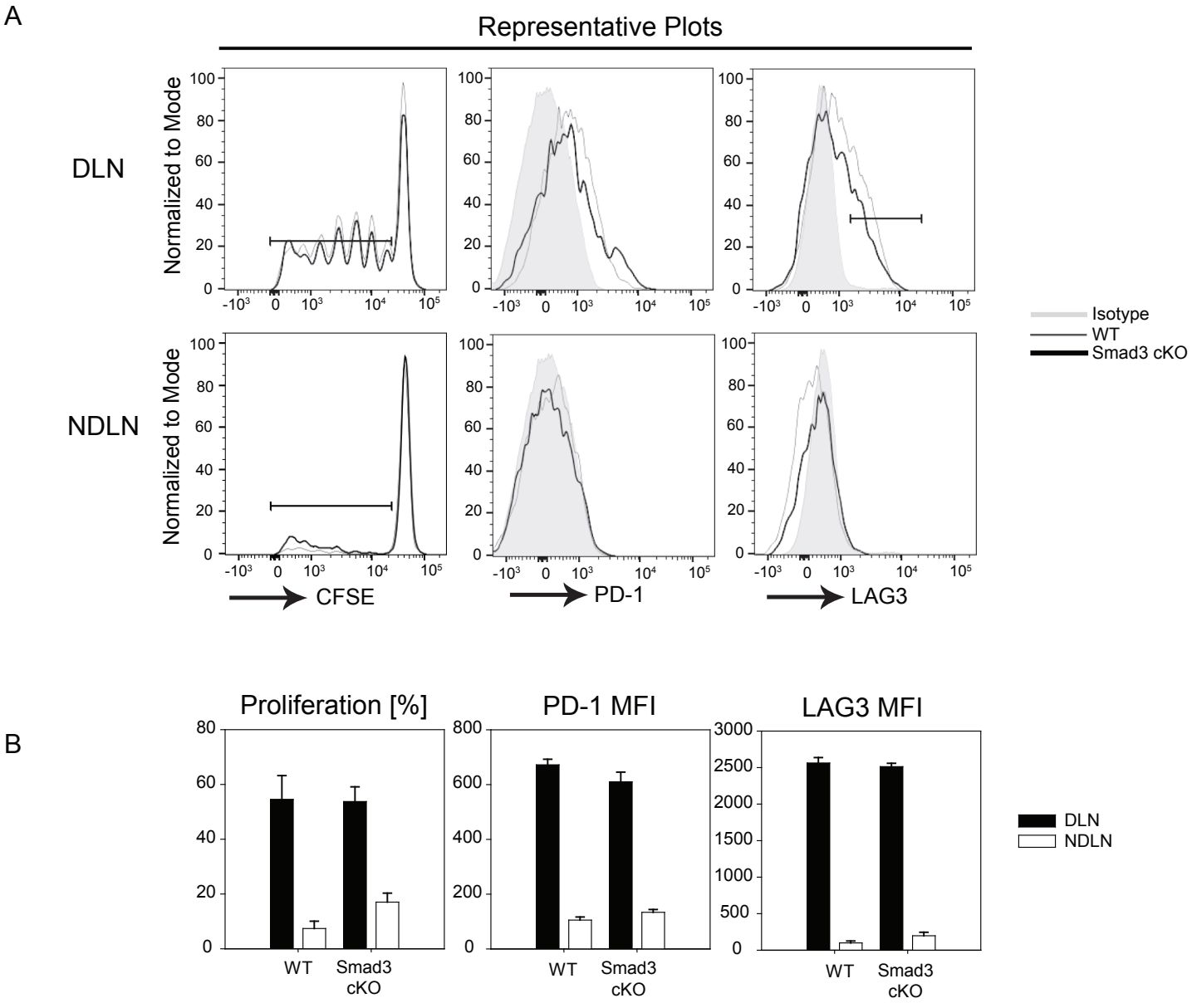
B

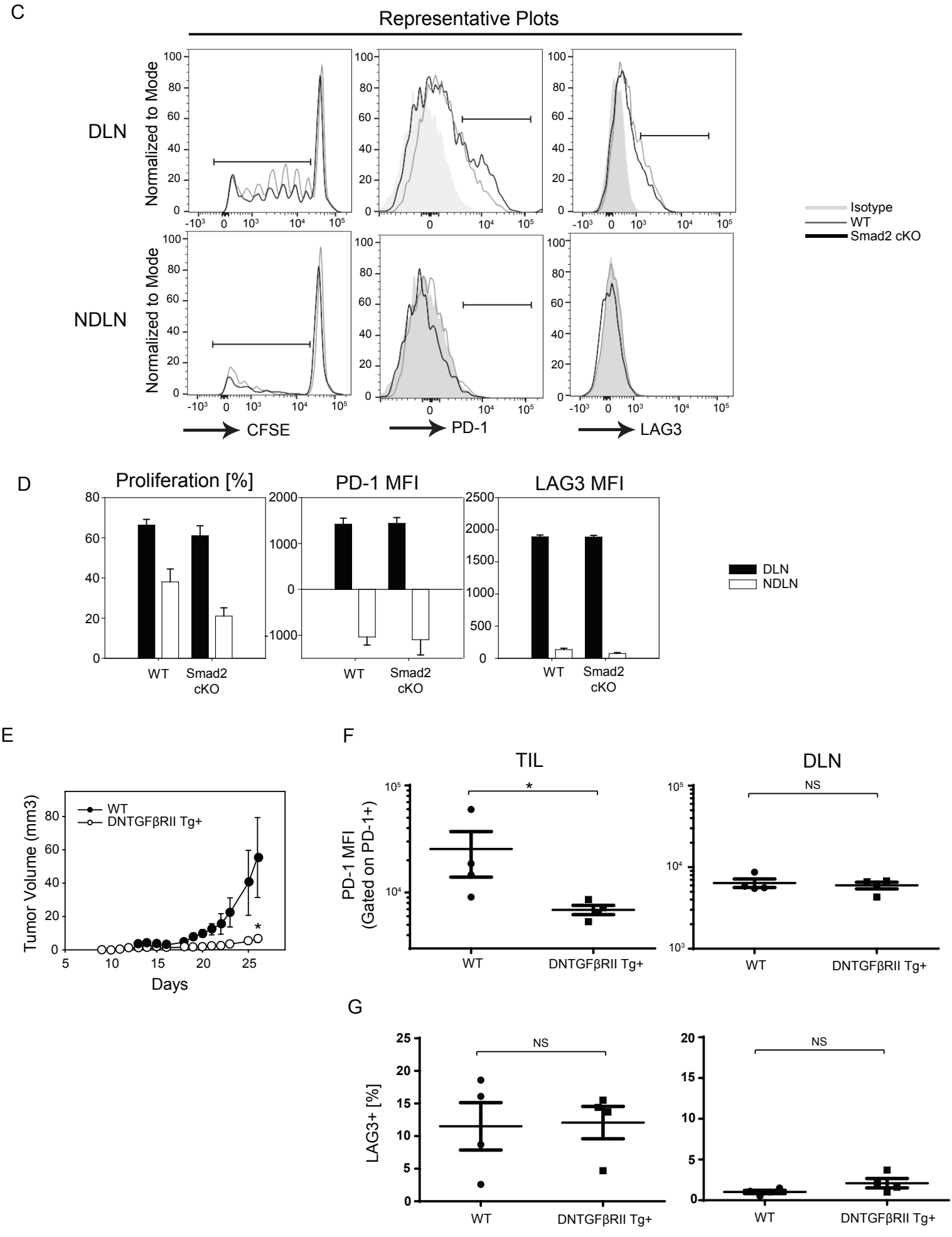


Supplemental Figure 3



Supplemental Figure 4




C
Representative Plots
DLN
NDLN
Normalized to Mode
CFSE
PD-1
LAG3
Isotype
WT
Smad2 cKO
D
Proliferation [%]
PD-1 MFI
LAG3 MFI
WT
Smad2 cKO
DLN
NDLN
E
Tumor Volume (mm3)
Days
WT
DNTGFβRII Tg+
F
TIL
DLN
PD-1 MFI (Gated on PD-1+)
NS
G
LAG3+ [%]
NS

Supplementary Information

Supplemental Figure 1. (a) The effect of various cytokines on PD-1 expression is shown as fold changes in MFI relative to the αCD3/αCD28 condition with no cytokines in both CD4+ (black bars) and CD8+ (white bars) T cells. CD3+ T cells were enriched using magnetic isolation kits from the peripheral blood of healthy donors. The cells were activated with αCD3/αCD28-conjugated beads in the presence of an individual cytokine (data from 500ng/ml is shown) with a cell to bead ratio of 1:1. In some experiments, a 1:3 cell to bead ratio was used with no changes in the relative effect of cytokines observed. After 72 hr, the cells were harvested and CD3+ CD4+ or CD3+ CD8+ were gated in order to assess respective PD-1 surface expression via flow-cytometry. (b) TGF-β induced proportionate increases in intracellular and surface PD-1 in the presence of αCD3/αCD28 stimulation as described in (a). (c) Human peripheral CD3+ T cells were isolated and activated with αCD3/αCD28-conjugated beads in the absence or presence of TGF-β1 for 72 hr. Cyclosporine A (CsA) was added after 24 hr of activation at varying concentrations and PD-1 expression was assessed by flow-cytometry: medium alone (filled circles); αCD3/αCD28 (open circles); αCD3/αCD28+TGF-β1 (filled triangles). The result is representative of two independent trials. (d) A putative NFATc1 binding site relative to Smad-binding elements (SBE) on a human *Pdcd-1* promoter region. (e) Jurkat T cells were transfected with a luciferase vector containing wild-type (WT) or mutant (Mut) NFATc1 site of the human *Pdcd-1* promoter as described in the Method section, and luciferase activity was measured after activation with αCD3/αCD28. The result is shown as the mean +/- SEM of technical replicates and is representative of at least two independent trials. (f) Jurkat T cells were transfected with a 1.9 kb long human *Pdcd-1* promoter-driven luciferase vector together with different amounts of TGF-βRI and RII expression plasmids. Subsequently, the cells were activated with αCD3/αCD28 with (white bars) or without TGF-β1 (grey bars) and luciferase activity was measured. The result is shown as mean +/- SEM of technical replicates and representative of at least two independent trials. (g) Transfection efficiency of TGF-βRI and RII expression plasmids on Jurkat T

cells by flow-cytometry, as shown in SSC (Y-axis) and TGF-βRII (X-axis) (right). EV: empty vector. The result is representative of two independent trials. (g) Chromatin immunoprecipitation analysis of NFATc1 on the human *Pdcd-1* promoter. Human peripheral CD3+ T cells were isolated and activated with αCD3/αCD28-conjugated beads in the absence (black bars) or presence (hatched bars) of TGF-β1 for 24 hr and the ChIP assay was performed as described in the Method section. The degree of enrichment is shown as fold-change (Y-axis) relative to non-specific binding by an isotype control in a human *Gapdh* or *Pdcd-1* promoter region. The result is shown as the mean +/- SEM of technical replicates and representative of at least two independent trials.

Supplemental Figure 2. (a) qPCR analysis of *Smad2* and *Smad3* mRNA levels in Jurkat T cells. Jurkat T cells were transfected with scramble, *Smad2* and *Smad3* siRNA as described in the Method section. After resting overnight, the cells were harvested and cellular RNA was isolated in order to assess *Smad2* and *Smad3* transcript levels. The result is shown as mean +/- SEM of technical replicates and representative of at least two independent trials. (b) siRNA transfected Jurkat T cells were harvested and lysed for western blot analysis of Smad2 and Smad3.

Supplemental Figure 3. (a) Western blot analysis of *Cre*-mediated gene knock-out in *Smad2* and *Smad3* cKO CD4+ T cells. Naïve CD4+ T cells (CD4+ CD25- CD62L+) were flow-sorted from WT, *Smad2* and *Smad3* cKO mice and were activated with αCD3/αCD28 for 72 hr. The cells were harvested and lysed for western blot analysis of Smad2 and Smad3 expression as described in the method section. Numbers represent biological replicates of each group. (b) Ovalbumin-specific CD8+ (OT-I) (top) and CD4+ (OT-II) (bottom) T cells were enriched by magnetic isolation from the spleen and activated for 72 hr with Type1 Ovalbumin (Ova) and Type II Ova (10 μg/ml) in the presence of irradiated splenocytes under different conditions. PD-1 (left) and LAG3 (right) expressions are shown in representative histograms: isotype (shaded histogram), peptide alone (thin histogram), peptide with

TGF-β1 (50 ng/ml) (bold histogram). (c) Foxp3 expression in CD4+ PD-1+ T cells infiltrating the tumor microenvironment in WT, *Smad2* cKO and *Smad3* cKO mice. A representative Foxp3 expression histogram is shown (left) and percentage of Foxp3 among PD-1+ CD4+ T cells in each group is shown as mean +/- SEM. (d) CD4+ T cells were magnetically isolated from Foxp3-GFP transgenic mice, and were activated with αCD3/αCD28 for 72 hr with or without TGF-β1. PD-1 expression was separately assessed on GFP+ and GFP- subsets as shown in representative histograms: isotype (light shade); αCD3/αCD28 (dashed line); GFP+ subset from αCD3/αCD28+TGF-β1 (dark shade); GFP- subset from αCD3/αCD28+TGF-β1 (black line).

Supplemental Figure 4. The effects of TGF-β1 signaling on PD-1 expression

(a) Representative histograms of CFSE (left), PD-1(middle) and LAG3 (right) expression on WT (thin grey histogram) and *Smad3* (thin black histogram) cKO OT-I cells originating from the draining lymph nodes (DLN) (top) and non-draining lymph nodes (NDLN) (bottom). (b) CFSE-, PD-1+ and LAG3+ OT-I subsets in DLNs were gated based on OT-I cells from NDLNs. The percentage of CFSE- (left) and MFI of PD-1 (middle) and LAG3 (right) of each subset are shown as mean +/- SEM, and the data represent two independent trials. (c,d) The same analysis was performed on WT and *Smad2* cKO OT-I cells and the data represent two independent trials. (e) WT and DNTGFβRII Tg+ mice were injected with 10^5 B16 melanoma cells in foot-pads, and tumor volume (mm^3) is shown as mean +/- SEM on different days. (f) PD-1 MFI of PD-1+ CD8+ subset originating from the tumor microenvironment (left) and DLN (right) is assessed from each mouse on Day 27. (g) The percentage of the LAG3+ CD8+ subset originating from the tumor microenvironment (left) and DLN (right) is assessed from each mouse on Day 27. The data were analyzed using Student's t-test and considered significant if *P<0.05.