TGF-BETA1/SMAD3 SUPPRESS ANTI-TUMOR IMMUNITY BY ENHANCING PD-1 EXPRESSION ON ANTIGEN-SPECIFIC CD8+ T CELLS IN CANCER

by

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Abstract

Programmed Death-1 (PD-1) is a co-inhibitory receptor that is highly expressed on exhausted tumor-infiltrating lymphocytes (TILs) in cancers and on virus-specific T cells in chronic HBV, HIV and HCV infection. Signaling through PD-1 inhibits T cell effecter function and limits effective anti-tumor and anti–viral immune responses. Despite its importance in both cancers and infectious diseases, the molecular mechanisms driving high PD-1 expression are not fully understood. In this study, we found that transforming growth factor-β1 (TGF-β1) enhances antigen-induced PD-1 expression on activated human and murine T cells *in vitro* and *in vivo*. A transcription factor, Smad, transduces signals from TGF-β superfamily ligands that regulate cell proliferation, differentiation and death through activation of receptor serine/threonine kinases. Inhibition of Smad3 phosphorylation or genetic deletion of Smad3 abrogates PD-1 enhancement on human and murine T cells, demonstrating that Smad3 is a critical mediator of TGF-β1-effects on PD-1 regulation. In addition, we found that Smad3 enhances *Pdcd-1* transcription by binding to its proximal promoter region. Finally, a PD-1^{hi} subset in Smad3-deficient CD8+ TILs was significantly decreased compared to wild-type T cells in a murine model of melanoma in association with enhanced anti-tumor function. In addition to TGF-β1's previously known effects on T cell function, our findings suggest that TGF-β1 mediates suppression via PD-1 upregulation in the tumor microenvironment. Thus, Smad3 may represent an additional target in therapeutic modulation of PD-1.

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1. Introduction

Programmed death-1 (PD-1) is a member of co-inhibitory receptor on T cells that is induced by antigenic stimulation[1]. PD-1 expression on functional memory CD8+ T cells declines upon the resolution of inflammation and the clearance of antigens during acute infections[2]. On the other hand, PD-1 expression is maintained on exhausted T cells in chronic infections. In cancer, tumor-infiltrating and tumor-specific CD4+ and CD8+ T cells express high levels of PD-1[3] and blockade of PD-1 has been effective in prolonging patient survival in melanoma, renal-cell cancer and non-small-cell lung cancers [4]. Similarly, chronic hepatitis C virus (HCV), hepatitis B virus (HBV) and human immunodeficiency virus (HIV) infection sustains high levels of PD-1 on viral-specific CD8+ T cells[5-7].

PD-1 on T cells can bind to its ligands, programmed death-1-ligand-1 (PD-L1) and -ligand-2 (PD-L2), and its binding can inhibit T cell effector functions[8]. Pathogen- or tumor-driven inflammation can induce PD-L1 and –L2 expression. For example, PD-L1 is highly expressed on many human tumors[9] and its expression is highly co-localized with infiltrating CD8+ T cells in human melanoma patients[10]. Similarly, patients with chronic liver diseases from HCV and HBV infection also show increased levels of PD-L1 on hepatocytes and Kupffer cellsin the liver[11]. Elevated PD-L1 and –L2 expression can possibly enhance its engagement with PD-1 on T cells and provide pathogens survival benefits over host immune responses[9,12-16]. While interferon-γ (IFN-γ, one of effector cytokines from T cells is known to enhance PD-L1 expression on tumor cells[12], a molecular mechanism that enhances PD-1 expression on T cells is not fully understood.

Transforming growth factor-β (TGF-β) is another immunosuppressive component in cancers and chronic viral infections[17-20]. It is a regulatory cytokine that transduces signals through a transcription factor, Smad, and regulates cell proliferation, differentiation and death through activation of receptor serine/threonine kinases. High serum levels of TGF-β1 are associated with poor prognosis in cancer[21,22] and tumor microenvironment-derived TGF-β can suppress anti-tumor T cell responses[23,24]. Accordingly, the blockade of TGF-β signaling on T cells has been effective in restoring effector functions[25] although the dominant source for TGF-β (tumor or T-cells) needs to be further determined[26]. The suppressive mechanisms of TGF-β1 include Smad2/3 dependent inhibition of effector cytokines by CD8+ T cells in cancer[27]. Also, in CD4+ T cells, TGF-β1 plays a critical role in development of regulatory T cells (Tregs) that suppress neighboring effector cells through both contact-independent and -dependent mechanisms[28,29].

Here, we report a novel molecular mechanism by which TGF-β enhances activation-induced PD-1 expression through Smad3 and that the Smad3/PD-1 axis suppresses T cell function. Utilizing mice conditionally deleted of Smad2 or Smad3 in T cells, we found that Smad3-deficent T cells do not demonstrate enhanced PD-1 expression, while Smad2-deficient T cells minimally do. This suggests that PD-1 expression on T cells is predominantly regulated by Smad3. Most notably, this effect of Smad3 was specific to PD-1 because other inhibitory receptors were differentially regulated. Thus, our findings suggest that tumor cells not only adapt through enhanced ligand (PD-L1) expression, but

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also increase PD-1 expression on TILs through TGF-β1 in order to further suppress anti-tumor immune responses.

2. Results

2.1 NFATc1 is critical for TCR-mediated induction of human PD-1 expression

It is known that TCR-mediated NFATc1 activation induces PD-1 expression on murine T cells[30]. Thus, we tested whether human PD-1 expression requires NFATc1 by utilizing cyclosporine A (CsA), a calcineurin inhibitor that exerts its immunosuppressive effects by keeping the transcription factor NFATc1 inactive. We found that CsA abrogated αCD3/αCD28-dependent PD-1 expression on human CD3+ T cells (Fig. 1a). In addition, we found that a 1.9kb-long human PD-1 promoter region contains a strong NFATc1 binding site (Fig. 1b). We cloned the sequence into a luciferase reporter vector and luciferase activity was measured after transfecting it into Jurkat T cells. αCD3/αCD28 activation induced PD-1 promoter activity (Fig. 1c, WT grey bar) and mutation in the NFATc1 binding site abrogated such induction (Fig. 1c, NFATm grey bar). When we further examined NFATc1-mediated PD1 regulation utilizing the chromatin immunoprecipitation (ChIP) assay, we found that αCD3/αCD28 induced strong NFATc1 binding to the proximal region of the human PD-1 promoter (Fig. 1d, αNFATc1 black bar under *Pdcd-1*). In contrast, NFATc1 shows minimal binding to a *Gapdh* promoter region in activated T cells (Fig. 1d, αNFATc1 black bar under *Gapdh*). Collectively,

these data suggest that activation-mediated PD-1 induction is conserved in both human and murine T cells.

2.2 TGF-β1 enhances PD-1 surface expression on human T cells in a dose dependent manner

To assess the effects of cytokines on activation-induced PD-1 expression, we isolated CD3+ T cells from healthy donor peripheral blood mononuclear cells (PBMC) and activated them with αCD3/αCD28-conjugated beads for 72hrs in the presence of one of 16 cytokines for 72hrs (Fig. 2). While most of the cytokines tested had no effect on PD-1 expression upon T cell activation, IL-2, IL-6, and IL-12 modestly increased PD-1 expression, consistent with previously published work[31,32]. However, the most dramatic effect on PD-1 expression was observed with TGF-β1 (Fig. 2). Thus, we further investigated TGF-β1 mediated PD-1 expression on T cells. The cells were also labeled with carboxyfluorescein succinimidyl ester (CFSE) to monitor cellular proliferation (Fig. 3a, representative plot). αCD3/αCD28 induces increased PD-1 expression relative to resting CD8+ and CD4+ T cells, demonstrating TCR and co-stimulation dependent PD-1 expression (Fig. 3a, left and middle graphs). The co-culture of cells with TGF-β1 (Fig.3b, open circle line graphs) further enhanced PD-1 expression on both CD8+ (Fig.3b, left panel) and CD4+ (Fig. 3b, right panel) T cells versus αCD3/αCD28 (Fig.3b, filled circle line graphs) on all CFSE generations. TGF-β1 did not have any effects on cellular proliferation as measured by the fraction of T cells in each CFSE division (Fig. 3b, black

and white bar graphs), suggesting that enhanced PD-1 expression is not due to altered cellular proliferation.

While human memory populations such as CMV and EBV-specific T cells express intermediate levels of PD-1, naïve T cells do not express PD-1[33]. To test whether TGF-β1 mediated enhancement of PD-1 expression depends on the basal level of PD-1 expression, we isolated naïve T cells (phenotype CCR7+ CD45RA+) and memory T cells (phenotype CCR7+ CD45RA- or CCR7- CD45RA+) from healthy donor PBMC. The cells were activated with αCD3/αCD28-conjugated beads for 72hrs with or without TGF-β1. Although TGF-β1 increased PD-1 expression on both αCD3/αCD28 stimulated memory and naïve CD4+ and CD8+ T cells (Fig. 3c, representative plot), the effect was more pronounced on naïve T cells than on memory T cells for both CD8 (Fig. 3d, left) and CD4 subsets (Fig. 3d, right). Furthermore, we found that TGF-β1 increased PD-1 surface expression in a concentration-dependent manner (Fig.4a, left). In contrast, TGF-β1 did not affect expression of the T cell activation marker HLA-DR on T cells (Fig. 4a, right), suggesting that the TGF-β1-mediated effect is specific to PD-1 and not activation specific. Finally, enhanced surface expression of PD-1 was preceded by increased transcription of PD-1, as shown as kinetic changes of *pdcd-1* mRNA levels across different time points (Fig. 4b).

2.3 Anti-TGF-β1 neutralizing antibody and a TGF-βRI kinase inhibitor negate TGF-β1-mediated PD-1 enhancement

Next, we investigated whether the blockade of TGF-β1 signaling can abrogate TGF-β1-dependent PD-1 enhancement. TGF-β1 binds TGF-βRI and RII and acts through Smad-dependent and -independent mechanisms. Upon binding of the high affinity TGF-βRII by TGF-β1, TGF-βRI and RII heterodimerize and TGF-βRI, a serine-threonine kinase, phosphorylates Smad2/3. To address the role of TGF-β receptor signaling, the cells were activated in the presence of TGF-β1 with varying concentrations of an antibody that blocks the activity of TGF-β1 but not TGF-β2 or TGF-β3 (neutralizing antibody, nAb) (Fig. 5a) or TGF-βRI kinase inhibitor (SB431542) (Fig. 5b). Both TGF-β1 nAb and SB431542 decreased TGF-β1 dependent PD-1 expression in a dose-dependent manner, although SB431542 was more effective than TGF-β1 nAb. SB431542-mediated TGF-βR signaling inhibition was also shown by diminished phosphorylation levels of Smad2 (Fig. 5c). Analogous to the effects on surface expression, SB431542 blocked a TGF-β1-dependent increase in *pdcd-1* mRNA levels (Fig. 5d).

2.4 Smad3 directly binds to Smad Binding Elements (SBE) and regulates PD-1 promoter activity

Our data suggest that human PD-1 expression is under transcriptional control by TGF-β1, and we hypothesized that TGF-β1 directly modulates human PD-1 promoter activity. Jurkat T cells were transfected with PD-1 promoter-driven luciferase vector together with TGF-βRI and RII plasmids because Jurkat T cells express minimal levels of TGF-β receptors (Fig. 6a, b). The addition of TGF-β1 to αCD3/αCD28 enhanced NFATc1-dependent PD-1 promoter activity when Jurkat T cells were co-transfected with TGF-βRI

and RII plasmids (Fig. 6a). The expression of TGF-βRI and RII were confirmed by

flowcytometry (Fig. 6b). Supporting our hypothesis, we identified putative Smad-Binding

Elements (SBEs), one distal to (SBE-D) and the other proximal to (SBE-P) the previously

identified NFATc1 binding site (Fig. 7a). The introduction of site-directed mutations in

SBEs (shown in bold letters in Figure 7a, named SBE-D and SBE-P) significantly

diminished PD-1 promoter activity and introduction of both mutations (SBE-D/P) further

decreased the effect (Fig. 7b). The mutations in SBEs had no significant effect on PD-1

promoter activity upon αCD3/αCD28 stimulation in the absence of TGF-β1.

TGF-βR1 has serine/threonine kinase activity that phosphorylates Smad2 and Smad3[34].

Smad2 and Smad3 bifurcate the signaling pathway by forming heterodimers with Smad4

(co-smad)[35,36]. Thus, we further investigated whether Smad2 or Smad3 is a dominant

regulator of PD-1 promoter activity by using siRNA (Fig. 8a, b). We found that knock-

down of Smad3 expression (but not Smad2) abrogated TGF-β1 enhancement of PD-1

promoter activity (Fig.9a). Similarly, the Specific Inhibitor of Smad3 (SIS3) inhibited

TGF-β1-enhanced PD-1 promoter activity (Fig. 9b, white bars) without altering

NFATc1-dependent PD-1 promoter activity (Fig. 9b, grey bars). Thus, our data

collectively showed that Smad3 is a key mediator of enhanced PD-1 promoter activity

and increased *pdcd-1* transcription levels.

To further validate our luciferase-based reporter system, we utilized the chromatin

immunoprecipitation (ChIP) assay to verify Smad3 binding to the human PD-1 promoter.

While αCD3/αCD28 did not induce Smad3 binding, the addition of TGF-β1 significantly

induced its binding to the human PD-1 promoter (Fig. 9c, dark grey bar on right panel).

This binding was specifically due to TGF-β1 receptor signaling as it was abrogated by

TGF-βRI kinase inhibitor treatment (Fig. 9c, light grey bar on right panel). In contrast,

there was no effect of TGF-β1 on binding of Smad3 to a *Gapdh* promoter region (Fig. 9c,

left panel).

2.5 TGF-β1 dependent Smad3 enhances PD-1 expression on human and murine T cells

We addressed whether TGF-β1 dependent PD-1 regulation is conserved in murine T cells

utilizing ovalbumin-specific CD8+ (OT-I) and CD4+ (OT-II) T cells. CD8+ T cells were

enriched from OT-I or OT-II mice and activated with cognate ovalbumin peptide in the

presence of irradiated antigen presenting cells. Activation in the presence of TGF-β1

enhanced PD-1 expression on both OT-I (Fig. 10, top left) and OT-II (Fig. 10, bottom

left). In contrast, lymphocyte-activation gene3 (LAG3) expression, another inhibitory

receptor, decreased on OT-I and OT-II cells in the presence of TGF-β1 suggesting that

TGF-β1 has differential effects on inhibitory receptors (Fig. 10, right).

Next, we investigated whether Smad3 deficiency can abrogate TGF-β1 dependent PD-1

expression on murine T cells. CD4+ T cells were isolated from wild-type (WT), *Smad2*

f/f; CD4-cre (*Smad2* cKO), and *Smad3* f/f; CD4-Cre (*Smad3* cKO) mice and activated

with αCD3/αCD28 with or without TGF-β1. Cre-mediated gene knock-out of Smad2 and

Smad3 in CD4+ T cells was confirmed by Western blot analysis (Fig. 11). TGF-β1

minimally increased PD-1 expression on Smad3 cKO CD4+ T cells (Fig. 12a, right)

compared to WT CD4+ T cells (Fig. 12a, left). In contrast, *Smad2* cKO CD4+ T cells

maintained high PD-1 expression in response to TGF-β1 (Fig. 12a, middle). Similarly,

when WT, Smad2 cKO, and Smad3 cKO OT-1 cells were activated with type-I OVA in

the presence of TGF-β1, Smad3 cKO OT-1 showed decreased PD-1 expression (Fig. 12b,

top) without significant changes in cellular proliferation (Fig. 12b, bottom). To further

investigate the role of Smad3 in PD-1 regulation, we treated human CD3+ cells with

Smad3 inhibitor (SIS3)[37] and found that SIS3 treatment decreased PD-1 surface

expression in a dose-dependent manner (Fig. 12c).

2.6 Tumor-infiltrating Smad3 cKO CD8+ T cells have decreased PD-1 expression in B16 melanoma

PD-1 is highly expressed on tumor infiltrating lymphocytes (TILs) in cancer[3]. Given that

TGF-β1 production is associated with advanced stage cancer[38,39], we hypothesized that

Smad3 contributes to such high PD-1 expression. It is known that B16 melanoma secrets

TGF-β1[25] and this makes it an ideal model system to test our hypothesis. In order to

investigate whether TGF-β1 regulates PD-1 expression through Smad3 *in vivo*, WT,

Smad2 cKO, and Smad3 cKO mice were challenged with B16 melanoma and PD-1

expression on tumor infiltrating lymphocytes were assessed on day 24. Smad2 and

Smad3 are known suppressors of T cell function[27]. Consistent with this, growth of B16

melanoma in both Smad2 and Smad3 cKO mice was significantly delayed (Fig. 13).

Although Smad2 cKO and Smad3 cKO mice had comparable volumes of B16 melanoma,

the PD-1^{hi} subset population was significantly lower on Smad3 cKO CD8+ TILs, but not on Smad2 cKO CD8+ TILs (Fig. 14a, representative histogram). On average, Smad3 cKO mice lost approximately 40 percent of the PD-1^{hi} subset, while Smad2 cKO mice maintained a PD-1^{hi} subset comparable to that of WT mice. Interestingly, the PD-1^{hi} subset population on Smad2 cKO CD8+ TILs was slightly increased (although not statistically significant) and LAG3 expression was significantly enhanced (Fig. 14b, representative histogram and right panel).

Interestingly, PD-1 expression on CD4+ TILs was comparable among all groups (data not shown). One potential explanation for such discrepancy in CD4+ T cells could be due to a minimal fraction of antigen-specific CD4+ T cells in the tumor microenvironment. Alternatively, because PD-1 expression is a marker of Tregs[40,41], a dominant fraction of CD4+ T cells could be PD-1+ Foxp3+ Tregs that are not specific for antigen but recruited to B16 melanoma. Supporting this notion, we found that the majority of CD4+ TILs express Foxp3 and intermediate levels of PD-1 in contrast to higher levels of PD-1 on CD8+ T cells (Fig. 15a). We further found that Foxp3+ CD4+ T cells are capable of enhancing PD-1 expression in the presence of TCR signaling *in vitro*. CD4+ T cells were isolated from Foxp3-GFP transgenic mice and activated in the presence of TGF-β1. Both Foxp3+ and Foxp3- CD4+ T cells are capable of enhancing PD-1 expression to the same extent in response to TGF-β1 (Fig. 15b). Although Foxp3+ cells are capable of enhancing PD-1 expression in the setting of TGF-β1 and TCR signaling, it is possible that the majority of CD4+ T cells in the tumor had not undergone TCR signaling, limiting PD-1 upregulation.

In order to test whether these observations on the effects of Smad3 cKO were consistent on antigen-specific CD8+ T cells, we utilized B16 melanoma cells stably expressing ovalbumin (B16-ova). CD45.1 WT mice were challenged with B16-Ova on Day 0. After 12 days, CD45.2 OT-1 cells from WT and *Smad3* cKO OT-1 mice were adoptively transferred into the tumor-bearing mice. Tumor growth was monitored and lymphocytes infiltrating into tumors were harvested after 5 days. We found that transferred Smad3 cKO OT-1 cells limit tumor growth more effectively than WT OT-1 cells do (Fig. 16). Consistent with this enhanced anti-tumor immune function, *Smad3* cKO TILs show increased cellular proliferation by CFSE (Fig. 17a, histogram) when gated on CD45.2+ donor cells. At the same time, *Smad3* cKO TILs have significantly fewer of the PD-1^{hi} expressing cells highly characteristic of WT TILs (Fig. 17b, top). This effect of Smad3 was specific to PD-1 since there was no reduction in LAG3^{hi} CD8+ T cells among *Smad3* cKO TILs (Fig. 17b, bottom). On the other hand, Smad2 cKO TILs show comparable cellular proliferation by CFSE (Fig. 17c, histogram) and PD-1^{hi} expressing cells did not decrease in Smad2 cKO TILs (Fig. 17d, top), further supporting that Smad3 is a critical mediator of PD-1 expression in the tumor microenvironment. In contrast, Smad2 cKO TILs maintained high levels of PD-1 and LAG3 (Fig. 17d, bottom), which is consistent with our observation in polyclonal CD8+ T cells (Fig. 14). Conversely, PD-1 and LAG3 expression on T cells in draining lymph nodes (DLN) was comparable between WT and *Smad2* (Fig. 18) or *Smad3* cKO (Fig. 19) OT-1 suggesting that the effect of TGF-β1 is specific to the tumor-microenvironment.

2.7 TGF-β1/Smad3-dependent enhanced anti-tumor effects involve PD-1 expression

Smad3 is known to inhibit CD8+ T cell effector function, which may occur through many different mechanisms. Our data provide evidence that enhancement of PD-1 expression represents a newly defined mechanism through which Smad3 suppresses T cell function. In order to address how significant the impact of Smad3-mediated PD-1^{hi} T cell loss is on tumor evasion of T cell responses, we treated Smad3 cKO mice bearing B16-melanoma with an anti-PD-1 blocking antibody previously shown to have therapeutic efficacy in WT mice bearing B16-melanoma. If the effect of Smad3 cKO on tumor growth is mediated through a mechanism other than PD-1 or if the effects of Smad3 cKO on PD-1 expression are not sufficient to negate that mechanism of tumor evasion, treatment with anti-PD1 blocking antibody would confer additional therapeutic benefits in Smad3 cKO mice. To assess this, WT and Smad3 cKO mice were challenged with 5×10^5 B16 melanoma cells and were given either isotype-matched IgG or anti-PD-1 blocking antibody. We found that the tumor volume of WT mice treated with anti-PD1 blocking antibody was decreased compared to IgG treated WT mice (Fig. 20). In contrast, anti-PD-1 blocking antibody had no effect on tumor growth in Smad3 cKO mice. Differential CD8+ T cell effector function was observed as well. Anti-PD-1 antibody treated WT mice had enhanced CD8+ T cell secretion of IFN-γ+ (single-positive) and IFN-γ+/TNF-α (double positive) compared to WT mice, while anti-PD-1 failed to increase CD8+ T cell secretion of IFN-γ and TFN-α in Smad3 cKO (Fig. 21). Collectively, the data suggest that PD-1 enhancement represents a novel mechanism through which TGF-β1/Smad3

suppresses effective anti-tumor immune responses and that Smad3 is a major regulator of the suppressive effects of PD-1.

3. Discussion and Conclusions

PD-1 expression can be differentially regulated by the environmental context in which a T cell encounters antigen. Upon activation, nuclear factor of activated T cell (NFATc1) transiently induces PD-1 expression on T cells[30,42]. Once PD-1 expression is induced, it is sustained in chronic infections or toleragenic environments[2], but high level expression is not maintained in certain inflammatory conditions, such as *Listeria monocytogenes* infection[43]. Further supporting the notion of dynamic PD-1 expression, there has been emerging evidence that cytokines can regulate NFATc1-induced PD-1 expression. IFN-α promotes PD-1 expression on murine T cells through STAT1 mediated transcriptional regulation of PD-1 gene expression[44,45]. IL-6 also increases PD-1 expression through a STAT3-dependent mechanism in murine CD8+ T cells[31], and we found the similar regulation in human CD4+ and CD8+ T cells (Fig. 2). IL-12 has differential effects on PD-1 *in vivo* and *in vitro*. IL-12-conditioned tumor-specific memory CD8+ T cells have lower PD-1 expression *in vivo* with stronger anti-tumor immune responses[44]. In contrast, we have found that IL-12 increases PD-1 expression on naïve human CD4+ and CD8+ T cells *in vitro,* consistent with other's findings on murine CD8+ T cells[31]. Thus, while our data agree with the literature that IFN-α, IL-6, and IL-12 modulate PD-1 expression, TGF-β1 has the greatest effect on PD-1 expression, which has not been shown previously.

The data on the effect of other cytokines on PD-1 expression also collectively show that the regulatory mechanisms of PD-1 expression are highly conserved between human and mouse. This is further supported by high sequence homology between human and murine PD-1 proximal promoter regions including the NFATc1 binding site[31]. We demonstrate that Smad3-dependent PD-1 regulation is also conserved in showing that TGF-β1 has the greatest effects on PD-1 expression on both human and murine T cells. TGF-β1 requires antigenic stimulation in order to enhance PD-1 expression, supporting the previous findings that NFATc1 is critical for PD-1 induction in mice[30] and that mutation of antigens such that the TCR is no longer engaged results in decline in PD-1 expression in human chronic infection with HCV or HIV[6,46]. Furthermore, TGF-β1 enhances PD-1 expression on both CD4+ and CD8+ T cells regardless of their naïve or memory status, although its effect was more pronounced on naïve T cells than on memory T cells. Our proliferation assays showed that TGF-β1-mediated PD-1 enhancement is independent of cellular proliferation. Even though TGF-β1 induced phosphorlyation of Smad2 (Fig. 5c), we did not observe significant suppression of T cell proliferation by TGF-β1 in contrast to other's findings in which TGF-β1 was proposed to play a role in T cell proliferation[47]. TGF-β1-mediated suppression can be overcome by CD28-mediated co-stimulation, and it is possible that our *in vitro* culture system may mask such inhibition[48]. This is supported by our data that Smad3 cKO OT-1 did not show significantly altered proliferation *in vitro*, but had a small proliferation advantage *in vivo* (Fig. 17a). We also observed that isolated Smad3 cKO CD4+ T cells have increased IL-2 expression compared to WT litter mates when activated with αCD3/αCD28, consistent with previous reports[49].

Others have suggested a potential association between TGF-β1 production and high PD-1

expression, but its molecular mechanism and biological implications have not been fully

addressed[26,50,51]. TGF-β1 signaling consists of Smad and non-Smad dependent pathways

and Smad-dependent gene regulation (Smad2 and Smad3) has been well-

characterized[52,53]. Some genes are preferentially and exclusively regulated by Smad2 or

Smad3 as in *Id1* and *C-myc*[54,55]. On the other hand, Smad2 and Smad3 can redundantly

regulate expression of many genes that are under control of TGF-β1[56]. More recently, a

novel role of Smad4 was discovered in which it is required for T cell proliferation and

anti-tumor immunity[57]. Our luciferase assay and *in vitro* data suggest that PD-1

regulation is predominantly under the control of Smad3. Although our *in vitro* data

support a minor role for Smad2 in TGF-β1-dependent PD-1 enhancement, our *in vivo*

data clearly demonstrated no enhancement of PD-1 expression through Smad2 with

Smad2 cKO mice showing a small increase in PD-1 expression. The *in vivo* data could

reflect enhanced Smad3 expression in compensation for Smad2 deletion in T cells.

The minor contribution of Smad2 to PD-1 regulation *in vitro* could be due to conserved

DNA binding domains in Smad2 and Smad3[58]. Even though our ChIP analysis suggests

that Smad3 binds to the human *Pdcd-1* promoter, Smad2 may co-bind to those regions as

well and regulate PD-1 promoter activity. Identification of the transcriptional complexes

formed by Smad3 at the human *Pdcd-1* promoter might address this. NFATc1 may form

part of the transcriptional complex given that TGF-β1 treatment results in enhanced

NFATc1 binding with Smad3 to the human *Pdcd-1* promoter (data not shown). It is

possible that Smad3 directly interacts with NFATc1[59], and NFATc1-mediated

recruitment of co-activators and epigenetic status change lead to increased PD-1 promoter activity[59-62]. Alternatively, Smad3 could directly bring co-activators independently of NFATc1 to the proximal region of the PD-1 promoter[63].

Although we observed similar effects of TGF-β1 on both CD4+ and CD8+ T cells *in vitro*, our *in vivo* studies mainly focused on CD8+ T cells because the PD-1 expression difference was greater in CD8+ T cells than in CD4+ T cells *in vivo*. Furthermore, the effect of TGF-β1 on CD8+ T cells was specifically on TILs, but not on those originating from the draining or nondraining lymph nodes. We did not find the percentage of PD-1^{hi} T cells to be significantly different among CD8+ T cells originating from the tumor-draining lymph nodes of Smad3 cKO mice (Fig. 18). This may be due to the fact that the PD-1^{hi} CD8+ T cell population prominent in the tumor microenvironment was absent in the draining lymph nodes, and suggests that TGF-β1 levels could be much lower outside of the tumor microenvironment.

In sum, our data demonstrate a novel immunosuppressive function of TGF-β1 in regulating high-level PD-1 expression on T cells encountering cognate antigen. In addition to other suppressive roles for TGF-β1, tumors that secrete TGF-β1 may induce high levels of PD-1 on T cells as they encounter antigens on the tumor surface, reducing T cell effector function and limiting the anti-tumor T cell response. In addition, our data provide mechanistic understanding of the regulation of high-level PD-1 expression. While it is well known that T cells against intact antigen in the setting of chronic viral infections such as HCV and HIV or malignancy express very high levels PD-1, it is not

known how those high levels are induced. This study elucidates a mechanism through which the highest levels of PD-1 are induced. Indeed, high TGF-β1 levels are associated with worse disease outcome in HCV infection[64,65], and TGF-β1 is commonly secreted by tumors in advanced disease stages of cancer[38,39], which may further limit the efficacy of T cells against disease in those settings. Given the potential for autoimmunity with PD-1 therapy, it is worth investigating whether inhibitors of Smad3 used in combination with other immunotherapeutic agents activate T cells expressing the highest levels of PD-1 rather than all T cells bearing PD-1.

4. Materials and Methods

Mice

All animals were housed and handled in compliance with Johns Hopkins Animal Care and Use policy. *Smad2* floxed/floxed (fl/fl) and *Smad3* fl/fl mice were generated by Se-Jin Lee's Laboratory at Johns Hopkins University and backcrossed to C57BL/6 at least 6 generations. *Smad2* fl/fl and *Smad3* fl/fl mice were crossed to C57/BL6 CD4- Cre transgenic mice purchased from the Jackson Laboratory. CD45.1 congenic mice were purchased from National Cancer Institute (NCI) at Frederick. OT-I and OT-II mice were generous gifts from Drs. Charles Drake and Hyam Levitsky at Johns Hopkins University.

Human and murine primary T cell Isolation and Culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from leukopheresis by Ficoll-Hypaque density gradient. Isolated human PBMCs were subjected for CD3+ T cell isolation by using a pan T cell isolation kit (Miltenyi) as instructed in the manual.

Isolated CD3+ T cells were cultured in RPMI+10% Fetal Bovine Serum (supplemented with HEPES buffer, Penicillin/Streptomycin, and L-glutamine). The isolated cells were activated with αCD3/αCD28-conjugated Dynabeads (Invitrogen) at a cell to bead ratio of 1:1. Murine CD4+ and CD8+ T cells were isolated from the spleen and lymph nodes using CD4 Negative Selection Kit (Invitrogen), and were activated with plate-coated αCD3 (10ug/ml) and soluble αCD28 (2ug/ml) for 72hrs. OT-I and OT-II cells were activated with type I and II OVA in the presence irradiated antigen-presenting cells (APCs) for 72hrs.

Transient Transfection and Luciferase Assay

Jurkat T cells (clone E6-1) were maintained in RPMI+10% Fetal Bovine Seurm. 1.5×10^7 Jurkat T cells were transfected with 10ug of pGL-3 Firefly luciferase vector (Promega) and 1ug of pRL-TK (Promega) by electroporation using Nucleofector II (Amaxa/Lonza). The cells were rested in a 6-well plate overnight and activated with plate-coated αCD3 (10ug/ml) and soluble αCD28 (5ug/ml) with or without rhTGF-β1 (50ng/ml). After 24hrs, the cells were harvested and lysed followed by luminescence measurement using Dual-Luciferase Assay (Promega). Where indicated, the cells were co-transfected with empty vector (pSG-V5), TGF-βRI-His (Addgene plasmid #19161), TGF-βRII (Addgene plasmid #11766). For siRNA-mediated knock-down, the cells were co-transfected with 1.5uM of siRNA for Smad2 and Smad3 (Santa Cruz Biotechnology).

Cytokines and Drug Treatments

Human recombinant IL-1α, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IL-15, IL-17, IL-18, IL-21, IL-23, INF-α, IFN-γ, TGF-β1, TNF-α, were purchased from Peprotech and used at the indicated concentrations *in vitro*. Primary T Cells were treated with neutralizing TGF-β1 antibody (Abcam) and small molecule inhibitors SB431542 (Sigma-Aldrich), SIS3 (Calbiochem), and CsA (Sigma-Aldrch) for 1hr before activation at a concentration range of 0.1uM to 100uM.

Flow Cytometry

After 72hrs of activation, human T cells were harvested and centrifuged at 400g (or 1500rpm) for 5 minutes. The cells were washed in FACS buffer (PBS+2% Fetal Bovine Serum) and stained with Aqua Viability Dye (Invitrogen) as instructed in the manual. After wash, the cells were stained with PD-1 PE (Biolegend), CD8 PerCP (eBioscience), CD4 Pacific Blue (eBioscience), CD3 FITC (eBioscience), HLA-DR APC (eBioscience), or HLA-DR qDot605 (Invitrogen). The similar protocol was used in murine T cells and PD-1 PE (eBioscience), CD4 or CD8 PerCP (eBioscience), LAG3 APC or PacBlue (Biolegend), CD3 AF700 (Biolegend) were used for flowcytometery. For intracellular staining, the cells were incubated with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence of Golgiplug (BD Biosciences) for 5-6 hours. It was followed by fixation/permeabilization steps using Foxp3/transcription factor staining buffet set (eBioscience). IFN-γ FITC or APC and TNF-α PacBlue or APC were used to assess cytokine expression.

Real-time qPCR assay

Total RNA was extracted from human CD3+ T cells under the indicated conditions using RNEasy Plus Kit (Qiagen). 100ng of extracted RNA was reverse-transcribed using SuperScriptIII First-Strand Synthesis System (Invitrogen). Generated cDNA was subjected for real-time PCR assay using *Pdcd1* primers (IDT). All target genes were normalized to 18s rRNA or 28s rRNA as previously described.

Molecular Cloning and Site-directed Mutagenesis

Human PD-1promoter (1.9kb) was cloned from genomic DNA of isolated CD3+ T cells and the sequence was confirmed. The amplified clone was ligated to SacI and XhoI digested pGL3-Basic (Promega) using In-fusion cloning kit (Clonetech). Site-directed mutagenesis was carried out for NFATm, SBE-D, SBE-P using QuickChange Lightning Kit (Agilent Technologies).

Chromatin Immunoprecipitation (ChIP) assay

ChIP assay was performed according to the manufacturer's guidance (Invitrogen MAGnify ChIP system). Briefly, isolated CD3+ T cells were activated with αCD3/αCD28-conjugated beads for 24hrs and fixed with 2% formaldehyde. Sonicated DNA was immunoprecipiated with αSmad3 (Cell Signaling Technology) and αNFATc1 (Santa Cruz Biotechnology). The immunoprecipated chromatin was analyzed on Roche Light Cycler 480 by SYBR green using the following primers for PD-1 promoter. Forward 5'-CCTCACATCTCTGAGACCCG-3'; Reverse 5'CCGAAGCGAGGCTAGAAACC-3'

Western Immunobloting

Human or murine T cells were activated as indicated above and harvested and lysed in RIPB Buffer (Cell Signaling Technology). Protein-extract concentration was measured using the BCA protein assay kit (Thermo Scientific) and followed by heating under reducing conditions. The equal amount of extracts was loaded/run on NuPAGE Precast gels (Invitrogen) and transferred membranes were blotted with following antibodies: p-Smad2, total-Smad2, total-Smad3 (Cell Signaling Technology), and β-actin (Sigma).

B16 melanoma and adoptive T cell transfer experiments

1×10^5 B16 melanoma cells were injected on a flank for each mouse in 100ul volume. Tumor volumes were measured every other day using a caliper and assessed using the formula ½ ($L\times W^2$). For adoptive transfer experiments, CD45.1 host mice were injected with 1×10^5 B16-OVA melanoma cells on a flank. 8×10^6 WT OT-1 or cKO OT-1 were labeled with CellTrace CFSE Cell Proliferation Kit (LifeTechnologies) and were adoptively-transferred into tumor-bearing mice by retroorbital injection on Day12. The tumors were harvested on Day5 after the adoptive transfer and lymphocytes were purified using Percoll (GE Healthcare) gradient. In a blocking experiment, 5×10^5 B16 melanoma cells were injected on a flank and armenian hamster IgG isotype control (Rockland) or anti-PD1 antibody (G4) were injected intraperitoneally two times a week each 100ug/mouse from Day0.
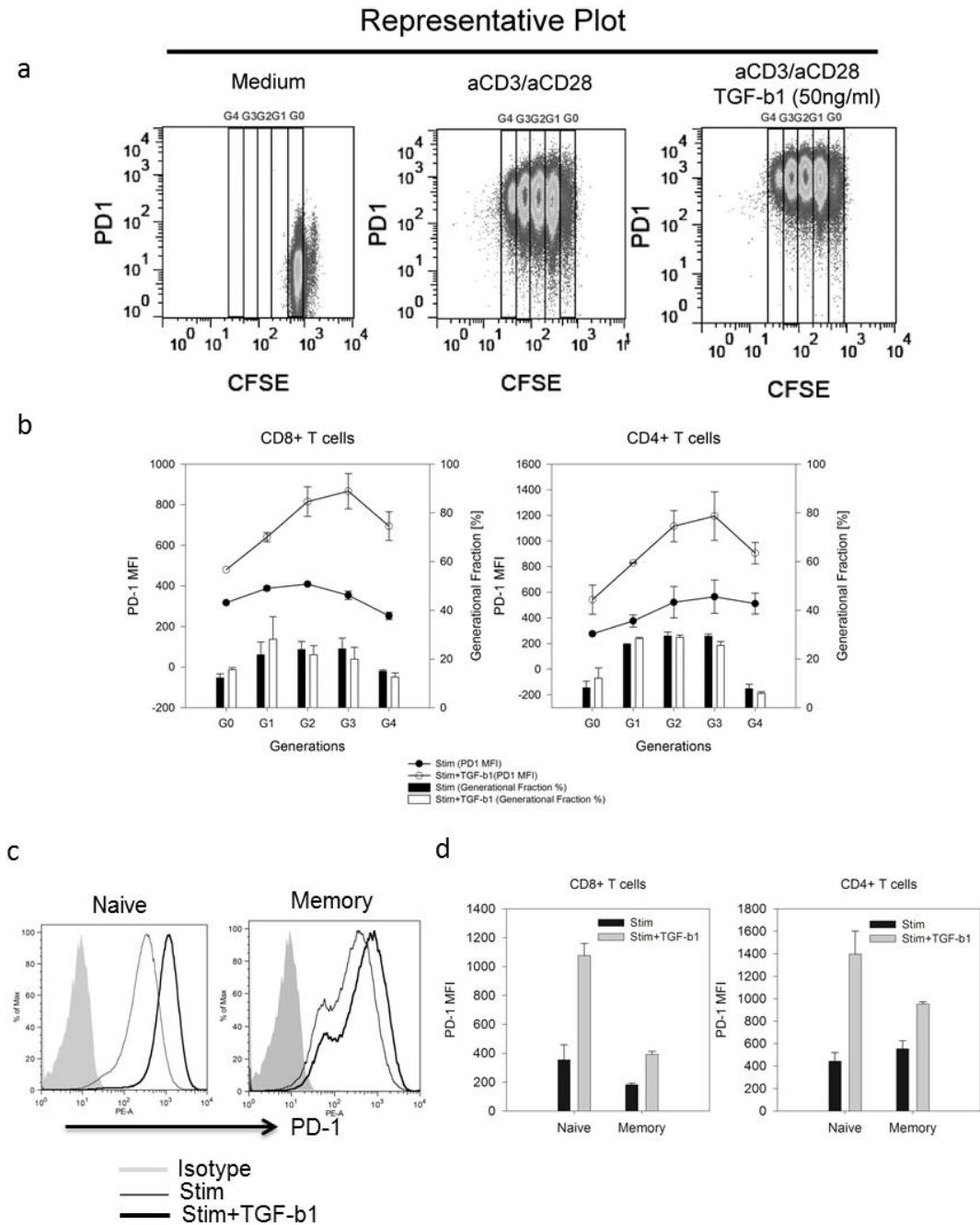
5. Figures
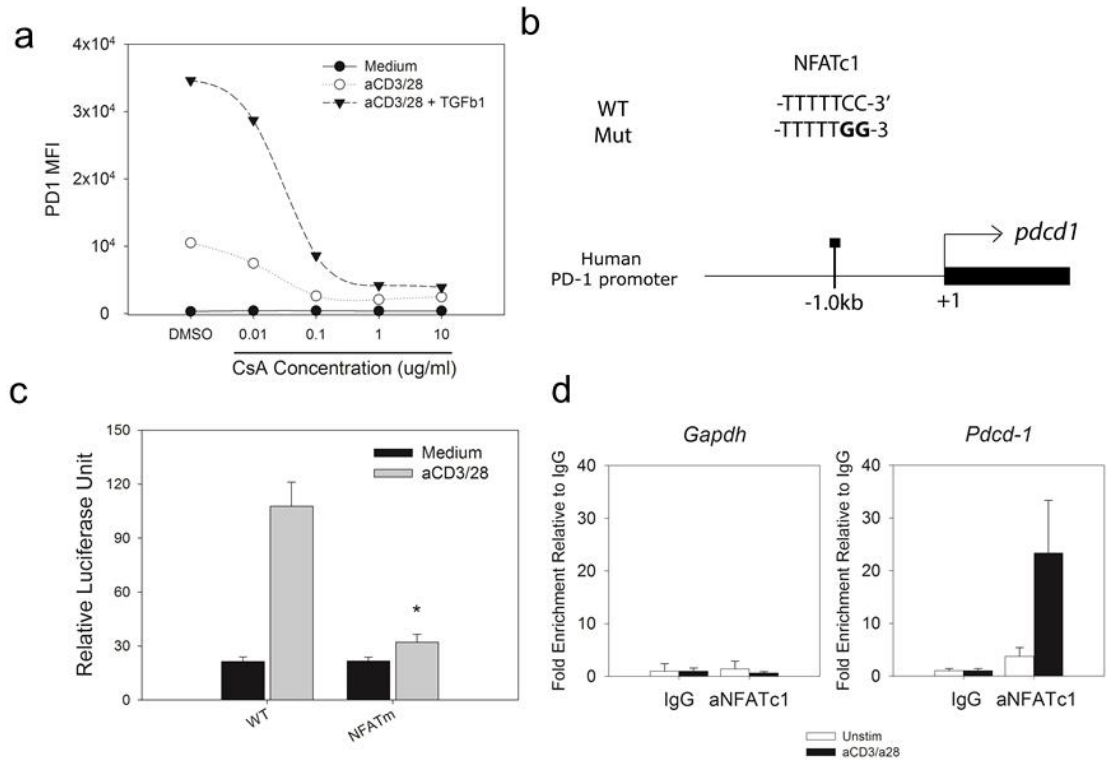
Figure 1. NFAT-c1 is critical for TCR-mediated PD-1 induction (a) Human peripheral

CD3+ T cells were isolated and activated with αCD3/αCD28-conjugated beads for 72hrs.

Cyclosporine A (CsA) was added after 24hrs of activation at varying concentrations and

PD-1 expression was assessed by flowcytometry: medium alone (filled circles);

αCD3/αCD28 (open circles). MFI is mean fluorescence intensity. (b) A putative

NFATc1 binding site on a human PD-1 promoter region. (c) Jurkat T cells were

transfected with a luciferase vector containing either the wild-type (WT) or mutant (Mut)

NFATc1 site of the human PD-1 promoter as described in the method section and

luciferase activity was measured after activation with αCD3/αCD28. (d) Chromatin

Immunoprecipitation analysis of NFATc1 on human PD-1 (*Pdcd-1*) or *Gapdh* promoter.

Human peripheral CD3+ T cells were isolated and activated with αCD3/αCD28-conjugated beads for 24hrs 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 control promoter region (*gapdh*) or the human *pdcd-1* promoter.
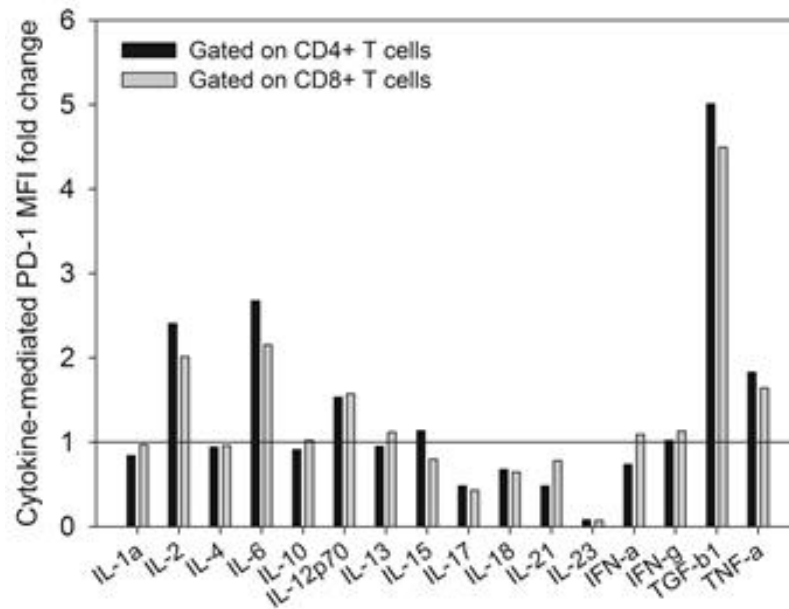
**Figure 2. Effects of multiple cytokines on T-cell PD-1 expression**

The effect of various cytokines on PD-1 expression is shown as fold changes in MFI relative to the αCD3/αCD28 condition with no cytokine in both CD4+ and CD8+ 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 the individual cytokine (500ng/ml or 500IU/ml) with a cell to bead ratio of 1:1 or 1:3. After 72hrs, the cells were harvested and CD3+ CD4+ or CD3+ CD8+ were gated in order to assess respective PD-1 surface expression via flow cytometry.

Figure 3. TGF-β1 enhances PD-1 expression on human naïve and memory T cells.

Human CD3+ T cells were isolated from healthy donor peripheral blood mononuclear

cells (PBMCs) and were activated with αCD3/α28-conjugated beads for 72hrs with or

without TGF-β1 (50ng/ml). (a) Representative plots PD-1 (Y-axis) vs. CFSE (X-axis) are

shown for different conditions. (b) PD-1 MFI is shown with αCD3/α28 activation in the absence of (filled circle lines) or with TGF-β1(open circle lines). The percentage of cells in each CFSE generation is shown with αCD3/α28 activation (black bar graphs) and αCD3/α28 activation in the presence of TGF-β1 (white bar graphs). (c) Naïve and memory of T cell subsets of both CD4+ and CD8+ T cells were isolated based on CCR7 and CD45RA expression. A representative histogram plot of PD-1 is shown. Shaded histogram: Isotype; thin histogram (αCD3/α28); and solid histogram (αCD3/αCD28 and TGF-β1). (d) Average PD-1 MFI values and standard errors of naïve and memory subsets are shown from gated CD8+ (left) and CD4+ (right) T cells.
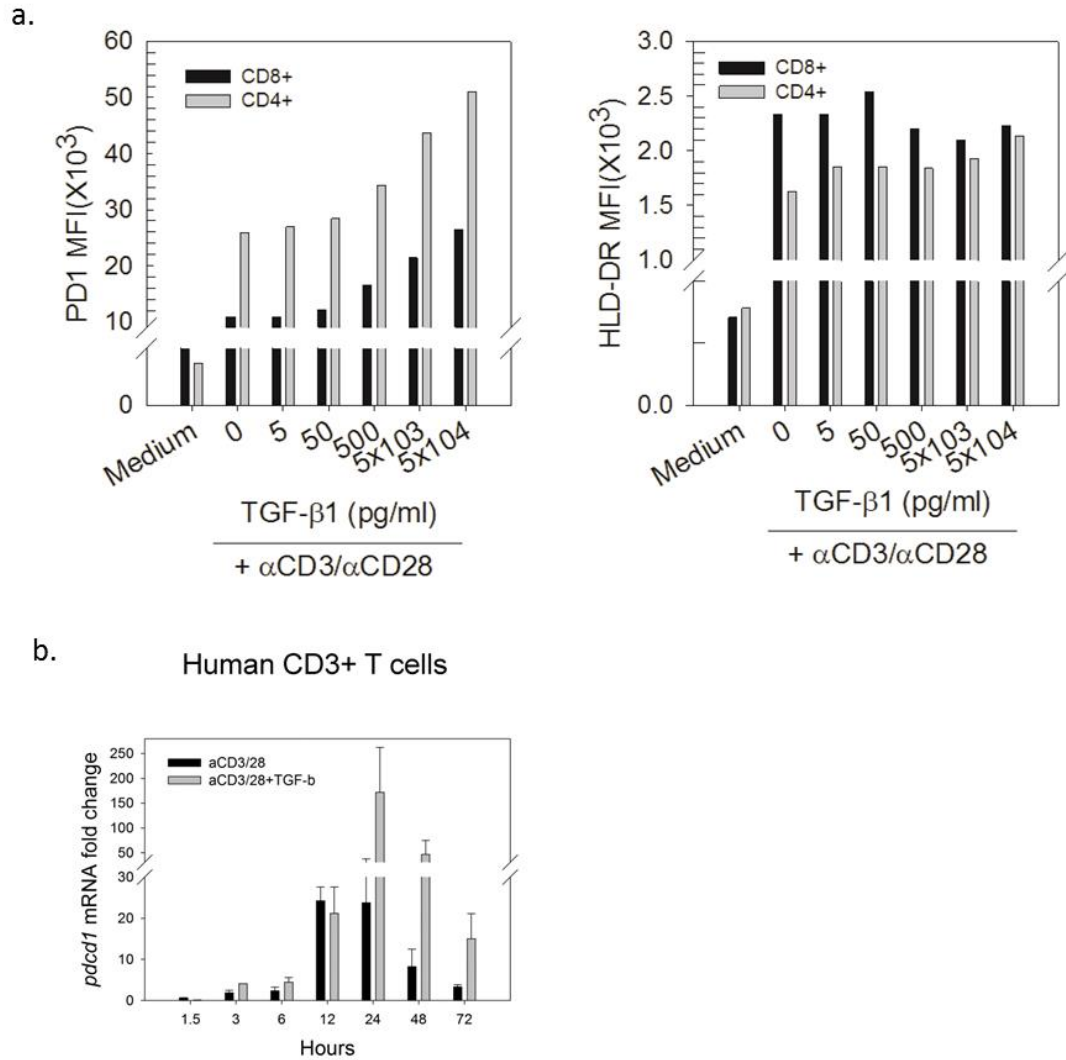
Figure 4. TGF-β1-enhances PD-1 expression in a dose-dependent manner through transcriptional regulation (a) Isolated human CD3+ T cells were activated with αCD3/αCD28-conjugated beads for 72hrs in the presence of varying concentrations of TGF-β1 (5 to 50,000 pg/ml). MFI of PD-1 (left) and HLA-DR (right) expression were assessed on CD4+ (light grey bars) and CD8+ (dark grey bars) T cells. The shown result is the representative of at least three independent trials. (b) Isolated human CD3+ T cells were activated with αCD3/αCD28-conjugated beads with or without TGF-β1 (50ng/ml) from 1.5 to 72 hours. *Pdcd-1* transcript levels in different conditions were normalized to

that of resting CD3+ T cells. The results are shown as means +/- SEM of duplicate

experiments and are representative of at least three independent trials.
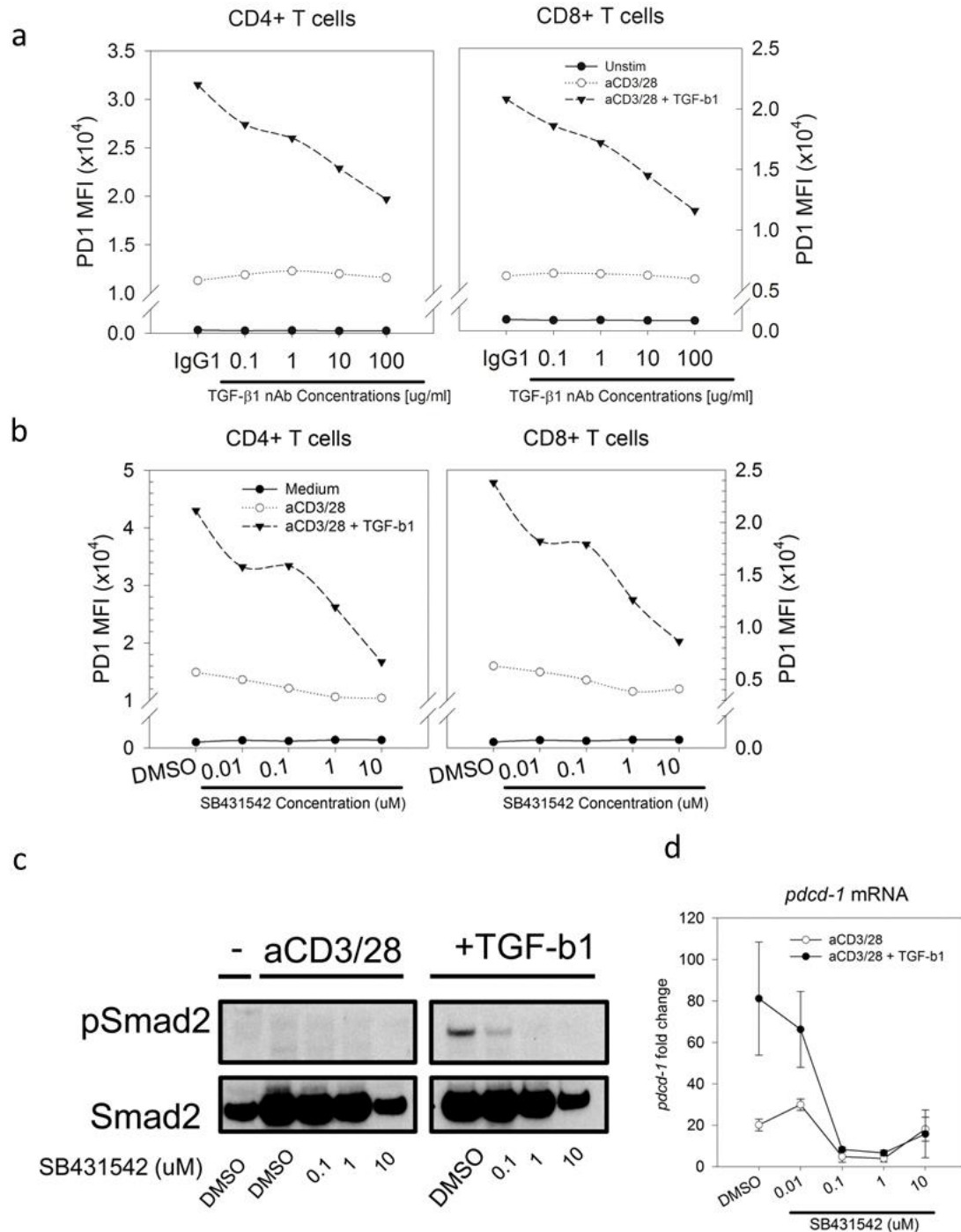
Figure 5. TGF-βR signaling is critical for PD-1 expression (a,b) Effects of anti-TGF-

β1 neutralizing antibody (nAb) and TGF-βRI kinase inhibitor (SB431542) on PD-1

expression. Enriched human CD3+ T cells were activated for 72hrs with αCD3/αCD28-conjugated beads and TGF-β1 under varying concentrations of TGF-β1 nAb (a) or SB431542 (b) and PD-1 MFI was assessed: medium alone (closed circles); αCD3/α28 only (open circles); αCD3/αCD28+TGF-β1 (closed triangles). The result shown is representative of at least three independent trials. (c) Western-blot analysis of phosphorylated-Smad2 (pSmad2) in human CD3+ T cells treated with varying concentrations of TGF-βRI kinase inhibitor (SB431542). (d) Effects of SB431542 on *pdcd-1* transcript levels. Enriched human CD3+ T cells were activated for 24hrs with αCD3/αCD28-conjugated beads and TGF-β1 under increasing concentrations SB431542. *Pdcd-1* transcript levels in each condition were normalized to that of resting human CD3+ T cells. The result is shown as means +/- SEM of duplicate experiments and representative of at least three independent trials.
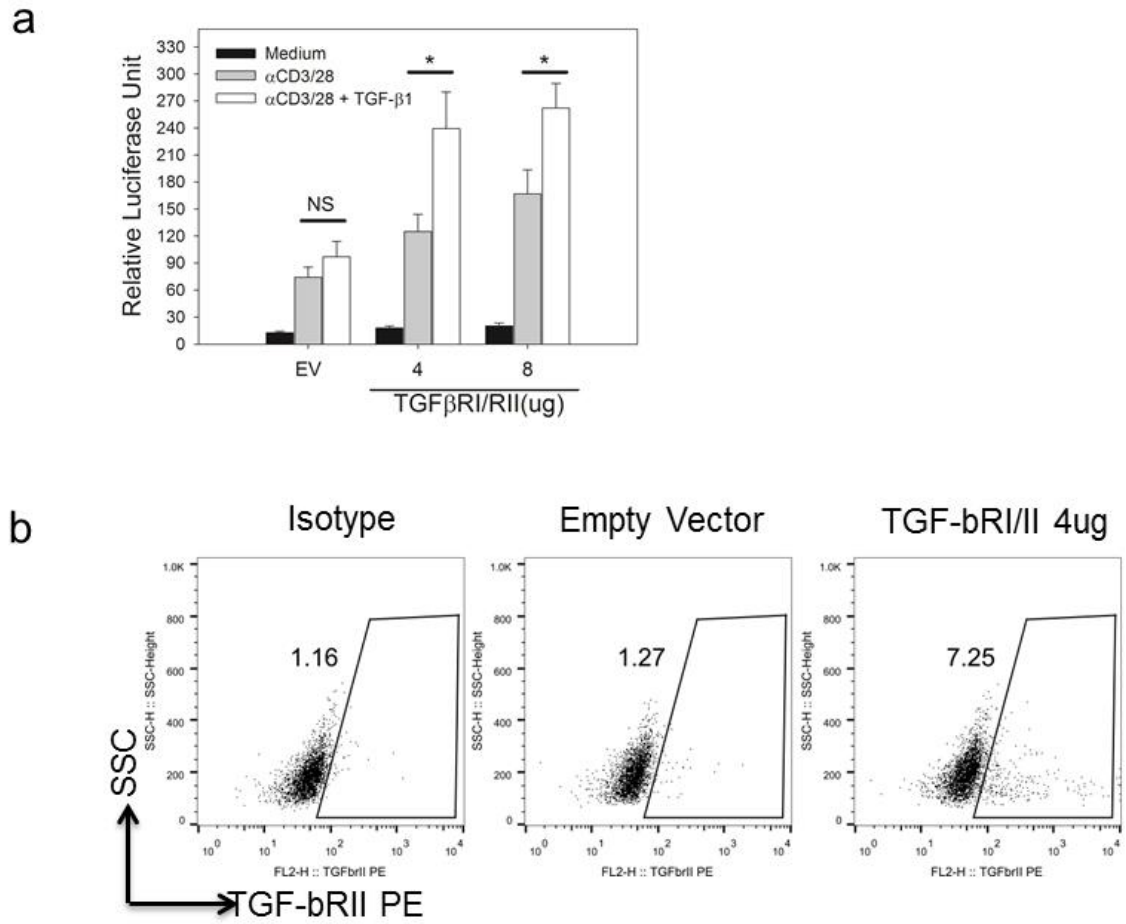
Figure 6. Overexpression of TGF-βRI and RII in Jurkat T cells (a) Jurkat T cells

were transfected with a 1.9kb long human PD-1 promoter-driven luciferase vector

together with different amounts of TGF-βRI and RII expression plasmids. Subsequently,

the cells were activated with αCD3/αCD8 with (white bars) or without TGF-β1 (grey

bars) and luciferase activity was measured. (b) Transfection efficiency of TGF-βRI and

RII expression plasmids on Jurkat T cells by flowcytometric analysis, as shown in SSC

and TGF-βRII.
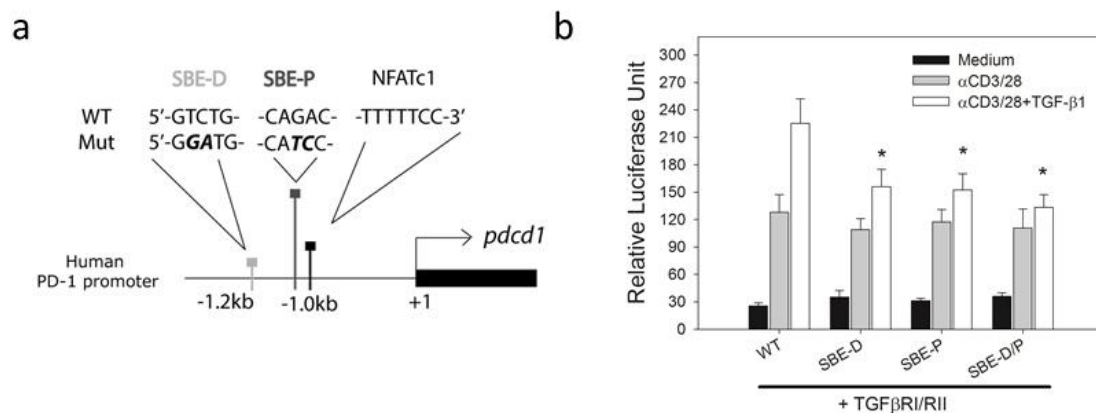
Figure 7. Smad-binding elements (SBEs) regulate PD-1 promoter activity

(a) Schematic illustration of the proximal region of the human PD-1 promoter. Two Smad-Binding-Elements (SBEs) are located at 1.2kb (SBE-D) and 1.0kb (SBE-P) upstream of the PD-1 transcription start site. NFATc1 consensus sequence is located in immediate proximity to SBE-P. Wild-type (WT) and mutated (Mut) sequences of both SBE-D and SBE-P are shown. (b) Effects of TGF-β1 on PD-1 promoter activity. Jurkat T cells were transfected with luciferase reporter vectors containing wild-type (WT), mutant SBE-D, mutant SBE-P, and mutant SBE-D/P sequences of PD-1 promoter (1.9kb). TGF-βRI and RII expression plasmids were co-transfected, and after 12hrs of resting, the cells were activated with plate-bound αCD3 and soluble αCD28 in the absence (grey bars) or presence (white bars) of TGF-β1 (50ng/ml). Luciferase activity was measured after 24hrs as described in the method.
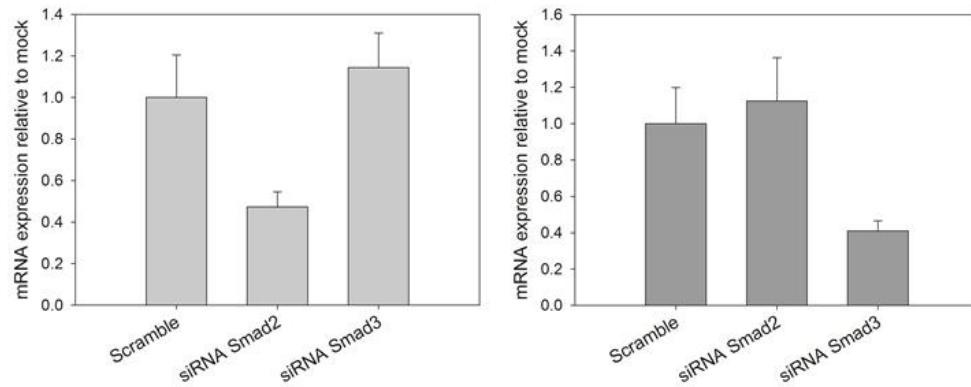
Figure 8. Efficiency of siRNA-mediated knock-down of Smad2 and Smad3

(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. (b) siRNA transfected Jurkat T cells were harvested and lysed for western blot analysis of Smad2 and Smad3.
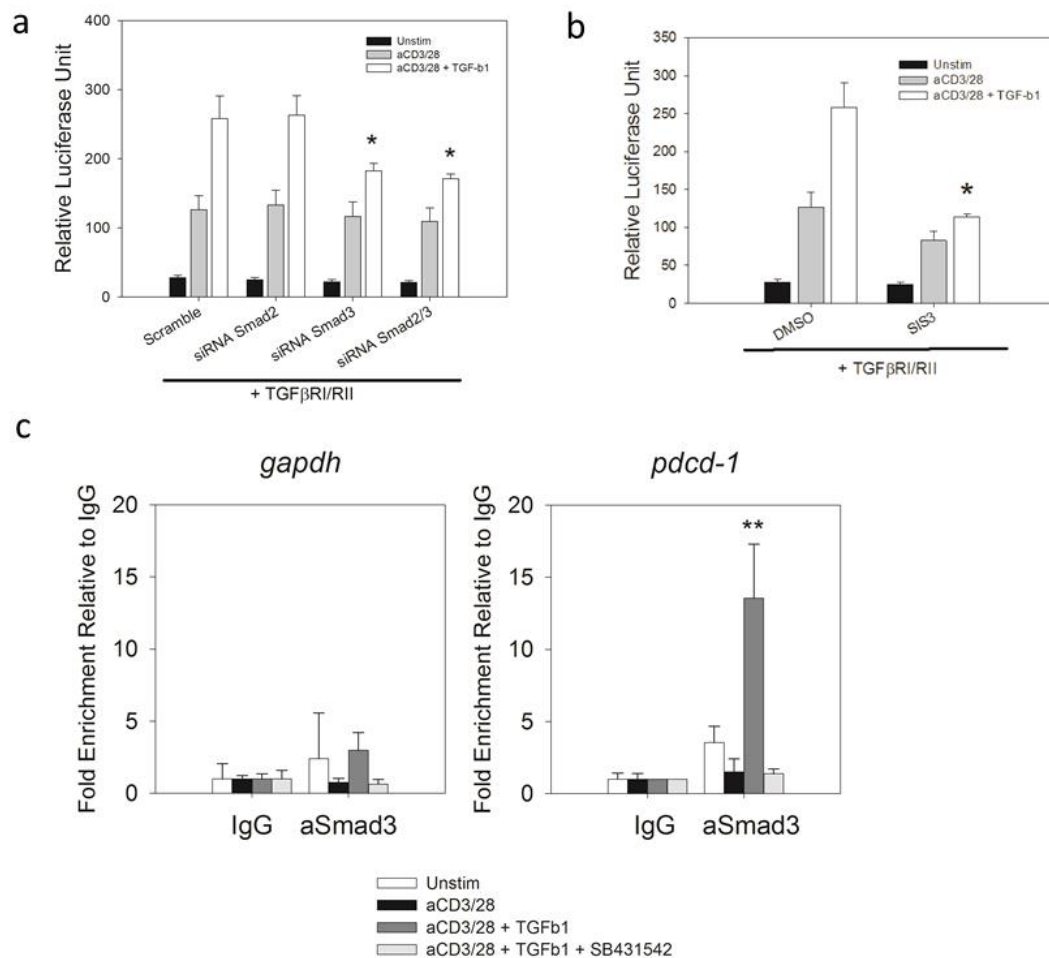
Figure 9. Smad3 regulates PD-1 promoter activity by direct binding

(a) Effects of Smad3 siRNA on PD-1 promoter activity. Jurkat T cells were co-transfected with 1.5uM of scrambled siRNA against Smad2 or Smad3 and PD-1 promoter-driven luciferase activity was measured in relative luciferase units (firefly/renilla luciferase activity). (b) Effects of Smad3 Inhibitor on PD-1 promoter activity. Transfected Jurkat T cells were treated with 10uM of Specific Inhibitor of Smad3 (SIS3) and PD-1 promoter-driven luciferase activity was measured after 24hrs of activation. The result is shown as mean +/- SEM of duplicate experiments and is representative of at least three independent trials. *p<0.05, **p<0.001. (c) TGF-β1

induced Smad3 binding to human PD-1 promoter. Isolated human CD3+ T cells were activated for 24hrs under different conditions: medium alone (white bars); αCD3/α28 alone (black bars); αCD3/α28 with TGF-β1 (dark grey bars); αCD3/αCD28 + TGF-β1 with SB431542 (light grey bars). Immunoprecipitated DNA was subjected for qPCR and fold enrichment of binding relative to IgG is shown as mean +/- SEM of triplicate results.
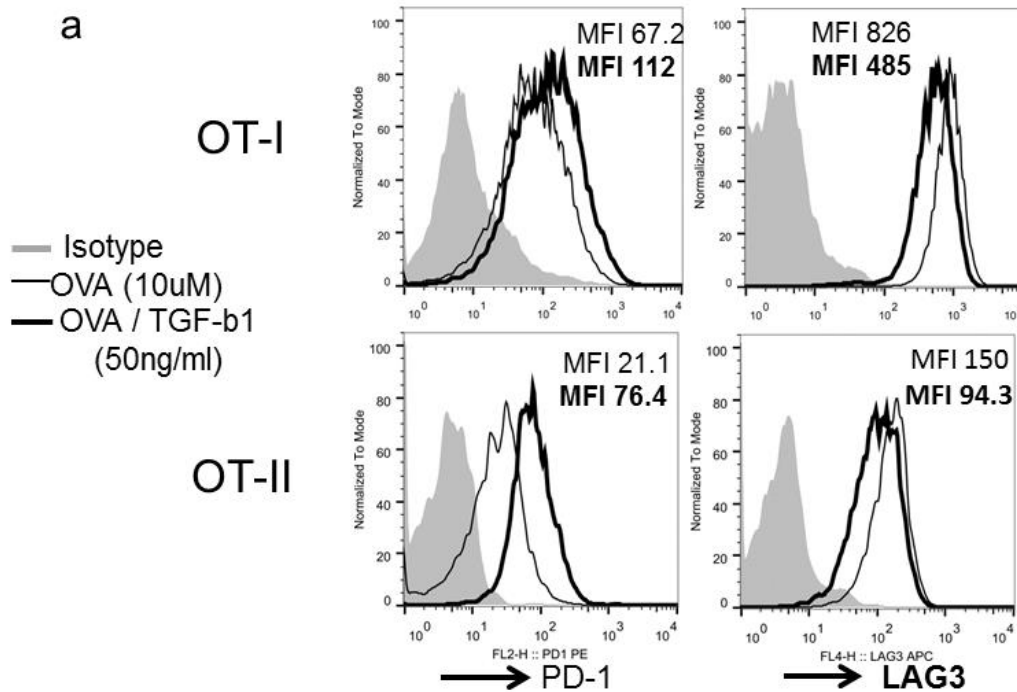
Figure 10: Differential effects of TGF-β1 on inhibitory receptors on murine T cells
Ovalbumin-specific CD8+ (OT-I) (top) and CD4+ (OT-II) (bottom) T cells were enriched by magnetic isolation from the spleen and activated for 72hrs with type-I ovalbumin (Ova) or type-II Ova (10ug/ml) in the presence of irradiated splenocytes under different conditions. PD-1 (left) and LAG3 (right) expression are shown in representative histograms: peptide alone (thin line histogram), peptide with TGF-β1 (50ng/ml) (bold line histogram), isotype (shaded histogram).
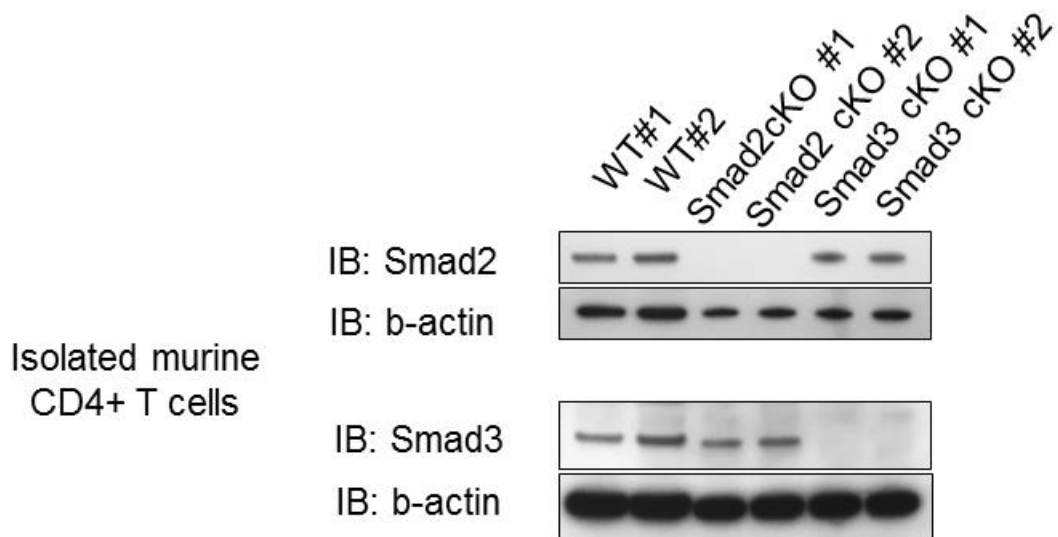
Figure 11: Western blot analysis of cre-mediated gene knock-out in Smad2 and Smad3 cKO CD4+ T cells. Naïve CD4+ T cells (CD4+ CD25- CD62L+) were flow-sorted from WT, Smad2 and Smad3 cKO mice and were activated with αCD3/αCD28 for 72hrs. The cells were harvested and lysed for western blot analysis of Smad2 and Smad3 expression as described in the method section.

a

Polyclonal CD4+ T cells

WT   Smad2 cKO   Smad3 cKO

Isotype
Stim
Stim + TGF-b1

PD-1

aCD3/aCD28
aCD3/aCD28 + TGF-b1

b

OT-I

WT   Smad2 cKO   Smad3 cKO

Isotype
OVA
OVA + TGF-b1

MFI 60   MFI 39.3   MFI 40.6
MFI 149   MFI 116   MFI 79.1

PD-1

MFI 93.0%   MFI 96.0%   MFI 93.0%
MFI 97.5%   MFI 97.8%   MFI 96.3%

CFSE

c

CD4+ T cells   CD8+ T cells

Stim
Stim + TGF-b1

PD-1 MFI (x10⁴)

DMSO 2.5 5 10 20
SIS3 Concentration (uM)
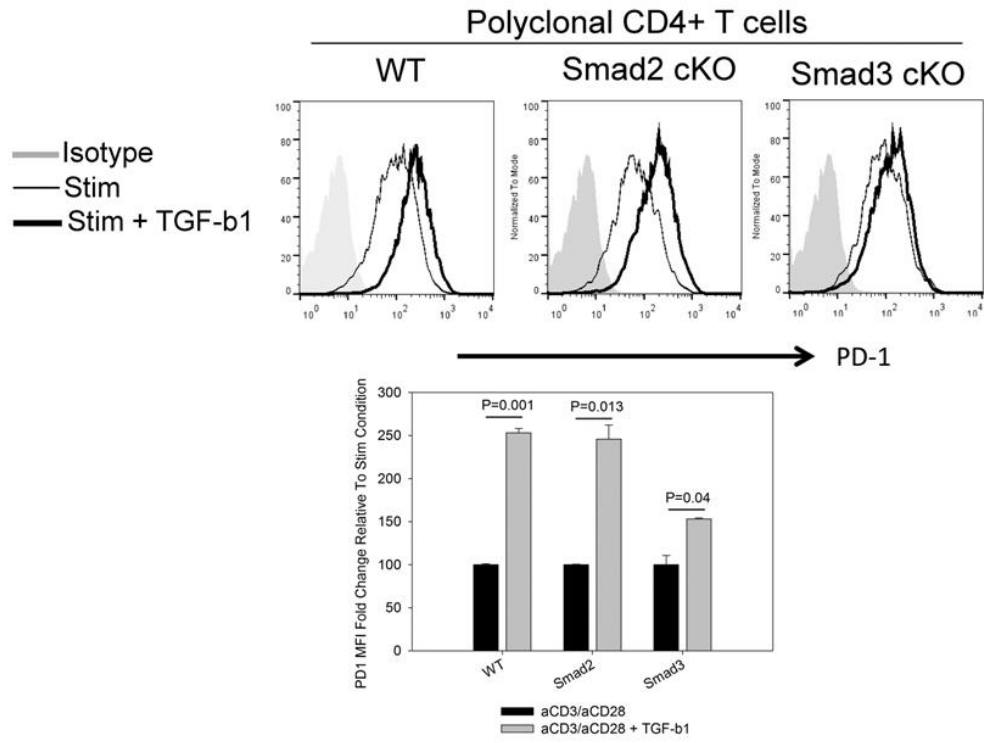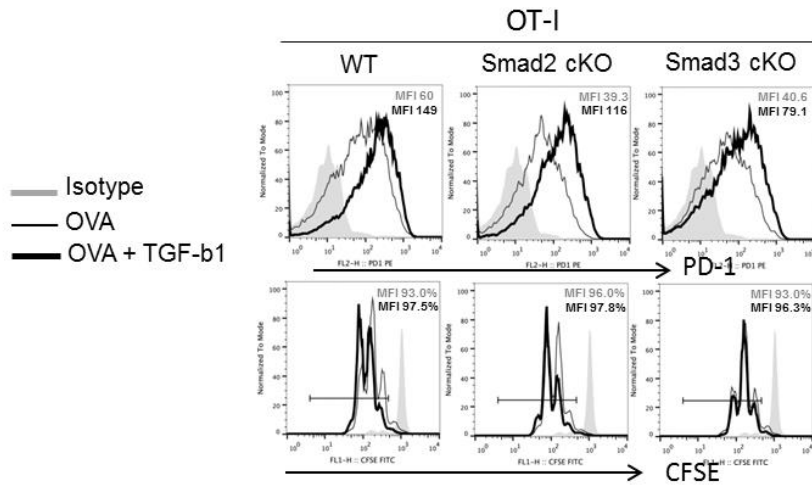
Figure 12. Smad3 is a major contributor of TGF-β1 dependent PD-1 expression

(a) Smad3-mediated PD-1 expression on murine CD4+ T cells. CD4+ T cells were isolated from wild-type (WT), Smad2 f/f; CD4-cre (Smad2 cKO), Smad3 f/f; CD4-cre (Smad3 cKO) mice and activated with plate-coated αCD3 and soluble αCD28 with (bold histogram) or without TGF-β1 (50ng/ml) (thin histogram) for 72hrs. PD-1 expression is shown as overlaid histograms with isotype control (shaded histogram), αCD3/αCD28 (thin histogram),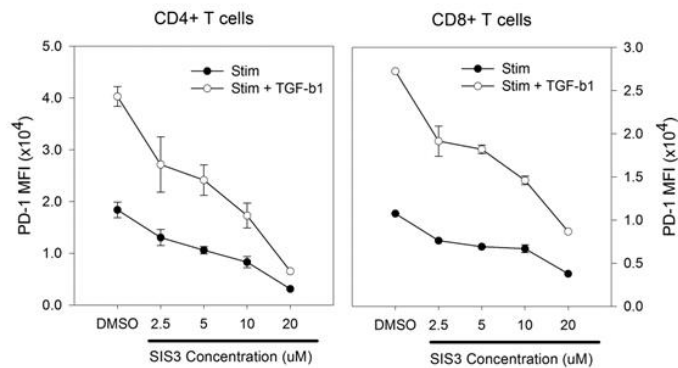 αCD3/αCD28+TGF-β1 (bold histogram). PD-1 MFI is also shown as the mean +/- SEM of two independent trials (bar graphs). (b) WT, Smad2 cKO, and Smad3 cKO OT-1 cells were magnetically isolated from the spleens and activated with type-I Ova in the presence of irradiated splenocytes for 72hrs. Representative PD-1 (top) and CFSE (bottom) expression are shown as overlaid histograms for different conditions: isotype control or resting conditions (shaded histogram), αCD3/αCD28 (thin histogram), αCD3/αCD28+TGF-β1 (bold histogram). (c) Effects of Specific Inhibitor of Smad3 (SIS3) on human CD3+ T cells PD-1 expression. Human CD3+ T cells from healthy donors were isolated and pretreated with SIS3 at varying concentrations. After 1hr, the cells were activated with αCD3/αCD28-conjugated beads for 72hrs with or without TGF-β1. MFI of PD-1 express in different conditions was assessed: αCD3/αCD28 (closed circle line); αCD3/αCD28 with TGF-β1 (open circle line).The data are representative of three independent trials.
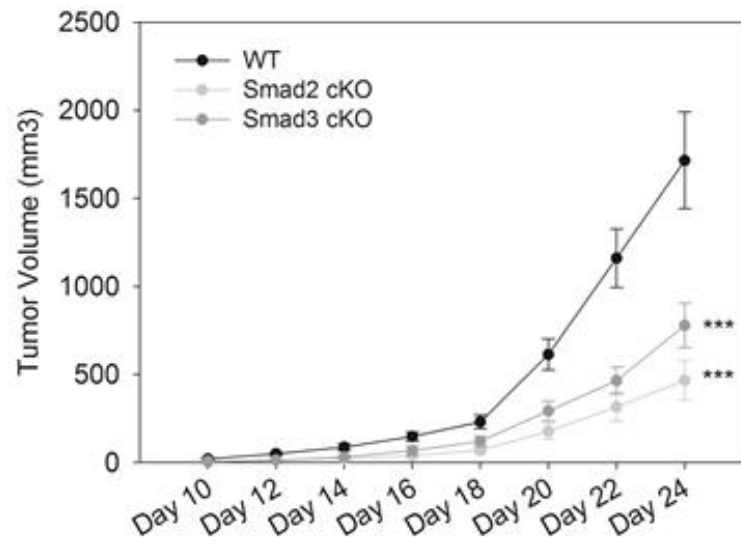
Figure 13. Growth kinetics of B16-melanoma in Smad2 and Smad3 cKO mice

Growth kinetics of B16 melanoma in WT, Smad2 cKO, and Smad3 cKO mic are shown

as the average volume +/- SEM on different days. A minimum of 6 mice per group was

used and the data shown are the combined results of two independent experiments.
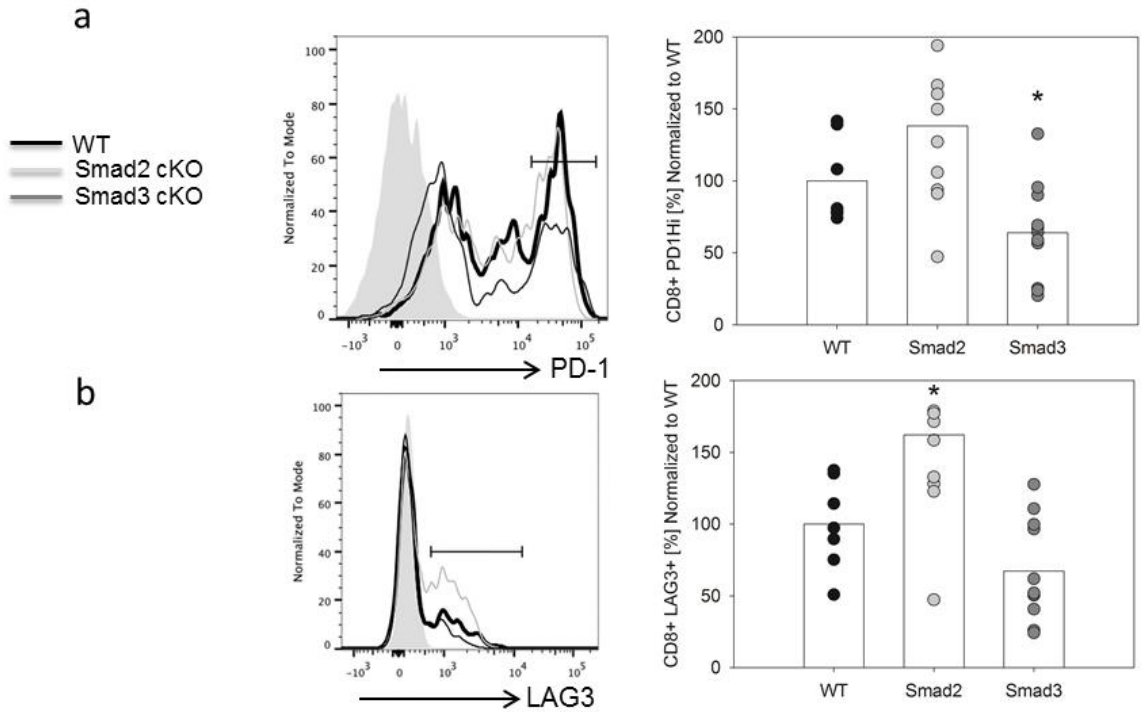
Figure 14. Smad3 regulates PD-1 expression on CD8+ T cells in B16-melanoma

PD-1 (a) and LAG3 (b) expression on tumor-infiltrating lymphocytes from WT (black), Smad2 cKO (light grey) and Smad3 cKO (dark grey) is shown as an overlaid histogram (left). Average CD8+ PD-1^{hi} or LAG3+ percentages in Smad2 cKO and Smad3 cKO TILs are shown as normalized values to WT CD8+ PD-1^{hi} and LAG3+ percentages (right).
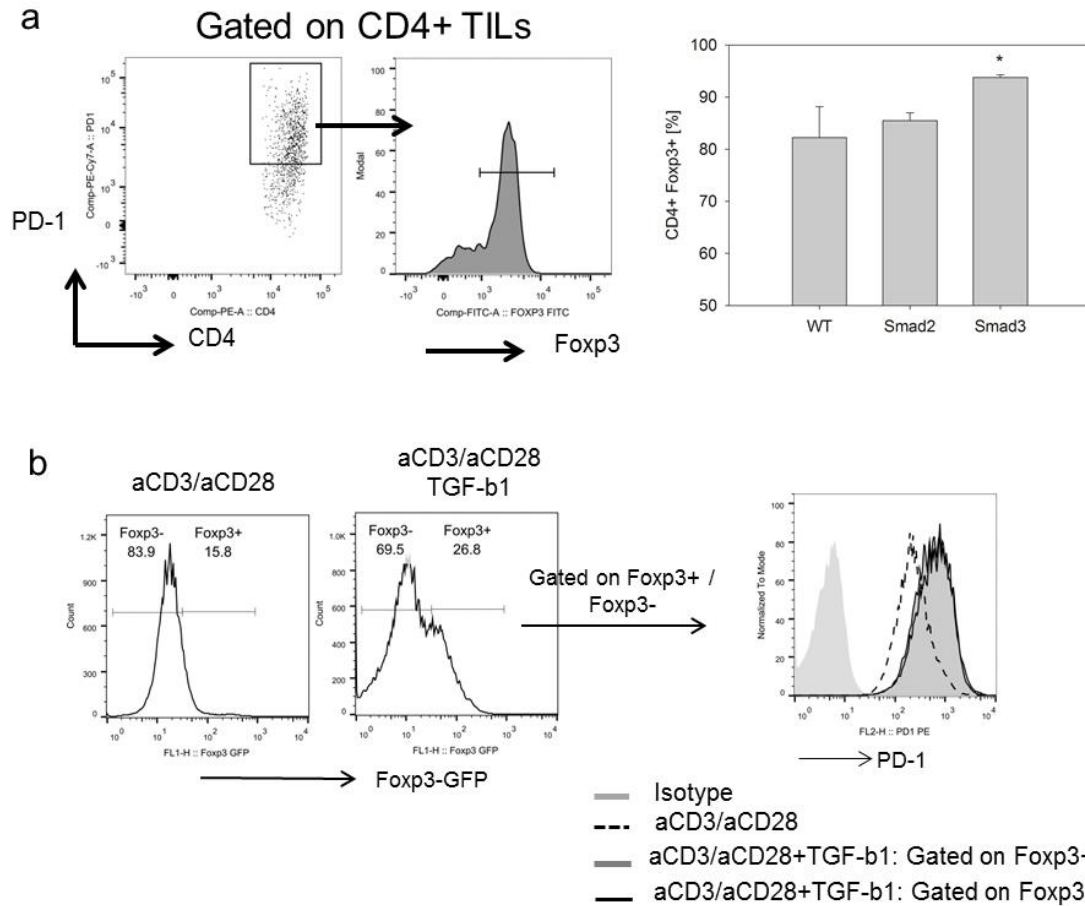
Figure 15. Regulatory CD4+ T cells and PD-1 expression

(a) 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 Foxp3+ (%) in each group is shown as average and standard errors. (b) CD4+ T cells were magnetically isolated from Foxp3-GFP transgenic mice, and were activated with αCD3/αCD28 for 72hrs with or without TGF-β1. PD-1 expression was separately assessed on GFP+ and GFP- subsets as shown in overlaid histograms: isotype (light shade); αCD3/αCD28 (dashed line); GFP+ subset from αCD3/αCD28+TGF-β1 condition (dark shade); GFP- subset from αCD3/αCD28+TGF-β1 condition (black line).
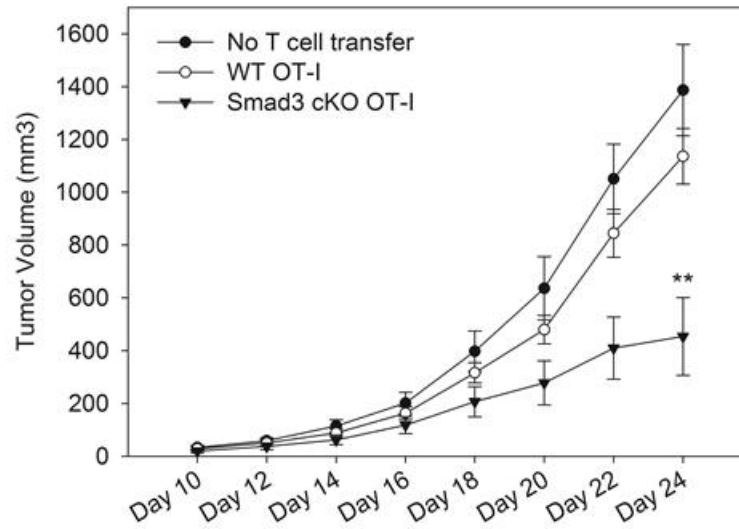
Figure 16. Growth kinetics of B16-Ova in mice that received WT and Smad3 cKO OT-1 adoptive T cell transfer. C57/BL6 expressing CD45.1 congenic markers were challenged with $1X10^5$ B16-Ova melanoma cell line on Day 0. On Day10, PBS (closed circles) or $1x10^7$ CD45.2 CD8+ OT-1 T cells from WT (open circles) or Smad3 cKO (triangles) mice were adoptively transferred into the mice with comparable tumor sizes. Average tumor volume (mm^3) is shown as mean +/- SEM on different days with at least n=6 mice per group from two independent experiments.
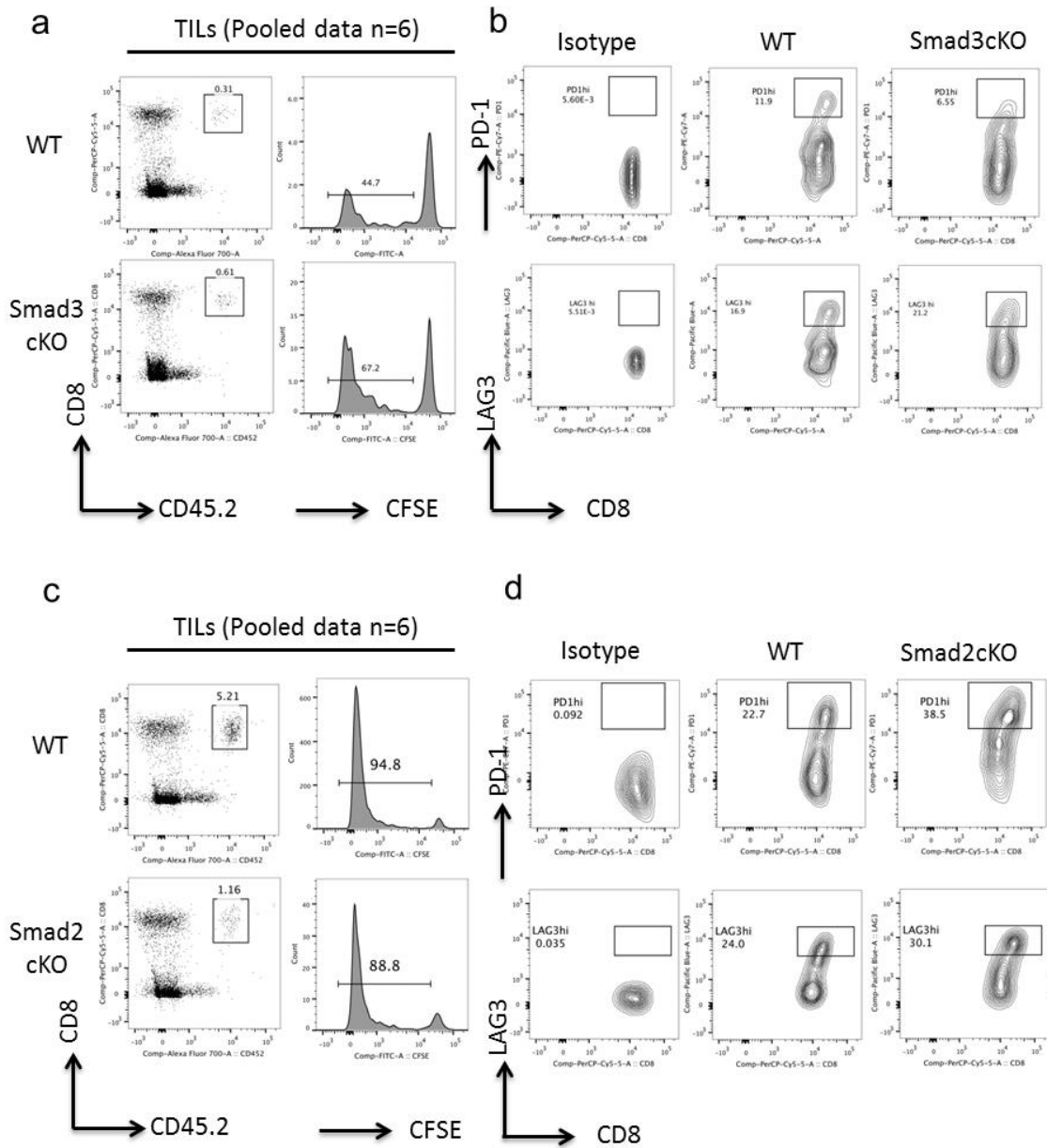
Figure 17. Inhibitory receptor expression on WT and Smad3 or Smad2 cKO OT-1 infiltrating the tumor microenvironment. (a,c) CFSE-labelled tumor infiltrating WT OT-1 (top) or cKO OT-1 (bottom) T cells were isolated from B16-Ova 5 days after adoptive transfer, and tumor infiltrating lymphocyte (TILs) proliferation was assessed for Smad3 cKO (a) and Smad2 cKO OT-1 (c) T cells. The CD45.2+ donor population was

gated from a plot of CD8 (Y-axis) and CD45.2 (X-axis) (left), and a representative

histogram of CFSE (right) is shown from pooled TILs from n=6 mice per group. (b,d)

Contour plots of PD-1 (top) and LAG3 (bottom) among the proliferated cells (i.e CFSE

negative populations) are shown as isotype (left), WT (middle) and cKO (right): Smad3

cKO (b) and Smad2 cKO (d). The data are representative of two independent experiments.
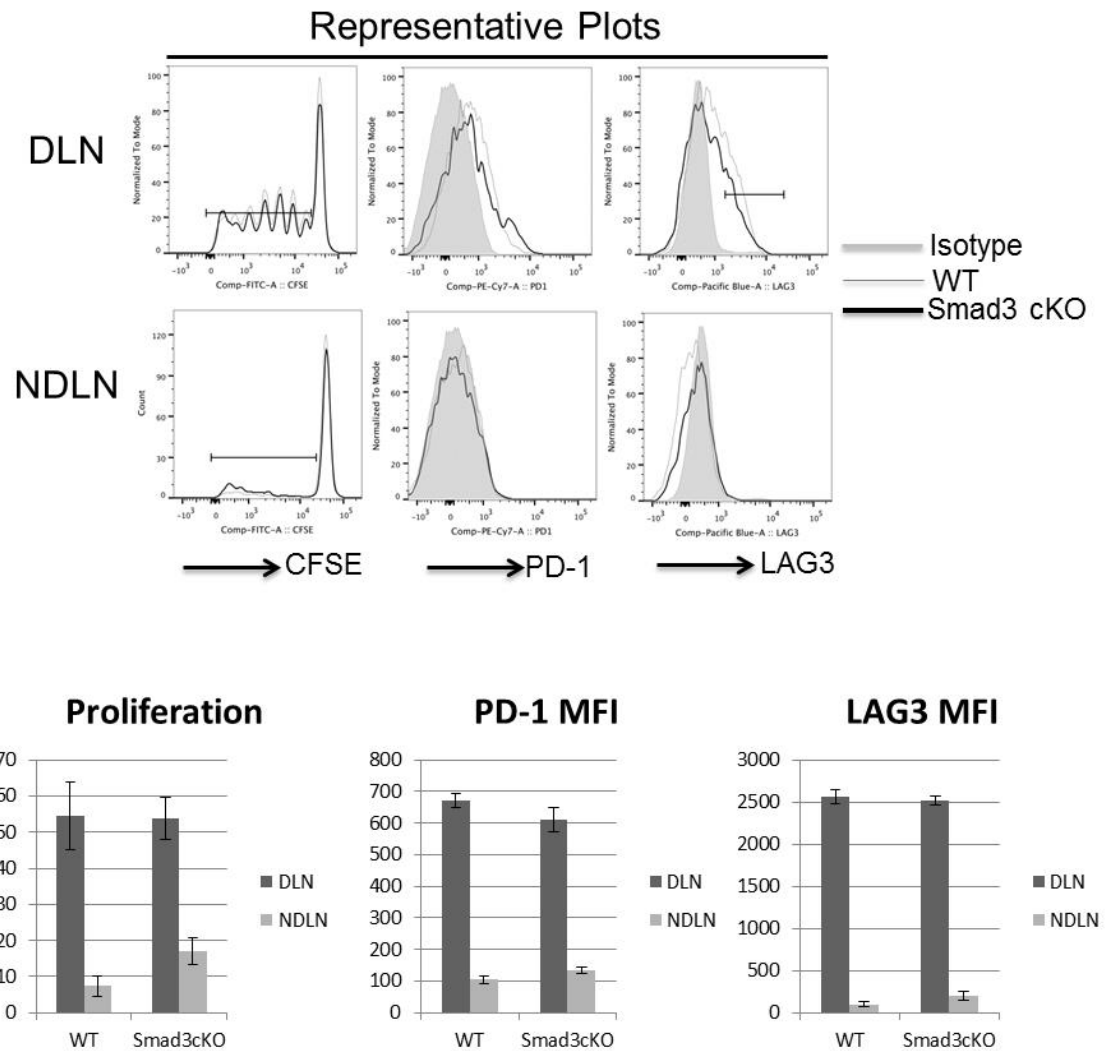
Figure 18. Inhibitory receptor expression on WT and Smad3 cKO OT-1 in the

lymph nodes

Representative histograms of CFSE, PD-1 and LAG3 expression on WT and Smad3 cKO

OT-1 T cells originating from the draining lymph nodes (top) and non-draining lymph

nodes (bottom). The result is shown as mean +/- SEM and is representative of two

independent trials.
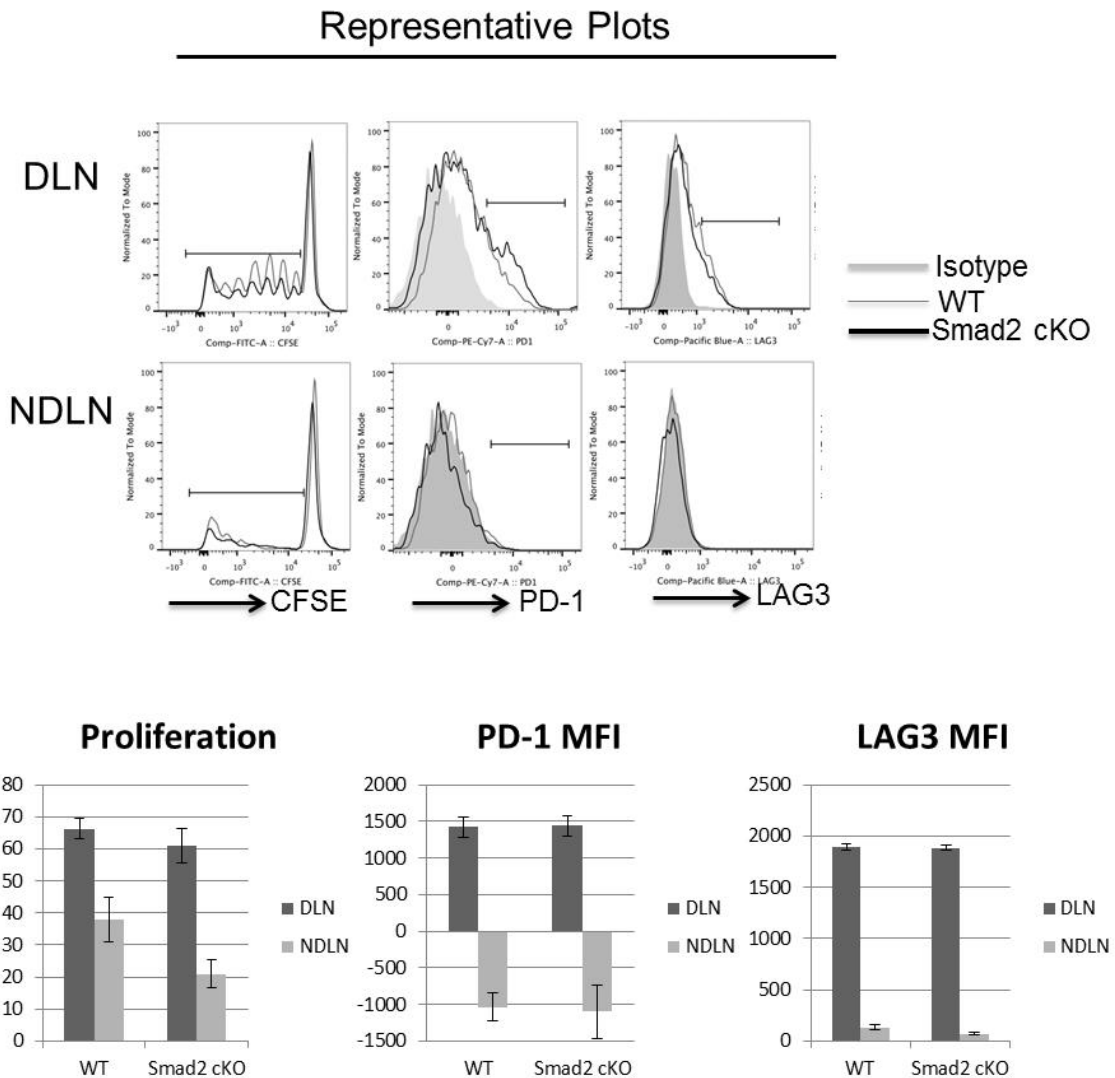
Figure 19. Inhibitory receptor expression on WT and Smad2 cKO OT-1 in the

lymph nodes

Representative histograms of CFSE, PD-1 and LAG3 expression on WT and Smad2 cKO

OT-1 T cells originating from the draining lymph nodes (top) and non-draining lymph

nodes (bottom). The result is shown as mean +/- SEM and is representative of two independent trials.
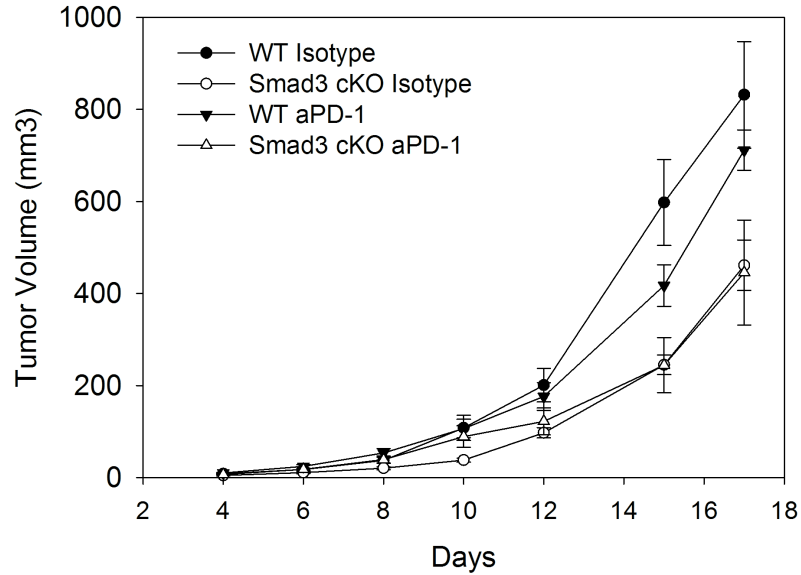
Figure 20. Effects of anti-PD-1 blocking antibody in Smad3-mediated immune suppression in B16-melanoma

Growth kinetics of B16 melanoma in WT (filled graphs) and Smad3 cKO mice (open graphs). WT and Smad3 cKO were challenged with 5×10^5 B16-melanoma cell line on Day 0. WT and Smad3 cKO mice were treated with either isotype-matched control IgG (circles) or anti-PD-1 antibody (triangles) from the day of tumor implantation until Day17. Average tumor volume (mm^3) is shown as mean +/- SEM on different days with at least n=6 mice per group from two independent experiments.
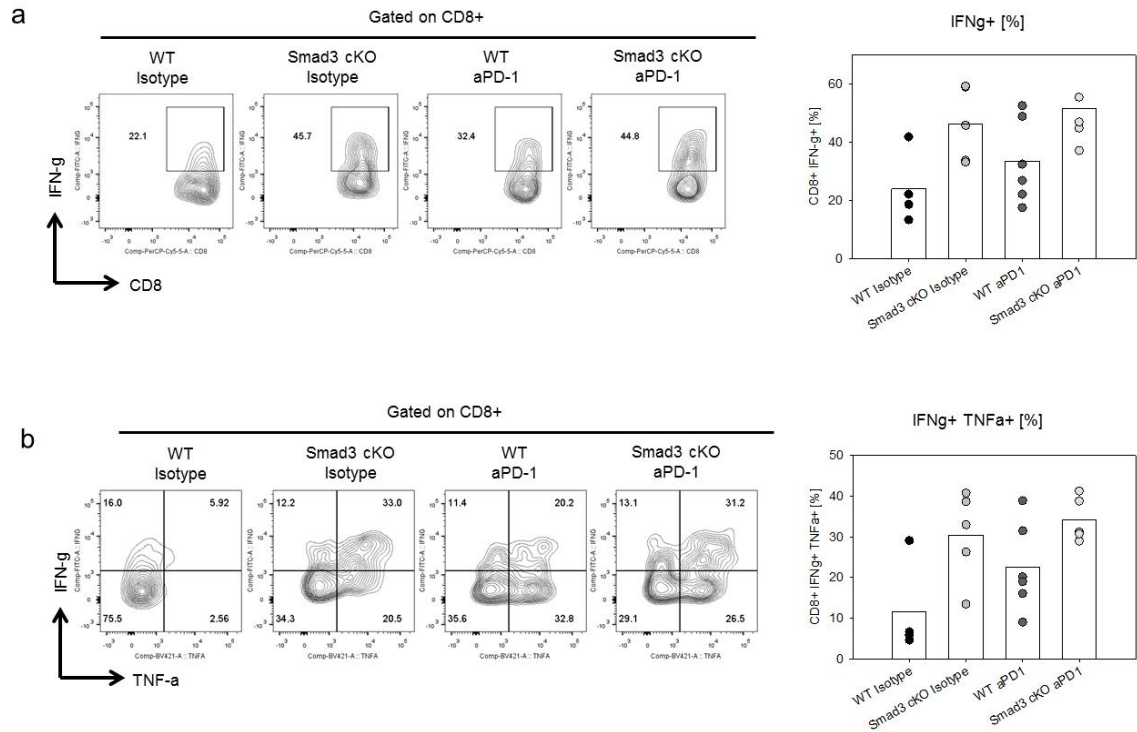
**Figure 21. Effector function of CD8+ TILs in B16-melanoma**

Tumor infiltrating lymphocytes were isolated from the tumor bearing mice of each group and were incubated with PMA/ionomycin in the presence of Golgi-inhibitor for 5hrs. The cells were extracellularly stained for CD3 and CD8 followed by intracellular staining of effector cytokines IFN-γ and TNF-α. Representative plots of IFN-γ+ single-positive subset (b) and IFN-γ+TNF-α+ double positive subset (c) among CD8+ T cells. The data are representative of two independent experiments.

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Senior Undergraduate Research	June 2006-June 2007	Laboratory of Ellen Rothenberg, California Institute of Technology

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ACADEMIC HONORS

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PEER-REVIEWED PUBLICATIONS

Park BV, Chattergoon MA, Rutebemberwa A, Levine J, Winter M, Sebald SM, Lee SJ, Pan F, Pardoll DM, Cox AL (2015) TGF-β1 mediated Smad3 enhances PD-1 expression on antigen-specific CD8+ T cells in cancer: Manuscript in preparation.

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SCIENTIFIC PRESENTATIONS

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Park, B., Chattergoon, M., Cox, A, Pardoll, D., and Pan, F. (2014). PPAR-a negatively regulates Th17 development. 12th Annual Immunology Retreat at Johns Hopkins School of Medicine.

PATENTS

Pan F, Pan D, Pardoll DM, Barbi J, **Park BV.** Targeting YAP Activity in Combination with Immunomodulation to Alter Regulatory T Cell-mediated Immune Regulation in order to Treat Cancer. JHU reference #C13353