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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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Abstract

The receptor programmed death 1 (PD-1) inhibits T cell proliferation and plays a critical role in suppressing self-reactive T cells, and it also compromises antiviral and antitumor responses. To determine how PD-1 signaling inhibits T cell proliferation, we used human CD4⁺ T cells to examine the effects of PD-1 signaling on the molecular control of the cell cycle. The ubiquitin ligase SCF^{Skp2} degrades p27^{kip1}, an inhibitor of cyclin-dependent kinases (Cdks), and PD-1 blocked cell cycle progression through the G₁ phase by suppressing transcription of *SKP2*, which encodes a component of this ubiquitin ligase. Thus, in T cells stimulated through PD-1, Cdks were not activated, and two critical Cdk substrates were not phosphorylated. Activation of PD-1 inhibited phosphorylation of the retinoblastoma gene product, which suppressed expression of E2F target genes. PD-1 also inhibited phosphorylation of the transcription factor Smad3, which increased its activity. These events induced additional inhibitory checkpoints in the cell cycle by increasing the abundance of the G₁ phase inhibitor p15^{INK4} and repressing the Cdk-activating phosphatase Cdc25A. PD-1 suppressed *SKP2* transcription by inhibiting phosphoinositide 3-kinase–Akt and Ras–mitogen-activated and extracellular signal–regulated kinase kinase (MEK)–extracellular signal–regulated kinase (ERK) signaling. Exposure of cells to the proliferation-promoting cytokine interleukin-2 restored activation of MEK-ERK signaling, but not Akt signaling, and only partially restored *SKP2* expression. Thus, PD-1 blocks cell cycle progression and proliferation of T lymphocytes by affecting multiple regulators of the cell cycle.

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Author contributions: N.P. performed experiments in all phases of the studies, analyzed the data, and contributed to the preparation of the manuscript. J.B. established the method for the preparation of antibody-coated magnetic beads, performed experiments to optimize the culture conditions, performed the biochemistry studies of cell cycle analysis, and contributed to the preparation of the manuscript. V.P. was involved in the planning, execution, and analysis of the quantitative PCR studies. F.L. provided the Smad3 Ser213 phosphospecific antibody. L.L. performed flow cytometric analysis for the assessment of TGF- β receptor binding, prepared the schematic overview of the findings shown in Fig. 7, and contributed to the preparation of the manuscript. V.A.B. was responsible for the overall design, planning, and analysis of the studies and for the preparation of the manuscript.

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INTRODUCTION

Peripheral tolerance is a mechanism that suppresses the activation of self-reactive lymphocytes that escaped central tolerance, a process that occurs in the thymus and results in the elimination (deletion) of T lymphocytes that bear receptors for self-antigens. Although mechanisms of central tolerance result in the deletion of most self-reactive T cells, some T lymphocytes that are specific for self-antigens escape this process, leave the thymus, and circulate in the periphery (1). To control the development of autoimmunity, multiple mechanisms of peripheral tolerance have evolved, including the induction of T cell anergy, the deletion of self-reactive T cells, and the suppression of effector T cells by regulatory T cells (T_{regs}) (2). Extensive experimental evidence indicates that the recently characterized pathway of the B7-CD28 family of co-receptors, which consists of the receptor PD-1 (programmed death 1, also known as CD279) and its ligands PD-L1 (also known as B7-H1 and CD274) and PD-L2 (also known as B7-DC and CD273), plays a vital role in the induction and maintenance of anergy and peripheral tolerance. This pathway also regulates the balance between the stimulatory and the inhibitory signals that are needed for effective immunity and the maintenance of T cell homeostasis (3).

PD-1 exerts its effects during the initial phase of the activation and expansion in number of autoreactive T cells by attenuating self-reactive T cells during presentation of self-antigen by dendritic cells (DCs) (4, 5). PD-1 also inhibits the functions of self-reactive effector T cells against nonhematopoietic tissues and mediates tissue tolerance by suppressing tissue-reactive T cells and protecting against immune-mediated tissue damage (6, 7). In addition to mediating T cell–intrinsic inhibitory effects, PD-1 inhibits T cell responses by promoting the induction and maintenance of inducible T_{regs} (iT_{regs}) (8). In contrast to its important beneficial role in maintaining T cell homeostasis, PD-1 mediates potent inhibitory signals after ligation with PD-1 ligands expressed on malignant tumors that prevent the expansion of effector T cells and have detrimental effects on tumor-specific immunity (9–12). Moreover, expression of PD-1 by “exhausted” virus-specific T cells that are characteristic of chronic viral infections prevents the proliferation and function of virus-specific effector T cells and clearance of the virus (13–16). Although the potent antiproliferative function of PD-1 is well established, it is unknown how PD-1 affects the molecular events of the cell cycle machinery, thereby leading to blockade of cell cycle progression and T cell proliferation.

Cell cycle progression is a tightly regulated process that depends on the abundance and activation of positive and negative regulators of the cell cycle machinery. Expression of D1-type cyclins occurs during entry into G_1 phase, induction of cyclin E occurs at the late G_1 phase, and expression of cyclin A occurs at the S phase. Cyclins associate with specific cyclin-dependent kinases (Cdks), which provide enzymatic activity to the cyclin-Cdk holoenzyme complexes and are inhibited by Cdk inhibitors. D-type cyclins associate with Cdk4 and its homolog Cdk6, whereas cyclin E and cyclin A associate with Cdk2. $p27^{\text{kip1}}$, a member of the Kip/Cip family of Cdk inhibitors, is abundantly expressed in T cells and suppresses the enzymatic activity of cyclin-Cdk complexes operative in all the phases of the cell cycle. In contrast, $p15^{\text{INK4}}$, a member of the INK family of Cdk inhibitors, selectively suppresses the enzymatic activity of the cyclin-Cdk complexes operative at the G_1 phase. Cdks promote cell cycle progression, in part by phosphorylating the transcription factor

retinoblastoma (Rb) and related pocket proteins, thereby reversing their ability to sequester E2F transcription factors, which then leads to the expression of E2F-regulated genes (17). Cdk2 also phosphorylates the signal transducers Smad2 and Smad3, transcription factors that transmit signals downstream of the transforming growth factor- β receptor (TGF- β R). Smad3 inhibits cell cycle progression from the G₁ to the S phase, and impaired phosphorylation of its Cdk2-specific site renders it a more effective inhibitor. In contrast, Cdk2-mediated phosphorylation reduces the transcriptional activity and antiproliferative functions of Smad3 (18). Cdks are also regulated by the tyrosine phosphatase Cdc25A, which reduces the extent of their tyrosine phosphorylation, enabling Cdk activation (19).

Here, we examined the effects of PD-1 on the regulation of cell cycle progression in primary human CD4⁺ T cells in response to growth signals mediated through stimulation of the T cell receptor (TCR) and CD3 complex and the CD28 costimulatory pathway. Our studies showed that PD-1 abrogated induction of the gene that encodes Skp2, the substrate-recognition component of the Skp1–Cullin–F-box (SCF) family ubiquitin ligase SCF^{Skp2} that leads to the ubiquitin-dependent degradation of p27^{kip1}, thereby resulting in the accumulation of p27^{kip1} and inhibition of Cdk2. Subsequent to the impaired activity of Cdk2, which resulted in the defective phosphorylation and enhanced transactivation of the transcriptional activator Smad3, PD-1 abrogated the production of the Cdk-activating phosphatase Cdc25A and increased the abundance of the G₁ phase inhibitor p15^{INK4B}. The inhibitory effect of PD-1 on Skp2 abundance was mediated by inhibition of the phosphoinositide 3-kinase (PI3K)–Akt and the Ras–mitogen-activated or extracellular signal-regulated kinase kinase (MEK)–extracellular signal-regulated kinase (ERK) pathways and was only partially reversed by interleukin-2 (IL-2) signaling, which restored activation of MEK-ERK signaling but not that of Akt. Thus, selective effects of PD-1 on Akt and MEK-ERK signaling pathways regulate components of the cell cycle and T cell proliferation.

RESULTS

PD-1 prevents progression of primary human T cells to the S phase of the cell cycle

Signaling through PD-1 inhibits T cell proliferation, but the molecular events that mediate blockade of the cell cycle in response to PD-1 have not been identified. To investigate these events, we performed experiments with highly purified human CD4⁺ T cells and magnetic beads conjugated with monoclonal antibodies (mAbs) against CD3 and CD28 with or without agonist mAb against PD-1 (20), similarly to an approach previously used in a mouse cell system (8). Primary T lymphocytes that naturally reside in the G₀ phase of the cell cycle (21–24) provide an ideal physiological model to study the cell cycle entry and S phase progression of T cells in response to growth signals generated through the TCR-CD3 complex in conjunction with the costimulatory and co-inhibitory receptors of the B7-CD28 superfamily, CD28 and PD-1, respectively. Ligation of PD-1 during TCR-CD3- and CD28-mediated activation inhibited DNA synthesis in primary human T cells (Fig. 1A), consistent with previous observations (20). As determined with ethynyl deoxyuridine (EdU) staining, we found that ligation of PD-1 resulted in the cell cycle arrest of CD4⁺ T cells at the G₀-G₁ phase (Fig. 1B and fig. S1). PD-1 did not delay the kinetics of cell cycle entry and cell cycle

progression, but instead markedly suppressed the number of cycling CD4⁺ T cells (Fig. 1, B and C).

PD-1 does not induce increased production of TGF- β or of TGF- β receptors

Previous studies indicated that PD-1 enhances the effects of TGF- β in converting naïve T cells into iT_{reg} cells (8). In that experimental system, PD-1 signaling reduced the threshold amount of TGF- β required to convert naïve CD4⁺ T cells into iT_{regs} and facilitated the generation of iT_{regs} in the presence of amounts of TGF- β that were too low to induce the conversion of naïve CD4⁺ T cells in the absence of PD-1 signaling. Because TGF- β is a well-established inhibitor of T cell proliferation and is produced during the stimulation of T cells (25, 26), we sought to determine whether PD-1-mediated signals might lead to the increased production of endogenous TGF- β . Analysis of TGF- β secretion showed that comparable amounts of TGF- β were produced by T cells during stimulation through the TCR-CD3 complex and CD28 in the presence or absence of PD-1 signaling (Fig. 2A). Production of comparable amounts of TGF- β was confirmed by intracellular staining with TGF- β -specific antibodies (Fig. 2B).

Next, we examined whether PD-1 might increase the sensitivity of CD4⁺ T cells to TGF- β as a result of differential expression of TGF- β Rs. TGF- β signals through type I and type II transmembrane serine and threonine kinase receptors by binding to and bringing together both receptor types into a complex. The constitutively active type II receptor phosphorylates and activates the type I receptor, which then transduces the signal to downstream components (27). Real-time, quantitative polymerase chain reaction (PCR) analysis showed that the abundances of TGF- β RI and TGF- β RII mRNAs in stimulated cells were increased compared to those in unstimulated cells but were comparable in T cells stimulated in the presence and absence of PD-1 stimulation (Fig. 2C). To examine whether PD-1 increased the binding of TGF- β to TGF- β Rs on the cell surface, we examined the TGF- β binding capacity of CD4⁺ T cells under distinct culture conditions. We found that there was an increase in the TGF- β -binding capacity of CD4⁺ T cells stimulated through the TCR-CD3 complex and CD28 either in the presence or in the absence of PD-1 signaling as determined by analysis of the proportion of TGF- β -binding cells (Fig. 2D). The increase in the proportion of TGF- β -binding cells was slightly lower in T cells receiving PD-1 signals (Fig. 2, D and E, top panel). Moreover, under the latter conditions, T cells displayed less TGF- β binding at 24 hours of culture, as determined by the mean fluorescence intensity (MFI). The MFI of activated T cells was 16.9 ± 2.5 , whereas that of activated T cells stimulated through PD-1 was 7.7 ± 1.1 (Fig. 2E, lower panel), which suggested a lower abundance of TGF- β Rs on a per-cell basis. Thus, blockade of cell cycle progression by PD-1 was not a result of increased TGF- β production, TGF- β R abundance, or extent of TGF- β binding.

PD-1 inhibits the activation of Cdk2

To investigate the effects of PD-1 on the molecular components of the cell cycle machinery that mediated cell cycle arrest, we examined the abundance of cyclins and Cdks. Expression of D1-type cyclins occurs during entry into the G₁ phase, whereas induction of cyclin E occurs at the late G₁ restriction point, and expression of cyclin A is observed at the S phase. Cyclins associate with specific Cdks, which provide enzymatic activity to the cyclin-Cdk

holoenzyme complexes and are inhibited by Cdk inhibitors. D-type cyclins associate with Cdk4 and its homolog Cdk6, whereas cyclin E and cyclin A associate with Cdk2 (17). Stimulation of T cells through the TCR-CD3 complex and CD28 increased the abundances of cyclin D1 and Cdk4, and PD-1 signaling had no effect on these events (Fig. 3, A and B). Similarly, during stimulation through TCR-CD3 and CD28 either in the presence or in the absence of PD-1 signaling, T cells showed increased abundance of Cdk2 and cyclin E, although cyclin E displayed slightly delayed kinetics of production in the presence of PD-1 signals (Fig. 3, A and B). In contrast, enhanced phosphorylation of Rb and the abundance of cyclin A, which is encoded by an E2F target gene and is a hallmark of the S phase, were markedly impaired in the presence of PD-1 (Fig. 3, A and B). Thus, although PD-1 blocked progression to the S phase of the cell cycle, it did not prevent the production of G₁ phase-specific cyclins and their associated Cdks, which suggests that cells undergoing PD-1 signaling enter the cell cycle but do not progress past the G₁ restriction point to the S phase.

Because PD-1 induced cell cycle arrest at the G₁ phase without affecting the abundances of cyclin E or Cdk2, we hypothesized that PD-1 might affect the activation of Cdk2, which mediates its effects at the G₁-S phase transition. The Cdk inhibitor p27^{kip1}, a member of the Kip family of Cdk inhibitors, physically interacts with Cdk2. Through this interaction, p27^{kip1} inhibits activation of the cyclin E-Cdk2 and cyclin A-Cdk2 complexes (17, 28). Although p27^{kip1} also associates with the cyclin D-Cdk4 complex (and with the cyclin D-Cdk6 complex), this interaction does not inhibit cyclin D-associated enzymatic activity, but instead acts to sequester p27^{kip1}, thereby reducing its association with cyclin E-Cdk2 and facilitating activation of the cyclin E-Cdk2 complex (29).

Stimulation of T lymphocytes through the TCR and CD28 results in the decreased abundance of p27^{kip1}. This event is regulated by ubiquitin-dependent degradation of p27^{kip1} protein (22, 23), which is mediated by the ubiquitin ligase SCF^{Skp2} (30). Stimulation through the TCR-CD3 complex and CD28 activates expression of the gene encoding Skp2, the substrate-recognition component of the SCF^{Skp2} (21). On the basis of those previous studies and our current findings that PD-1 induced arrest of the cell cycle at the G₁ phase without affecting the abundance of cyclin E and Cdk2, we examined the activation of Cdk2 and the abundances of p27^{kip1} and Skp2 in cells stimulated through TCR-CD3 and CD28 in the presence or absence of PD-1 signaling. Activation of Cdk2, as determined with an antibody specific for Cdk2 phosphorylated at Thr¹⁶⁰ (which is phosphorylated upon Cdk2 enzymatic activation), was substantially impaired in activated T cells cultured under conditions of PD-1 stimulation compared to that in activated T cells cultured without PD-1 stimulation (Fig. 3, C and D).

Assessment of Cdk2 activity by in vitro kinase reaction with histone H1 as an exogenous substrate confirmed these results (fig. S2). Moreover, activated T cells cultured in the presence of PD-1 stimulation displayed a markedly increased amount of p27^{kip1} compared to that of activated T cells cultured without PD-1 stimulation (Fig. 3, C and D). In contrast to the enhanced amounts of p27^{kip1}, an increase in the abundance of Skp2 was abrogated by PD-1 signaling (Fig. 3, C and D). Consistent with our previous results (21), the increase in Skp2 protein abundance in response to stimulation of TCR-CD3 and CD28 was mediated by increasing the abundance of *SKP2* mRNA, which was abrogated by PD-1 signaling (Fig.

3E). In addition, phosphorylation of p27^{kip1} on Thr¹⁸⁷, which lies within a Cdk and a MAPK (mitogen-activated protein kinase) consensus site and is required for recognition by SCF^{Skp2} (31–34), was substantially diminished by PD-1 signaling (Fig. 3, C and D). Together, these data suggest that PD-1 abrogates the increase in the abundance of Skp2, which then results in the accumulation of p27^{kip1}, the inhibition of Cdk2 activity, and a blockade in the G₁-S transition.

PD-1 increases the transactivation of Smad3

Cdks promote cell cycle progression, in part by phosphorylating the transcription factor Rb and related pocket proteins, thereby reversing their ability to sequester E2F transcription factors, which then leads to the expression of E432F-regulated genes (17). Cdk2 also phosphorylates the signal transducers Smad2 and Smad3. Smad3 inhibits cell cycle progression from the G₁ to the S phase, and impaired phosphorylation of its Cdk2-specific site renders it a more effective inhibitor. Impaired Cdk2-mediated phosphorylation also increases DNA binding and transactivation by Smad3. In contrast, Cdk2-mediated phosphorylation reduces the transcriptional activity and antiproliferative function of Smad3 (18). Because ligation of PD-1 resulted in impaired Cdk2 activation, we examined whether Smad3 might be differentially phosphorylated on the Cdk2-specific site in cells stimulated through TCR-CD3 and CD28 in the presence or absence of PD-1 signals. The extent of phosphorylation of Smad3 on Ser²¹³, a site specifically phosphorylated by Cdk2 (18), was increased after 48 hours of T cell stimulation; however, PD-1 signaling substantially diminished the phosphorylation of Smad3 on Ser²¹³ (Fig. 3, C and D).

On the basis of these findings, we examined whether ligation of PD-1 altered the transcriptional activity of Smad3. We used a Smad-dependent reporter (35) to assess Smad3 transcriptional activity by luciferase assay. T cells activated through TCR-CD3 and CD28 in the presence of PD-1 signaling displayed abundant Smad3 transcriptional activity, whereas activated T cells stimulated in the absence of PD-1 signaling exhibited much less transcriptional activity of Smad3 (Fig. 4A). These results suggested a direct coupling between PD-1 signaling and enhanced Smad3 activity, which is regulated by cell-intrinsic mechanisms and not by differential TGF- β production or binding to TGF- β Rs. To establish whether PD-1 functioned in this pathway independently of TGF- β , we performed experiments with a TGF- β RII-Fc chimera that neutralizes the effects of TGF- β by competing for binding to TGF- β Rs, and we examined the transcriptional activity of Smad3 and cellular proliferation in cultures of T cells stimulated through TCR-CD3, CD28, and PD-1. We found that neutralization of TGF- β signaling did not affect the transcriptional activity of Smad3 or the antiproliferative function mediated by PD-1 (fig. S3).

PD-1 signaling differentially regulates the expression of the genes encoding p15^{INK4B} and Cdc25A

The Cdk4 and Cdk6 inhibitor p15^{INK4B} is a well-established transcriptional target of Smad3, and expression of the gene encoding p15^{INK4B} is stimulated by TGF- β (18, 36). In contrast, the TGF- β –Smad3 axis mediates transcriptional repression of the gene encoding the tyrosine phosphatase Cdc25A (37, 38), which is induced in an E2F-dependent manner (39), and activates Cdk4, Cdk6, and Cdk2 by reducing the extent of their tyrosine phosphorylation

(19). To determine the functional consequences of altered Smad3 transactivation by PD-1, we examined the effects of PD-1 on both of these transcriptional targets of Smad3. At 24 hours of culture, the amount of *CDKN2B* mRNA, which encodes p15^{INK4B}, was increased in activated T cells that were stimulated through PD-1 compared to that in activated T cells that were not stimulated through PD-1 (Fig. 4B). Conversely, *CDC25A* mRNA was almost undetectable in activated T cells stimulated through PD-1 (Fig. 4B). At 24 hours of culture, activated T cells with or without PD-1 stimulation displayed comparable amounts of p15^{INK4B} protein (Fig. 4, C and D), consistent with the comparable amounts of TGF- β produced during culture, as shown earlier (Fig. 2B). However, as a consequence of the enhanced transcriptional activity of Smad3 and the increased abundance of mRNA for the gene encoding p15^{INK4B} at 24 hours of culture, activated T cells that were stimulated through PD-1 for 48 or 72 hours displayed increased amounts of p15^{INK4B} protein compared to that of activated T cells that were not stimulated through PD-1 (Fig. 4, C and D). In contrast, at the same time points, activated T cells that were stimulated through PD-1 displayed substantially reduced amounts of Cdc25A protein (Fig. 4, C and D), consistent with the decreased abundance of *CDC25A* mRNA at 24 hours of culture (Fig. 4B). Furthermore, enzymatic activation of Cdk4 at 48 and 72 hours of culture, as determined with an antibody specific for Cdk4 phosphorylated at Thr¹⁷², a marker of Cdk4 activation, was decreased in activated T cells stimulated through PD-1 compared to that in activated T cells that did not receive PD-1-dependent signals (Fig. 4, C and D).

In addition to inducing transcriptional repression of the gene encoding Cdc25A, the TGF- β -Smad3 pathway promotes ubiquitin-dependent degradation of Cdc25 by the SCF ^{β} -TrCP E3 ubiquitin ligase complex (40). For this reason, we examined whether PD-1 might affect the ubiquitin-dependent degradation of Cdc25, and so we performed experiments with the proteasome inhibitor MG132 or dimethyl sulfoxide (DMSO) as control. The abundance of Cdc25A protein increased in T cells stimulated through TCR-CD3 and CD28 either in the presence or in the absence of MG132. After incubation with MG132, the abundance of Cdc25A protein also increased in T cells stimulated by TCR-CD3, CD28, and PD-1 and was comparable to that of T cells stimulated without PD-1 ligation (Fig. 4, E and F). These results suggest that in addition to inhibiting expression of the gene encoding Cdc25A, ubiquitin-targeted degradation is an important mechanism to reduce the abundance of Cdc25A protein in response to PD-1 signaling. To determine whether the reduction in Cdc25A abundance had a causative role in the antiproliferative effect mediated by PD-1, we performed experiments in T cells expressing a Cdc25A mutant (Cdc25AD81A/S82A) that is resistant to ubiquitin-dependent degradation (41). Expression of Cdc25AD81A/S82A substantially reversed the antiproliferative effect of PD-1 signaling in these cells (Fig. 4G).

Smad3 has an active role in regulating gene expression downstream of PD-1

To determine whether Smad3 had a causative role in regulating gene expression downstream of PD-1, we knocked down endogenous Smad3 with short hairpin RNA (shRNA) specific for Smad3 and examined the effect of PD-1 ligation on p15^{INK4B} abundance. Smad3-specific shRNA induced a 75% reduction in the amount of Smad3 protein in Smad3 knockdown cells compared to that found in T cells transfected with an expression plasmid encoding a scrambled shRNA sequence that does not lead to the specific degradation of any

known cellular mRNA (Fig. 5, A and B). Culture of Smad3 knockdown and control knockdown T cells indicated that depletion of endogenous Smad3 substantially impaired the ability of PD-1 to increase the abundance of p15^{INK4B} in activated T cells stimulated through PD-1 compared to that in activated T cells that did not receive PD-1 stimulation (Fig. 5, C and D). Further, under conditions of Smad3 knockdown, the antiproliferative effect of PD-1 was substantially reduced (Fig. 5E). These results indicate that Smad3 is a critical component of a pathway downstream of PD-1 that mediates the arrest of T cells at the G₁ phase of the cell cycle.

PD-1 inhibits signaling through the Ras and the MEK-ERK pathways

We previously demonstrated that expression of the gene encoding Skp2, the substrate-recognition component of the SCF^{Skp2} ubiquitin ligase, which lies downstream of TCR-CD3 and CD28 signaling, requires activation of the PI3K-Akt and MEK-ERK pathways. In contrast, the p38 MAPK has no effect on the abundance of Skp2 or the degradation of p27^{kip1} upon T cell activation (21, 22). Moreover, activation of Ras is mandatory for the reduction in p27^{kip1} abundance that occurs upon T cell activation (42), and defective Ras activation in anergic T cells results in the accumulation of p27^{kip1} protein and arrest of the cell cycle (24, 43). Although it was previously determined that PD-1 inhibits TCR-mediated activation of Akt (44), the specific effects of PD-1 on the MAPK pathways have not been determined.

We examined the effects of PD-1 on these signaling pathways upon stimulation of the TCR in primary human T cells. We found that Akt activation occurred within 1 min of stimulation (Fig. 6, A and B), as determined by detection of the phosphorylation of Akt at Thr³⁰⁸, which resides in the activation loop and is phosphorylated by the kinase PDK1 downstream of PI3K (45–47), and gradually peaked over 1 hour (fig. S4, A and B). Activation of Akt in cells was confirmed by analysis of the phosphorylation of the endogenous glycogen synthase kinase-3β (GSK-3β), an Akt-specific substrate (fig. S4, A and B). Under these conditions, PD-1 suppressed the activation of PI3K-Akt signaling, as suggested by detection of the diminished phosphorylation of both Akt and GSK-3β (Fig. 6, A and B and fig. S4), consistent with previous observations (44).

Next, we examined the effect of PD-1 signaling on the MAPKKs (MAPK kinases) and MAPKs MEK1/2, ERK1/2, p38, and JNK (c-Jun N-terminal kinase). MEK1/2 was phosphorylated and activated upon activation of T cells through TCR-CD3 and CD28, which resulted in phosphorylation of its downstream target ERK1/2, as determined by Western blotting analysis with an antibody specific for ERK1/2 phosphorylated at Thr²⁰² and Tyr²⁰⁴, markers of activation. PD-1 substantially inhibited the activation of MEK1/2 and ERK1/2 (Fig. 6, A and B). In contrast, PD-1 had no effect on the activation of JNK or p38, which displayed comparable magnitude and duration of phosphorylation in the presence or absence of PD-1 signaling (Fig. 6, A and B).

Because the MEK-ERK pathway lies downstream of Ras, we examined Ras activation in T cells activated through TCR-CD3 and CD28 in the presence or absence of PD-1 signaling. We observed robust and sustained Ras activation when cells were stimulated through the TCR-CD3 complex and CD28 (Fig. 6, A and B). In contrast, concomitant ligation of PD-1

resulted in transient Ras activation that showed an early peak and rapidly declined (Fig. 6, A and B). These results indicate that PD-1 suppresses the generation of Skp2 because, in addition to inhibiting PI3K-Akt signaling, PD-1 inhibits Ras, thereby mediating selective inhibition of the MEK-ERK pathway. Because both of these pathways are indispensable for expression of the gene encoding Skp2 (21), by inhibiting both PI3K-Akt and Ras signaling, PD-1 blocks progression of the cell cycle at the level of Skp2.

IL-2 reverses the inhibitory effect of PD-1 on MEK-ERK, but not PI3K-Akt, signaling and partially restores Skp2 abundance

One important functional outcome of PD-1 signaling during T cell activation is the inhibition of IL-2 production. This effect might account for the blockade of cell cycle progression and the induction of an anergic or regulatory T cell phenotype of T cells receiving PD-1 signals during stimulation through their TCR (8, 48, 49). Consistent with this hypothesis, supplementation of cultures with exogenous IL-2 prevents the inhibitory effect of PD-1 on T cell proliferation in vitro and may reverse PD-1-dependent anergy induced by peptide injection in 2C Rag2^{-/-} mice in vivo (50, 51). IL-2 receptor (IL-2R)-mediated signals can activate the Ras-MEK-ERK pathway through recruitment of the Grb2-Sos-Shc complex (52, 53), as well as the PI3K-Akt pathway (54–56). For these reasons, we sought to determine whether IL-2 could overcome the inhibitory effects of PD-1 on the MEK-ERK or PI3K-Akt pathways and restore Skp2 abundance. Addition of exogenous IL-2 concomitantly with PD-1 ligation partially restored the activation of MEK1/2 and ERK1/2, which displayed a complete recovery 24 hours after addition of IL-2 and gradually declined, but remained detectable, for the remaining time of the assay (Fig. 6, C and D). In contrast, IL-2 had no detectable effect on the activation of Akt. The effects of IL-2 on these signaling pathways partially restored the abundance of Skp2 (Fig. 6, C and D) and T cell proliferation (Fig. 6G).

To determine whether simultaneous activation of MEK-ERK and PI3K-Akt signaling pathways during PD-1 ligation could restore Skp2, we stimulated T cells with the phorbol ester phorbol 12-myristate 13-acetate (PMA) that can directly activate Ras and Akt signaling. PMA resulted in robust activation of MEK-ERK and Akt signaling, restored the abundance of Skp2 (Fig. 6, E and F), and restored the proliferative response of the cells (Fig. 6G). These results indicate that the dual-inhibitory effect of PD-1 on the PI3K-Akt and MEK-signaling pathways has a critical role in mediating suppression of Skp2, which leads to blockade of cell cycle progression. During PD-1 signaling, IL-2 partially reconstituted activation of one pathway and partially restored the abundance of Skp2.

DISCUSSION

Here, we examined the mechanistic regulation of the cell cycle machinery PD-1. We determined that PD-1 induced blockade of cell cycle progression in the G₁ phase by increasing the abundances of the Cdk inhibitors p27^{kip1} and p15^{INK4} while suppressing expression of the gene encoding the Cdk-activating phosphatase Cdc25A. Our studies showed that ligation of PD-1 during the stimulation of T cells through the TCR-CD3 complex and CD28 inhibited activation of the PI3K-Akt and Ras-MEK-ERK pathways and blocked expression of the gene encoding Skp2, the substrate-recognition component of the

SCF^{Skp2} ubiquitin ligase that mediates degradation of p27^{kip1}, which then resulted in accumulation of p27^{kip1} and inhibition of Cdk2. Because of the impaired activity of Cdk2, T cells stimulated through PD-1 not only displayed decreased phosphorylation of Rb but also failed to phosphorylate the checkpoint inhibitor Smad3 on the Cdk2-specific site, leading to enhanced Smad3 transcriptional activity and resulting in the increased abundance of p15^{INK4B} and abrogation of the Cdk-activating phosphatase Cdc25A.

PD-1-mediated signals play a vital role in the induction and maintenance of anergy and peripheral tolerance (6, 7). Studies on the induction of anergy in vitro and tolerance in vivo have shown that tolerized T cells are blocked at the G₁ phase of the cell cycle. This event not only is correlative but also has a causative role in anergy induction (24, 57, 58). The importance of G₁ arrest for the induction of anergy in vivo is supported by the observation that mice expressing a mutant form of p27^{kip1} that lacks the Cdk-binding domain and is incapable of interacting with Cdk2 are resistant to tolerance induction by peptide antigen (59). Moreover, p27^{kip1}-deficient recipients reject cardiac allografts under conditions that enable long-term allograft survival in wild-type recipients (60). Our data explain how PD-1-mediated signals can regulate the molecular events that occur during the G₁ phase and the G₁-S transition, thereby resulting in G₁ arrest and determining whether T cells induce tolerance or productive immunity. We determined that in contrast to optimal activation through the TCR-CD3 complex and CD28, activation in the presence of PD-1 induced marked inhibition of Cdk2 activity, which resulted in diminished phosphorylation of Smad3 on the Cdk2-specific site and resulted in enhanced Smad3 transcriptional activity. As a consequence, two well-established transcriptional targets of Smad3, p15^{INK4B} and Cdc25A, were differentially affected.

Previous work indicated that PD-1 reduces the threshold of TGF- β -mediated signals, thereby synergizing with TGF- β to promote the conversion of naïve T cells into iT_{regs}, and can even induce the formation of iT_{regs} in the absence of exogenous TGF- β (8). On the basis of those findings and our results showing that PD-1 induced arrest at the G₁ phase of the cell cycle, we postulated that PD-1 might promote TGF- β production or enhance the abundance of TGF- β Rs. However, our studies revealed that PD-1 did not induce increases in TGF- β production or in the abundance of TGF- β Rs. Instead, PD-1 mediated the enhanced transactivation of Smad3 by altering the Cdk-mediated phosphorylation of Smad3. PD-L1-PD-1 interactions enhance the conversion of naïve T cells into iT_{regs} in the presence of TGF- β in vitro. PD-L1 induces and maintains iT_{regs} in vivo, indicating that PD-L1 may stabilize and sustain T_{regs} in the periphery similarly to the reported effects of TGF- β (8). Our findings here provide a mechanistic explanation for the reduced threshold of TGF- β -mediated conversion of T_{regs} when PD-1 signals are present and for the lack of conversion to iT_{regs} after adoptive transfer of wild-type naïve T cells into PD-L1^{-/-}PD-L2^{-/-}Rag2 recipients, which develop marked lymphoproliferation (8). Our results indicate that direct regulation of Smad3 transactivation is a central mechanism through which PD-1 synergizes with TGF- β to facilitate suppression of T cells and to maintain T cell tolerance and immune quiescence through both T cell-intrinsic and -extrinsic mechanisms.

A central finding of our studies was that induction of *SKP2* mRNA expression was abrogated in the presence of PD-1-mediated signals. We determined previously that the gene

encoding human *SKP2* contains a pair of consecutive Sp1- and Elk-1-binding sites, which function as activators of *SKP2* transcription upon binding Elk-1, after it is activated by phosphorylation by MAPKs (21, 61, 62). The *SKP2* promoter also contains two consecutive E2F sites in tandem with a TGF- β inhibitory site (TIE). This E2F-TIE element functions as a transcriptional repressor and is similar to a tandem E2F-TIE paired site in the promoter of *c-MYC*, which also functions as a repressor through binding of E2F4, E2F5, p107, and Smad (21, 63). Thus, it appears that PD-1 mediates suppression of Skp2 transcription by two mechanisms. First, by impaired activation of PI3K-Akt and Ras-MAPK signaling, which leads to impaired transactivation of Sp1/Elk-1, and second, by increased transactivation and binding of Smad to the E2F-TIE inhibitory element of the Skp2 promoter. Together, our observations support a model for PD-1-mediated blockade of cell cycle progression that is initiated by inhibition of PI3K-Akt and Ras-MEK-ERK signaling, which results in blockade of *SKP2* transcription, accumulation of p27^{kip1}, suppression of cyclin E-Cdk2 activity, impaired Cdk2-mediated phosphorylation of Rb and Smad3, and increased Smad3 transactivation. Subsequently, Smad3 trans-activation results in suppression of the gene encoding Cdc25A, increased p15 abundance, and further suppression of *SKP2* expression (Fig. 7).

An unexpected finding of our studies was that PD-1 regulated the abundance of Cdc25A both by suppressing the expression of Cdc25A mRNA and by promoting ubiquitin-dependent proteasomal degradation of Cdc25A protein. Studies in other cell types have shown that Cdc25A is controlled by multilayered mechanisms. Whereas the transcription of *CDC25A* is stimulated in an E2F-dependent manner and inhibited in a TGF- β - and Smad-dependent manner (37, 39), protein abundance is controlled more dynamically by ubiquitin-dependent proteasomal degradation. Two types of E3 ubiquitin ligase complexes mediate the ubiquitination of Cdc25A: the anaphase-promoting complex (APC) and the SCF complex (64, 65). The APC^{cdh1} complex mediates degradation of Cdc25A from the exit of mitosis through the G₁ phase of the cell cycle. Independently, the SCF ^{β -TrCP} complex plays a critical role in Cdc25A degradation during proliferation and also in response to DNA damage. APC recognizes specific KEN (Lys-Glu-Asn) box sequences in Cdc25A protein and induces phosphorylation-independent, ubiquitin-dependent degradation of Cdc25A. In contrast, phosphorylation of Cdc25A on Ser⁷⁶ or Ser⁷⁵ is a prerequisite for the subsequent phosphorylation of Ser⁸², which is critical for the binding of β -TrCP (41, 66, 67). The checkpoint kinase Chk1 phosphorylates serines at positions 75, 76, and 123 (66, 68–70), and p38 MAPK phosphorylates serines at positions 75 and 123 (71), whereas Smad3 regulates the phosphorylation of Ser⁷⁹ and Ser⁸², although the precise mechanism remains unclear (40).

Little is known about how Cdc25A is regulated by physiologic mechanisms involved in T cell responses. A previous study performed in T cells indicated that withdrawal of IL-3 or IL-7 activates p38 MAPK, resulting in the phosphorylation of Cdc25A on Ser⁷⁵ and Ser¹²³ and triggering its degradation (71). In that system, the effects of cytokine withdrawal on the abundance of Cdc25A mRNA were not addressed, and the ubiquitin-targeted degradation of Cdc25A was considered the primary mechanism by which abundance of the protein was controlled. Our studies indicated that Cdc25A is transcriptionally regulated during T cell activation through the TCR-CD3 complex and CD28 and that PD-1 inhibits this event. This

outcome is likely a result of the dual effects of PD-1 in inducing impaired phosphorylation of Rb and Smad3, thereby inhibiting E2F-regulated gene expression and enhancing the DNA binding capability and transactivation of Smad3, resulting in augmented Smad3-mediated suppression of *CDC25A* expression. However, PD-1 signaling substantially increased the extent of ubiquitin-mediated degradation of Cdc25A because inhibition of this pathway by the proteasome inhibitor MG132 resulted in comparable Cdc25A protein abundance in T cells activated in the presence or absence of PD-1 signals. Thus, Cdc25A degradation is also an active mechanism through which PD-1 mediates cell cycle arrest. Further studies will identify the specific ubiquitin ligase involved in ubiquitin-dependent proteasomal degradation of Cdc25A in response to PD-1 signaling.

We determined that PD-1 inhibited TCR-dependent MEK-ERK signaling and that this effect could be partially reversed by the addition of exogenous IL-2. PD-1 also inhibited TCR-dependent Akt activation; however, this effect was resistant to IL-2 supplementation. These results show that PD-1 mediates its inhibitory effects on T cell expansion by regulating the TCR signaling machinery through IL-2-dependent and -independent mechanisms. Furthermore, our results reveal that PD-1 mediates a potent inhibitory effect on the activation of signaling pathways initiated not only by the TCR but also by the IL-2R. Notably, CTLA4 may also block T cell expansion by mediating such dual-inhibitory effects on IL-2-dependent and -independent components of the T cell proliferative machinery (72). Although the precise molecular mechanisms through which CTLA4 mediates these effects have not been determined, these observations indicate that both CTLA4 and PD-1 activate direct, T cell-intrinsic inhibitory cues independently of their ability to suppress IL-2 production. In conclusion, our studies provide evidence that PD-1 targets both Ras and PI3K-Akt signaling to inhibit transcription of *SKP2* and to activate Smad3 as an integral component of a pathway regulating the blockade of cell cycle progression in T lymphocytes.

MATERIALS AND METHODS

Labeling of tosyl-activated magnetic beads with mAbs

mAbs were covalently attached to Dynabeads M-450 Tosylactivated beads according to the manufacturer's instructions (Invitrogen). The magnetic beads were coated with the following mAbs: anti-CD3 (UCHT1, R&D Systems Inc.), anti-CD28 (CD28.2, Biolegend), anti-human PD-1 (clone 17, provided by Pfizer Inc.), and control immunoglobulin G (IgG, Jackson ImmunoResearch Laboratories). In general, 2×10^8 magnetic beads were coated with anti-CD3 (8%) and anti-CD28 (10%), and either anti-PD-1 (anti-PD-1/CD3/CD28) or control IgG (IgG/CD3/CD28) constituted the remaining 82% of the total protein. All incubations were performed in 0.1 M sodium phosphate buffer for 18 hours at 37°C with constant rotation. The beads were then washed three times and stored at 4°C. Variations of this protocol have been previously described (8, 20, 73).

In vitro T cell cultures

Leukocytes were obtained from normal healthy blood donors. For short-term signaling experiments (in the order of minutes), leukocytes were first cultured in phytohemagglutinin (PHA) (5 µg/ml) for 48 hours to increase the abundance of PD-1. Subsequently, CD4⁺ T

cells were isolated by negative selection with the CD4⁺ T cell Isolation Kit II from Miltenyi Biotec and were brought to rest in culture medium overnight. For stimulation, CD4⁺ T cells were resuspended as 100×10^6 cells/ml in prewarmed RPMI 1640 containing 10 mM Hepes and mixed with an equal volume of RPMI/Hepes containing an equal amount of beads (conjugated either with anti-CD3, anti-CD28 mAbs, and IgG or with anti-CD3, anti-CD28, and anti-PD-1 mAbs). The cell-bead mixture was then centrifuged for 1 min at 300g at room temperature and placed immediately at 37°C. At the indicated time points, the reaction was stopped by addition of cold phosphate-buffered saline (PBS). For stimulation experiments over the course of 24 to 72 hours, CD4⁺ T cells were plated at 1.5×10^5 cells per well in 96-well flat-bottom plates with magnetic beads (1.5×10^5 beads per well) conjugated with anti-CD3, anti-CD28 mAbs, and IgG or with anti-CD3, anti-CD28, and anti-PD-1 mAbs. Cultures were performed in RPMI 1640 with L-glutamine (cellgro, Mediatech) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 10 mM Hepes, 1 mM sodium pyruvate, penicillin/streptomycin (50 U/ml; Cellgro/Mediatech), and gentamycin (80 µg/ml; Gibco/Invitrogen). Exogenous IL-2 was used at 50 U/ml, and PMA (Sigma) at 5 ng/ml. For the neutralization of TGF-β in the cultures, the recombinant human TGF-βRII-Fc chimera was used (R&D Systems Inc.). Where indicated, cells were cultured in the serum-free medium X-VIVO 20 (Lonza). DNA synthesis was assessed by [³H]thymidine incorporation for the last 16 to 18 hours of the culture. Proliferation index was expressed as the ratio of [³H]thymidine incorporation values in various culture conditions divided by the values of cells cultured with medium alone. For analysis of cell cycle progression, the Click-iT EdU flow cytometry assay kit (Invitrogen/Molecular Probes Inc.) was used according to the manufacturer's instructions.

Western blotting analysis, immunoprecipitations, and in vitro kinase reactions

For the preparation of lysates, cells were washed in PBS and lysed in lysis buffer containing 50 mM tris-Cl (pH 7.4), 150 mM NaCl, 2 mM MgCl₂, 10% glycerol, and 1% NP-40 supplemented with 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and Protease Inhibitor Cocktail (Thermo Fisher Scientific). Cell lysates were resolved by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and then analyzed by Western blotting with the indicated antibodies. Stripping and re-incubation of blots was performed with the Restore Plus Western Blot Stripping Buffer (Thermo Fisher Scientific). Antibodies against cyclin A, cyclin E, Cdk2, Cdk4, p15, β-actin, Skp2, Smad2/3, pERK1/2, and pJNK were from Santa Cruz Biotechnology. The anti-phospho-Cdk2 mAb (Thr¹⁶⁰), anti-phospho-Cdk4 (Thr¹⁷²), and antibodies against pAkt (Thr³⁰⁸), pAkt (Ser⁴⁷³), Akt, pp38, pMEK, and phosphor-GSK-3α/β (Ser^{21/9}) were from Cell Signaling Technology. The mAb against p27 was from BD Biosciences Pharmingen. The anti-phospho-p27^{kip1} (Thr¹⁸⁷) and the anti-ERK1/2 antibodies were from Upstate/Millipore. The anti-phospho-Smad3 (pSer²¹³) has been previously described (18). The mAb against cyclin D1 was from BD Biosciences, and the mAb against Rb was from eBioscience. To assess Ras activation by glutathione *S*-transferase (GST) pull-down assays, we purified GST-Raf1-RBD fusion proteins on glutathione-Sepharose (Amersham Pharmacia Biotech). Fusion protein (50 µg) coupled to glutathione-Sepharose beads (GammaBind Plus) was incubated with cell lysates (1 mg) in lysis buffer that did not contain NP-40. Eluted proteins were analyzed by SDS-PAGE and Western blotting with antibody against Ras. For in vitro kinase reactions,

immunoprecipitations were performed with mAb against Cdk2 that was conjugated to agarose (Santa Cruz) followed by in vitro kinase reactions with histone H1 as the exogenous substrate, as previously described (59). Reactions were analyzed by 10% SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane, and exposed to x-ray film.

Real-time quantitative PCR

Total RNA extraction was performed with the RNeasy Mini Kit from Qiagen according to the manufacturer's instructions, and 50 ng of RNA was subjected to quantitative PCR analysis for the target genes *TGF-βRI*, *TGF-βRII*, *CDC25A*, and *CDKN2B* (encoding for p15^{INK4B}). The FAM (carboxyfluorescein)-conjugated primers for all target genes, the TaqMan One-Step RT-PCR Master Mix reagents, and the VIC/TAMRA-conjugated primers for 18S RNA housekeeping were from Applied Biosystems/Roche. The reaction was performed with an AB 7000 quantitative PCR machine from Applied Biosystems.

Measurement of TGF-β binding and intracellular TGF-β concentrations

The TGF-β-binding capacity of CD4⁺ cells was determined with the Fluorokine Biotinylated Human TGF-β1 kit (R&D Systems) according to the manufacturer's instructions. To assess intracellular TGF-β, we cultured CD4⁺ cells in the serum-free medium X-VIVO 20 (Lonza) and determined the intracellular concentration of TGF-β by flow cytometry with phycoerythrin-conjugated antibodies against TGF-β1, -β2, and -β3 (R&D Systems) according to the manufacturer's instructions.

Measurement of cytokine production

Culture supernatants were harvested at the indicated time points and were subjected to enzyme-linked immunosorbent assay (ELISA) to quantify amounts of secreted TGF-β (R&D Systems) and IL-2 (eBioscience) according to the manufacturer's instructions. To assess TGF-β production in the presence of the indicated stimuli, we cultured CD4⁺ T cells in serum-free X-VIVO 20 medium (Lonza).

Mammalian expression plasmids, transfection of T cells, and reporter assays

CD4⁺ cells (5×10^6 cells per condition) were transfected by means of the Amaxa Nucleofector apparatus (Lonza) with the indicated plasmids. Transfected cells were immediately transferred to 2 ml of Lymphocyte Growth Medium-3 (LGM-3) (Lonza) and cultured for 5 hours. Subsequently, the cells were washed, resuspended, and stimulated as indicated in prewarmed LGM-3 medium. Plasmid encoding Smad3-specific shRNA and the control shRNA plasmid encoding a scrambled shRNA sequence that does not lead to degradation of any known mRNA were from Santa Cruz Biotechnology. The complementary DNAs for human Cdc25 and the degradation-resistant Cdc25 mutant D81A/S82A (41) were provided by J. Jin (University of Texas-Houston Medical School). To assess the extent of Smad-dependent transcription, we transfected CD4⁺ cells with 1 μg of the 4×Smad Binding Element (SBE)-luciferase reporter construct (35). Transfection efficiency was normalized by cotransfection with 1 μg of pEF-LacZ and assay for β-galactosidase activity. Transfected cells were immediately transferred to 2 ml of LGM-3 medium and cultured for 5 hours. Subsequently, the cells were washed, resuspended in prewarmed LGM-3, and stimulated

with beads coated with antibodies against CD3 and CD28 and with IgG or with beads coated with antibodies against CD3, CD28, and PD-1 for 5 or 24 hours before being harvested, lysed, and further analyzed for luciferase and β -galactosidase activity, as described previously (74).

Statistical analysis

In vitro assays and densitometry data were compared with the unpaired Student's *t* test or by analysis of variance (ANOVA) for more than two conditions. A *P* value of <0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

PD-1 prevents progression of primary human T cells to the S phase of the cell cycle. (A) Purified primary human CD4⁺ T cells were incubated with tosyl-activated magnetic beads conjugated with mAbs against CD3 and CD28 and with IgG or conjugated with mAbs against CD3, CD28, and PD-1 for the indicated times. DNA synthesis was determined by addition of [³H]thymidine for the last 16 hours of culture. Results are representative of seven independent experiments. **P* < 0.005 versus IgG control. CPM, counts per minute. (B) CD4⁺ T cells were cultured with the indicated tosyl-activated magnetic beads and were analyzed for cell cycle distribution at the indicated time points. Percentages of cells in the G₀-G₁ (lower left region), S (upper region), and G₂-M (lower right region) phases were identified by incubation with EdU, as described in Materials and Methods, followed by analysis with flow cytometry. (C) The proportions of cycling cells (in the S and G₂-M phases) at the indicated times of culture under each experimental condition are shown.

Fig. 2.

PD-1 does not induce the increased production of TGF- β or enhance the abundance of TGF- β Rs. **(A)** CD4⁺ T cells were cultured with medium alone (Unstim.) or were activated through CD3 and CD28 with or without PD-1 ligation, as described for Fig. 1. Under either stimulatory condition, TGF- β production at 24 and 48 hours was increased compared to that under unstimulated conditions. $*P < 0.005$, $n = 3$ experiments. **(B)** Intracellular TGF- β under the indicated culture conditions was examined by flow cytometry. Results are representative of three experiments. **(C)** TGF- β RI and TGF- β RII mRNAs were examined by real-time quantitative PCR. At all time points, TGF- β RI mRNA abundance was statistically significantly increased in T cells stimulated under either condition compared to that in unstimulated T cells ($*P < 0.005$). After 12 hours, TGF- β RII mRNA abundance was increased in T cells stimulated under either condition compared to that in unstimulated cells ($*P < 0.005$). **(D and E)** Biotinylated TGF- β was used to determine the extent of TGF- β binding to TGF- β Rs on cells. There was no increase in TGF- β binding to CD4⁺ T cells activated in the presence of PD-1 stimulation compared to that in cells lacking PD-1 stimulation. $P > 0.05$, $n = 3$ experiments. Data shown in (D) were from a single experiment and are representative of three experiments.

Fig. 3.

PD-1 inhibits activation of Cdk2. (A) T cells were activated through CD3 and CD28 with or without stimulation of PD-1. Cell lysates were prepared, and the amounts of the indicated proteins were examined by Western blotting. (B) Densitometric analysis of the abundance of each indicated protein normalized to that of β -actin, which were expressed as a fold increase relative to mean values before stimulation. Enhanced phosphorylation of Rb was determined by analysis of its electrophoretic mobility and densitometric analysis of the ppRb/pRb ratio at each time point. Fold increases in the abundance of each protein in activated T cells that were stimulated through PD-1 were compared to those in activated T cells without PD-1 stimulation at each time point. Data are presented as the means $\pm$ SEM. * $P < 0.05$, $n = 4$ experiments. (C) Cell lysates were examined for the indicated proteins by Western blotting. (D) Densitometric analysis of the abundance of each indicated protein normalized to that of

Smad3 and expressed as fold change relative to mean values of T cells activated in the absence of PD-1 stimulation for 24 hours, defined as 1. For pCdk2, values of T cells cultured under these conditions for 48 hours were defined as 1, because pCdk2 was undetectable at earlier times. Fold changes in the abundance of each protein at each time point were compared as described for (B). Data are presented as the means $\pm$ SEM. $^*P < 0.05$, $n = 4$ experiments. (E) PD-1-mediated signals block the induction of *SKP2* mRNA. T cells were cultured as indicated for 24 hours. The abundance of *SKP2* mRNA was assessed by real-time quantitative PCR and was compared between activated T cells in the presence or absence of PD-1 stimulation ($^*P < 0.05$).

Fig. 4.

PD-1 enhances the transactivation of Smad3. (A) T cells were transfected with a Smad3-dependent reporter and were cultured for 24 hours as indicated. Smad3 transcriptional activity, as determined by luciferase assay, was compared between T cells activated with or without stimulation through PD-1. $*P < 0.05$, $n = 3$ experiments. (B) The abundances of p15 and Cdc25 mRNAs, as assessed by real-time quantitative PCR, were compared between T cells activated with or without stimulation through PD-1. $*P < 0.05$, $n = 3$ experiments. (C) T cells were activated as shown and the abundances of the indicated proteins were analyzed by Western blotting. (D) Densitometric analysis of the abundance of each indicated protein normalized to that of β -actin at each time point and expressed as the fold change relative to mean values of activated T cells that were not stimulated through PD-1. Fold changes were compared between T cells activated with or without stimulation through PD-1. Data are presented as the means $\pm$ SEM. $*P < 0.05$, $n = 4$ experiments. (E and F) PD-1 promotes the ubiquitin-dependent degradation of Cdc25. (E) T cells were activated as indicated in the presence of either MG132 or DMSO, and Cdc25A protein abundance was examined by Western blotting. (F) Densitometric analysis of the abundance of Cdc25A protein normalized to that of β -actin and analyzed as described for (D). $*P < 0.05$, $n = 3$ experiments. (G) Expression of a degradation-resistant form of Cdc25A reduces the antiproliferative effect of PD-1. T cells were transfected with plasmid encoding wild-type (WT) Cdc25A or a degradation-resistant mutant of Cdc25A (Cdc25A^{81A/S82A}) and were stimulated through CD3, CD28, and PD-1 for 48 hours. DNA synthesis was determined by

the addition of [^3H]thymidine for the last 16 hours of culture. The stimulation index of cells expressing Cdc25A 81A/S82A was compared to that of cells expressing WT Cdc25A. $^*P < 0.05$, $n = 3$ experiments.

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Fig. 5.

Smad3 has an active role in the PD-1-mediated increase in p15^{INK4B} abundance. (**A** and **B**) T cells were transfected with Smad3-specific shRNA (Smad3-KD) or control shRNA (Control-KD), and the abundance of Smad3 was analyzed by Western blotting. (**B**) Densitometric analysis of Smad3 abundance normalized to that of β -actin. Data are presented as the means $\pm$ SEM. * $P < 0.05$, $n = 3$ experiments. (**C** and **D**) Smad3 is required for the increased abundance of p15^{INK4B} in response to PD-1. Smad3-KD and Control-KD T cells were activated for 24 hours as indicated, and the abundance of p15^{INK4B} was examined by Western blotting. (**D**) Densitometric analysis of the abundance of p15^{INK4B} protein normalized to that of β -actin and expressed as the fold change relative to mean values of activated T cells cultured in the absence of PD-1 stimulation for 24 hours. The abundance of p15^{INK4B} in control-KD cells and in activated Smad3-KD cells that were stimulated through PD-1 was compared to that in activated T cells without PD-1 stimulation. * $P < 0.05$, $n = 3$ experiments. (**E**) Knockdown of Smad3 reduces the anti-proliferative effect of PD-1. Smad3-KD and Control-KD T cells were stimulated through CD3, CD28, and PD-1 for 72 hours. DNA synthesis was determined by the addition of [³H]thymidine for the last 16 hours of culture, and the stimulation index of Smad3-KD cells was compared to that of Control-KD cells. * $P < 0.05$, $n = 3$ experiments.

Fig. 6.

PD-1 signaling inhibits activation of the PI3K-Akt and Ras-MEK-ERK pathways. **(A)** CD4⁺ T cells isolated from PHA blasts were stimulated as indicated, and the activation of Akt, MEK1/2, ERK1/2, JNK, and p38 was examined by Western blotting with phosphospecific antibodies. The abundance of ERK was analyzed as a control for equal loading. In the same samples, Ras activation was examined by pull-down assay with GST-Raf1-RBD and Western blotting analysis with a Ras-specific mAb. WCL, whole-cell lysate. **(B)** Densitometric analysis of changes in the abundance of phosphorylated substrates normalized to that of ERK1/2 and expressed as the fold change relative to mean values before stimulation. At each time point, the abundance of each protein was compared between T cells activated with or without stimulation through PD-1. * $P < 0.05$, $n = 3$ experiments. **(C)** PD-1 signaling inhibits the activation of Akt, MEK1/2, and ERK1/2 induced by mAbs against CD3 and CD28 during culture. Addition of exogenous IL-2 during T cell stimulation through the TCR-CD3 and PD-1 partially reversed the impaired activation of MEK-ERK signaling, but not of Akt signaling, and partially restored Skp2 protein abundance. In contrast, the addition of PMA induced activation of MEK-ERK and Akt signaling and restored Skp2 abundance. T cells were activated through CD3 and CD28 with or without stimulation through PD-1 and in the presence or absence of either IL-2 (50 U/ml) or PMA (5 ng/ml). The abundances of Skp2 and p27^{kip1} and the activation of Akt, MEK1/2, and ERK1/2 were examined by Western blotting analysis of cell lysates. **(D)** Densitometric analysis of changes in the abundance of the indicated proteins after stimulation normalized to that of β-actin and expressed as fold changes relative to the mean values before stimulation. For each time point, fold changes in the abundances of the indicated proteins in

activated T cells that were stimulated through PD-1 were compared to those in activated T cells without PD-1 stimulation ($^*P < 0.05$), in activated T cells with PD-1 stimulation and IL-2 ($^+P < 0.05$), and in activated T cells with PD-1 stimulation and PMA ($^oP < 0.05$). Fold changes in protein abundance in activated T cells that were stimulated through PD-1 and IL-2 were compared to those in activated T cells without PD-1 stimulation ($^*P < 0.05$). $n = 3$ experiments. (E) IL-2 partially restores T cell proliferation during stimulation through TCR-CD3 and PD-1, whereas PMA induces a hyperproliferative response even in the context of PD-1 signaling. CD4⁺ T cells were activated for 72 hours through CD3 and CD28 with or without PD-1 stimulation in the absence or presence of either IL-2 or PMA, and DNA synthesis was determined by the addition of [³H]thymidine for the last 16 hours of culture (m, medium). Data are representative of three experiments.

Fig. 7. Model for the effect of PD-1 on TCR-proximal signals and on regulation of the cell cycle, which leads to inhibition of T cell proliferation. (**A** and **B**) Our data support a model of PD-1-mediated blockade of cell cycle progression that is initiated by impaired activation of PI3K-Akt and Ras-MEK-ERK signaling, which results in defective induction of the gene encoding Skp2. This event results in the accumulation of p27^{kip1}, suppression of cyclin E-Cdk2 activity, impaired Cdk2-mediated phosphorylation of Rb and Smad3, and enhanced trans-activation of Smad3. Subsequently, Smad3-mediated transcriptional effects result in suppression of the expression of the gene encoding Cdc25A, an increase in the expression of the gene encoding p15, an increase in p15 abundance, and further suppression of *SKP2* expression. Thus, PD-1-mediated signals initiate and reinforce an inhibitory loop to suppress cell cycle progression. PY, tyrosine phosphorylation of Cdk that is removed by the phosphatase Cdc25A; P, sites of threonine phosphorylation induced during Cdk enzymatic

activation; pS²¹³, Cdk2-mediated phosphorylation of Smad3, which suppresses the transcriptional activity of Smad3.

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