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Programmed Death-1 (PD-1):PD-Ligand 1 Interactions Inhibit TCR-Mediated Positive Selection of Thymocytes[1]

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Positive selection during thymocyte development is driven by the affinity and avidity of the TCR for MHC-peptide complexes expressed in the thymus. In this study, we show that programmed death-1 (PD-1), a member of the B7/CD28 family of costimulatory receptors, inhibits TCR-mediated positive selection through PD-1 ligand 1 (PD-L1):PD-1 interactions. Transgenic mice that constitutively overexpress PD-1 on $CD4^+CD8^+$ thymocytes display defects in positive selection in vivo. Using an in vitro model system, we find that PD-1 is up-regulated following TCR engagement on $CD4^+CD8^+$ murine thymocytes. Coligation of TCR and PD-1 on $CD4^+CD8^+$ thymocytes with a novel PD-1 agonistic mAb inhibits the activation of ERK and up-regulation of bcl-2, both of which are downstream mediators essential for positive selection. Inhibitory signals through PD-1 can overcome the ability of positive costimulators, such as CD2 and CD28, to facilitate positive selection. Finally, defects in positive selection that result from PD-1 overexpression in thymocytes resolve upon elimination of PD-L1, but not PD-1 ligand 2, expression. PD-L1-deficient mice have increased numbers of $CD4^+CD8^+$ and $CD4^+$ thymocytes, indicating that PD-L1 is involved in normal thymic selection. These data demonstrate that PD-1:PD-L1 interactions are critical to positive selection and play a role in shaping the T cell repertoire. *The Journal of Immunology,* **2005, 175: 7372–7379.**

Peripheral T cell responses and tolerance rely upon the generation of an appropriate and diverse T cell repertoire during thymic selection (1). Developing thymocytes undergo rearrangement of germline TCR genes, creating a diverse pool of thymocytes with a broad range of specificities. TCR expression occurs at the $CD4^+CD8^+$ (double-positive (DP))[4] stage of thymocyte maturation and marks the beginning of thymic selection. Signal transduction through the TCR establishes thresholds for thymocyte selection. Thymocytes comprising the newly formed TCR repertoire are selected for their ability to recognize peptide in the context of self-MHC molecules. Positive selection tests the ability of the TCR to signal in response to self-MHC. Thymocytes that do not receive a TCR signal of sufficient strength fail to up-regulate cell survival genes and die through neglect. Negative selection eliminates thymocytes expressing TCRs that transmit a strong signal in response to self-MHC-peptide via active induction of apoptosis. Central tolerance is the result of thymic selection, whereby a mature T cell repertoire is generated that can respond appropriately to pathogens while displaying tolerance to peripheral self-Ags.

Costimulatory molecules are important to the initiation and termination of immune responses and modulate signaling through the TCR. The best-characterized T cell costimulatory pathway involves the B7-1 (CD80) and B7-2 (CD86) ligands that share two receptors, CD28 and CTLA-4 (CD152). CD28 provides signals that augment and sustain T cell activation in concert with TCR signaling, while CTLA-4 antagonizes TCR signals, serves to dampen secondary immune responses, and contributes to tolerance (2). The B7/CD28 family has expanded in recent years to include programmed death-1 (PD-1) (3) and its ligands, PD-1 ligand 1 (PD-L1) (4) and PD-1 ligand 2 (PD-L2) (5) (also known as B7-H1 (6) and B7-DC (7), respectively). PD-1 is induced on activated peripheral T and B cells and regulates T cell responses. PD-1 has an ITIM and a immunoreceptor tyrosine-based switch motif (8) motif in its cytoplasmic domain, and signaling through PD-1 leads to the recruitment of Src homology region 2 domain-containing phosphatase (SHP)-1 and SHP-2 (9) and subsequent inhibition of Zap70 and protein kinase Cθ activation (10). The functional significance of the PD-1-inhibitory signal is demonstrated by the phenotype of PD-1-deficient ($PD\text{-}1^{-/-}$) mice. C57BL/6 $PD\text{-}1^{-/-}$ mice display features of lupus (11), and BALB/c $PD\text{-}1^{-/-}$ mice develop a dilated cardiomyopathy (12), pointing to roles for PD-1 in inhibiting T and B cell activation and regulating peripheral tolerance.

Costimulation modifies TCR signaling and TCR signals, and it thereby may contribute to the regulation of thymocyte selection by determining thresholds for thymocyte selection. PD-1 is unique among members of the B7/CD28 family of costimulatory receptors because it has been directly implicated in thymocyte maturation. PD-1 is expressed on immature $CD4^-CD8^-$ (double-negative (DN)) thymocytes during TCRβ rearrangement. PD-L1 is expressed broadly in the thymic cortex and on thymocytes, while PD-L2 expression is limited to medullary stromal cells (13, 14). It is not yet clear whether PD-L1 and PD-L2 play overlapping or distinct roles in thymic selection. Studies in PD-1-deficient ($PD\text{-}1^{-/-}$) TCR transgenic (tg) strains first suggested a role for PD-1 in

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[4] Abbreviations used in this paper: DP, double positive; DN, double negative; MFI, mean fluorescence intensity; PD-1, programmed death-1; PD-L1, PD-1 ligand; PD-L2, PD-2 ligand; SHP, Src homology region 2 domain-containing phosphatase; SP, single positive; tg, transgenic; WT, wild type.

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thymic selection; H-Y tg$^+$ PD-1$^{-/-}$ mice have increased numbers of DP thymocytes (15). Loss of PD-1 facilitates TCRβ selection and modifies positive selection. Further work with RAG$^{-/-}$PD-1$^{-/-}$ 2C tg$^+$ mice shows an increase in peripheral DN T cells (16). These data demonstrate an important role for PD-1 in thymocyte development and are consistent with the hypothesis that PD-1 modifies TCR signaling thresholds and thus has an effect on thymic selection.

We undertook studies designed to analyze the role that PD-1 and its ligands play in positive selection. Because PD-1 is required at the DN stage of thymocyte maturation, studies in PD-1$^{-/-}$ mice cannot address whether PD-1 also plays an independent role in positive selection at the subsequent DP stage. To study how PD-1 affects DP thymocyte maturation, we overexpressed PD-1 on developing thymocytes and found decreased positive selection in vivo. Using a novel anti-PD-1 agonistic mAb, we show that PD-1 ligation strongly inhibits TCR-induced DP thymocyte maturation in an in vitro model of thymocyte maturation. PD-1 cross-linking has no effect on apoptosis or necrosis, but rather appears to act through down-regulation of TCR signaling. PD-1 ligation decreases phosphorylation of ERK and inhibits bcl-2 up-regulation, both of which are critical for thymocyte maturation. Furthermore, PD-1 signals overcome facilitation of positive selection by the positive costimulatory receptors CD2 and CD28. Finally, we show that PD-L1$^{-/-}$ mice have more DP and SP4 thymocytes than wild-type (WT) and PD-L2$^{-/-}$ mice, indicating that PD-L1 is the primary ligand for PD-1 in positive selection. Thus, PD-1:PD-L1 interactions inhibit TCR-mediated signaling, which results in altered positive selection of DP thymocytes.

Materials and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory. H-Y TCR tg mice (17) were purchased from Taconic Farms. PD-L1$^{-/-}$ mice (18) and PD-L2$^{-/-}$ mice (S. C. Liang et al., submitted for publication) were generated in our lab, as described. Harvard Medical School and Brigham and Women's Hospital are accredited by the American Association of Accreditation of Laboratory Animal Care. Mice were maintained and used according to institutional and National Institutes of Health guidelines in a pathogen-free facility. Mice were sacrificed between 5 and 8 wk of age, and the number of cells in the thymus and spleen was ascertained by counting cells that excluded trypan blue.

Generation of PD-1 T^c mice

Full-length murine PD-1 cDNA was generated from BALB/c-activated T cell cDNA using primers 5′-GGAATTCGCCACCATGTGGGTCCGGC AGGTAC-3′ and 5′-GGAATTCTTATCAAAGAGGCCAAGAACAA TG-3′ and cloned into the pEF6 mammalian expression vector. The mPD-1 cDNA was cloned into the VA hCD2 promoter vector (19) and sequenced, and the construct was excised and gel purified before injection into C57BL/6 mouse embryos. Two founder PD-1 tg (PD-1 A T^c and PD-1 B T^c) mice were identified by PCR and flow cytometry. Both founder strains were bred to PD-L1$^{-/-}$ mice (18) and PD-L2$^{-/-}$ mice (S. C. Liang et al., submitted for publication) as well as H-Y TCR tg mice.

Generation of PD-1 agonistic Ab

PD-1-specific mAbs were generated, as previously described (14). One (clone 29F.19G8; isotype rat IgG1, κ) of seven screened Abs was found to have agonistic activity, which was defined as decreased proliferation of PD-1T^c PD-L1$^{-/-}$ T cells, as measured by [^{3}H]thymidine incorporation of anti-CD3 and anti-PD-1 mAb-treated cells at 24 h in comparison with isotype-treated controls. Results were confirmed by assaying proliferation of purified CD4$^+$ and CD8$^+$ T cells from PD-1 A T^c PD-L1$^{-/-}$ mice on anti-CD3-coated plates in the presence or absence of plate-bound 29F.19G8 mAb (50 μg/ml) or isotype control (sodium azide-free/low endotoxin rat IgG1κ; BD Pharmingen). Experiments with soluble 29F.19G8 Ab show no effect on T cell proliferation unless the mAb is cross-linked.

Thymocyte maturation cultures

Thymocytes were isolated and washed with complete RPMI 1640 medium (5). CD8$^+$ thymocytes were isolated by positive selection with CD8 microbeads (Miltenyi Biotec), and were routinely >98% CD4$^+$CD8$^+$. Thymocyte maturation cultures were performed, as previously described (20). Briefly, plates were coated overnight with 10 μg/ml anti-TCR (clone H57-597), anti-CD2 (RM2-5), or anti-CD28 (37-51), as indicated in figure legends (all from BD Pharmingen), and either 50 μg/ml anti-PD-1 (29F.19G8) or an IgG1κ isotype. For apoptosis studies, 50 μg/ml anti-CD28 was used. Isotype control Ab was used to maintain a constant total Ab concentration. DP thymocytes (2×10^6) were plated on Ab-coated plates and incubated for 4, 18, or 20 h, as indicated, and then analyzed for apoptosis, bcl-2, and phospho-ERK. For analysis of CD69 and TCRβ expression, cells were washed, replated, and incubated overnight. Statistical analysis was performed using the unpaired Student's *t* test with Statview analysis software (SAS Institute).

Flow cytometry

Thymocytes were stained for 30 min on ice and analyzed on a FACSCalibur (BD Biosciences). The following Abs were used for staining: CD5 FITC, CD24 FITC, CD69 FITC, TCRβ PE, H-Y PE, and CD4 PerCP Cy5.5 (all from BD Pharmingen); CD8 allophycocyanin (Caltag Laboratories); and PD-1 PE (eBioscience). For annexin staining, thymocytes were isolated and stained with annexin V PE (BD Pharmingen), according to manufacturer's instructions. The nonvital dye 7-aminoactinomycin D (BD Pharmingen) was added to the samples before analysis to identify necrotic cells.

Intracellular phospho-ERK staining was adapted from a published protocol (21). Briefly, thymocytes were pelleted and resuspended in 2% paraformaldehyde and incubated at 37°C for 10 min. Cells were pelleted and resuspended in ice-cold 90% methanol and incubated on ice for 30 min. After incubation, cells were resuspended in anti-phospho-ERK Ab (Cell Signaling Technology), incubated for 20 min at room temperature, and then washed and stained with PE-conjugated secondary Ab. After washing, the cells were resuspended with Abs to CD4 PerCP Cy5.5 and CD8 allophycocyanin. Finally, the cells were washed and analyzed by flow cytometry.

For intracellular bcl-2 staining, thymocytes were isolated and stained for cell surface markers. Cells were resuspended in 1% paraformaldehyde plus 0.01% Tween 20 and incubated at room temperature in the dark for 1 h. Finally, cells were resuspended in anti-bcl-2 PE plus FcR blocking Ab (both from BD Pharmingen) for 30 min at room temperature. The cells were washed and analyzed by flow cytometry.

Results

Constitutive overexpression of PD-1 during thymopoiesis impairs thymocyte maturation

To examine the role of PD-1 in thymocyte maturation, particularly positive and negative selection, we made tg mice that express PD-1 specifically on thymocytes and T cells (PD-1 T^c). Distinct PD-1 expression patterns were seen in two founder lines. PD-1A T^c mice constitutively overexpress PD-1 on single-positive (SP) thymocytes and peripheral T cells, while PD-1B T^c mice overexpress PD-1 on both peripheral T cells and developing DN, DP, and SP thymocytes (Fig. 1*A*). Because PD-1 is normally expressed on DN thymocytes and PD-1 T^c B mice overexpress PD-1 on DN thymocytes, we looked for gross differences in early DN maturation. There was no detectable effect of PD-1 overexpression on the maturation of DN thymocytes. PD-1 B mice had similar overall numbers of DN thymocytes (Fig. 1*B*) and DN subsets in comparison with WT littermate controls (Fig. 2). PD-1 A T^c mice are identical to WT in DN cell numbers and subsets (Fig. 1*B* and data not shown).

The PD-1 B T^c strain provides a tool to assess the role of PD-1 in selection events at the DN to DP and DP to SP transitions. Studies in H-Y TCR tg$^+$ mice lacking PD-1 demonstrate increased DP cell numbers, while their DN cell numbers are normal (15). We find that PD-1B T^c mice display a substantially reduced percentage and number of DP and SP thymocytes at 6 wk of age (data not shown and Fig. 1*B*). The number of DP thymocytes is reduced

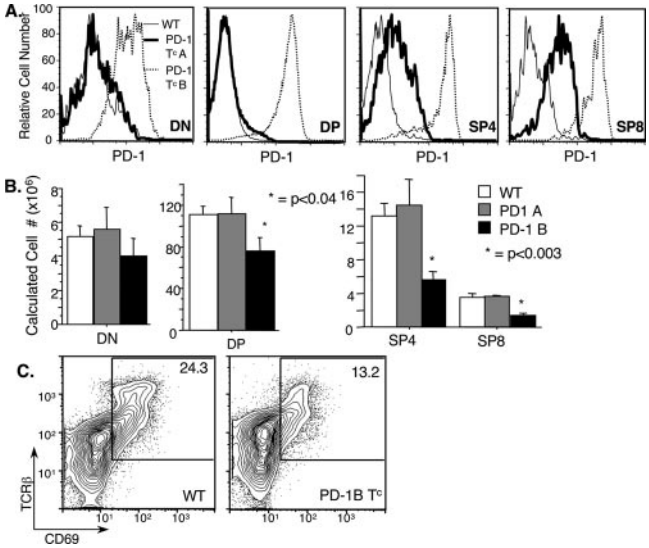

FIGURE 1. PD-1B T^c mice have impaired thymic selection. PD-1 T^c mice constitutively overexpress PD-1 under the control of the CD2 promoter/enhancer. Two separate lines of PD-1 T^c mice have distinct PD-1 expression patterns, resulting in specific effects on thymocyte selection. *A*, PD-1 is expressed at the DN, DP, and SP stages of thymocyte development in PD-1 B T^c mice, while PD-1 A T^c mice express PD-1 at the SP stage of maturation. Thymocytes from the indicated mouse strains were isolated and stained for CD4, CD8, and PD-1. Live cells were gated on CD4$^-$CD8$^-$ (DN), CD4$^+$CD8$^+$ (DP), CD4$^+$CD8$^-$ (SP4), or CD4$^-$CD8$^+$ (SP8) cells, as indicated. *B*, PD-1 B T^c ($n = 5$) mice have fewer SP thymocytes than WT ($n = 8$) or PD-1 A T^c ($n = 5$) mice. Cell yields were calculated by multiplying the subset percentage by the total thymocyte yield. *C*, PD-1B T^c ($n = 5$) mice have reduced CD69 expression on DP thymocytes in comparison with WT ($n = 4$) littermates.

20% in PD-1 B T^c mice in comparison with WT littermates. PD-1 B T^c SP thymocyte numbers are reduced 60% for both SP4 and SP8s in comparison with WT animals (Fig. 1*B*). PD-1A T^c mice have normal percentages and numbers of DP and SP thymocytes, and their peripheral T cell compartment is similar to WT (Fig. 1*B* and data not shown).

Thymocyte developmental markers are tightly regulated during positive selection and can be used to follow changes in the affinity and avidity of rearranged TCR for MHC-peptide ligand. CD5 has been shown to negatively affect TCR signaling, and is dynamically modulated on the surface of developing thymocytes in response to TCR affinity/avidity. Consistent with decreased TCR signals (22), CD5 is down-regulated on DP PD-1B T^c in comparison with WT littermate controls (WT CD5 mean fluorescence intensity (MFI) = 83 ± 4, PD-1B T^c CD5 MFI = 59 ± 4; $p < 0.0001$). CD69 is an activation marker that is up-regulated upon productive TCR-MHC peptide interaction on developing thymocytes. In PD-1B T^c mice, the percentage of DP thymocytes is comparable to WT littermates, but the proportion of CD69$^+$ DP thymocytes is significantly reduced (Fig. 1*C*, $p < 0.0001$). The reduced cell surface expression of CD5 and CD69 is consistent with an overall decrease in TCR signaling and an increased positive selection threshold as a result of constitutive PD-1 expression. These findings provide evidence that the higher signaling threshold that results from constitutive PD-1 expression causes fewer DP thymocytes to transition to mature SP cells.

PD-1 expression is induced upon TCR cross-linking of developing thymocytes

PD-1 is primarily expressed on thymocytes in the early DN stage of maturation (23), which is consistent with TCRβ selection defects in PD-1$^{-/-}$ mice. However, PD-1 mRNA expression is enhanced by i.v. injection of anti-CD3 Abs (3), suggesting that PD-1 expression may be induced by TCR cross-linking and also functions later during thymocyte development as a negative regulator of TCR signaling. Consistent with this finding, PD-1 also has been shown to be up-regulated in microarray analyses of several models of thymic selection (24, 25). For these reasons, we examined PD-1 expression upon TCR stimulation in developing thymocytes.

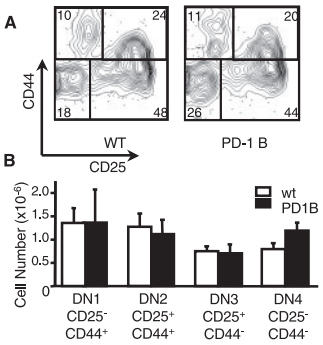

FIGURE 2. DN subsets are unchanged in PD-1 B T^c mice. Overexpression of PD-1 on DN thymocytes does not alter subset frequencies or cell numbers. *A*, CD3$^-$CD4$^-$CD8$^-$ thymocytes from PD-1 B T^c mice ($n = 4$) and WT ($n = 3$) littermates were analyzed for expression of CD25 and CD44. *B*, Thymocyte numbers for the WT and PD-1 B T^c mice were calculated by multiplying the absolute cell yield by the subset percentage. No significant difference was seen in DN thymocyte numbers.

To examine PD-1 expression after TCR cross-linking, we used an in vitro culture system in which DP thymocytes can be induced to undergo maturation upon stimulation with anti-TCR cross-linking Abs (20). Maturation was assessed by CD69 up-regulation and down-regulation of CD4/CD8 coreceptor expression (20). We found that TCR cross-linking on DP thymocytes induces PD-1 up-regulation on 30% of stimulated DP thymocytes (Fig. 3*A*). PD-1 expression was increased on DP thymocytes as early as 4 h after TCR cross-linking (data not shown). It is important to note that not all CD69$^+$ thymocytes express PD-1. It is unclear what differentiates thymocytes that do and do not up-regulate PD-1 after TCR cross-linking, as cells displayed a similar surface phenotype and most likely encountered TCR Abs at a similar time. A low level of PD-1 expression is found on a very small subset of freshly isolated WT CD69$^+$ DP thymocytes (Fig. 3*B*), a population that has productively interacted with selecting MHC-peptide ligand (26). These results are in contrast to published results showing no PD-1 expression on DP thymocytes and may reflect the use of a more specific (CD4$^+$CD8$^+$CD69$^+$ instead of CD4$^+$CD8$^+$) gating strategy (15). These data demonstrate that PD-1 is inducibly expressed by DP thymocytes and lend support for a role for PD-1 in thymic selection events.

Ligation of PD-1 with an agonistic Ab impairs thymocyte maturation

To further investigate the role of PD-1 in DP thymic selection, we used a novel anti-PD-1 agonistic mAb in thymocyte maturation studies in vitro. A panel of anti-PD-1 mAbs was screened for agonistic activity by in vitro stimulation of T cells with anti-CD3 and candidate anti-PD-1 mAbs. We used a T cell population that lacks PD-L1 expression (18) and constitutively expresses PD-1 (PD-1 A T^c PD-L1$^{-/-}$ mice) to eliminate potential interactions between PD-L1 and PD-1 on T cells. PD-1 A T^c mice were used to eliminate the possibility of thymocyte selection defects. Agonistic activity was assessed by evaluating proliferation and cytokine production. One Ab (29F.19G8) of seven tested showed significant

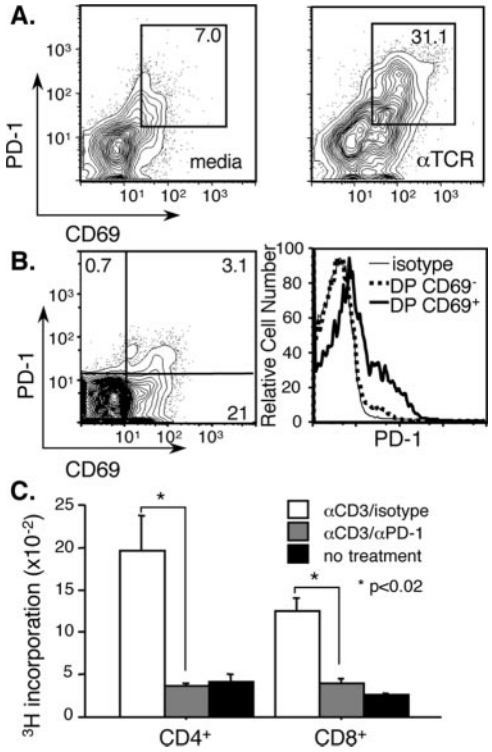

FIGURE 3. PD-1 is expressed on DP thymocytes, and expression is up-regulated after TCR stimulation. *A*, PD-1 expression is enhanced on DP thymocytes from WT mice after TCR stimulation. DP thymocytes were isolated from WT mice and incubated overnight on uncoated plates (medium) or plates coated with anti-TCRβ Abs. After overnight incubation, thymocytes were washed and rested for an additional 18 h before analysis of CD4, CD8, PD-1, and CD69 expression. *B*, CD69$^+$ DP WT thymocytes have increased PD-1 expression in comparison with CD69$^-$ DP thymocytes. Thymocytes were isolated from WT animals and stained for CD4, CD8, CD69, and PD-1. CD4$^+$CD8$^+$ thymocytes were analyzed for CD69 and PD-1 expression (*left*). Increased expression of PD-1 on CD69$^+$ DP thymocytes is shown in a histogram (*right*). *C*, PD-1 agonistic mAb inhibits anti-CD3-induced proliferation of PD-1 T^c PD-L1$^{-/-}$ CD4$^+$ or CD8$^+$ peripheral T cells. Purified CD4$^+$ or CD8$^+$ peripheral T cells were stimulated with plate-bound anti-CD3 and anti-PD-1 mAbs, as indicated. At 24 h, proliferation was assessed by ^{3}H incorporation. Data are representative of three experiments.

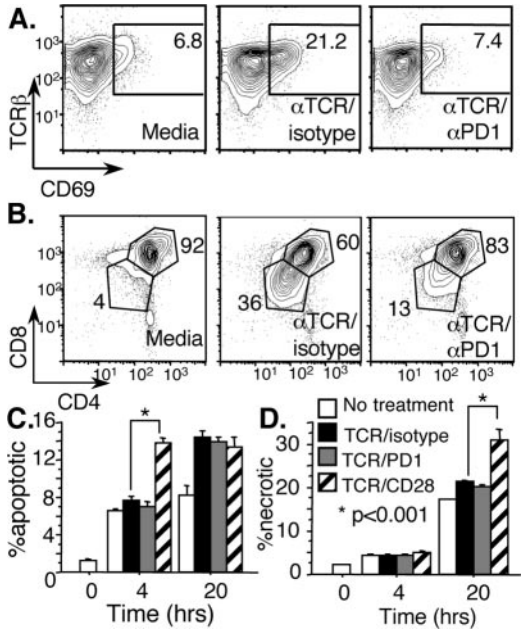

FIGURE 4. Cocross-linking of TCR and PD-1 inhibits TCR-induced CD69 up-regulation and CD4/CD8 coreceptor down-regulation without inducing apoptosis in thymocytes. Immature DP (>98% CD4$^+$CD8$^+$) thymocytes were isolated from WT mice and plated on untreated or Ab-coated plates, as indicated. After culture, thymocytes were stained for CD4, CD8, CD69, and TCRβ or annexin and analyzed by flow cytometry. *A*, Developing thymocytes up-regulate CD69 after culture on anti-TCR-coated plates, but coligation with anti-PD-1 and anti-TCR mAb inhibits CD69 up-regulation. *B*, Immature DP thymocytes down-regulate CD4 and CD8 after TCR stimulation. CD4 and CD8 down-regulation is inhibited by coligation with anti-PD-1 mAb treatment (50 μg/ml). *C*, Cross-linking of TCR and PD-1 does not increase apoptosis in immature thymocytes. Immature DP thymocytes were plated on Ab-coated plates, as indicated, and analyzed for apoptosis by flow cytometry (apoptotic cells were defined as annexin$^+$7-aminoactinomycin D$^-$) after 4 or 20 h. There was no increase in annexin staining on anti-TCR-treated thymocytes cotreated with anti-PD-1 mAb, while thymocytes treated with anti-TCR and -CD28 mAbs showed a significant increase in apoptosis at 4 h. *D*, Cross-linking of TCR and PD-1 does not increase the accumulation of necrotic thymocytes. Thymocytes treated with anti-TCR and anti-CD28 mAbs showed significant increases in necrotic cells at 20 h. Immature DP thymocytes were treated as in *C*, and necrotic cells were defined as annexin$^+$7-aminoactinomycin D$^+$ cells. Data are representative of three independent experiments.

inhibition of PD-1 A T^c PD-L1$^{-/-}$ T cell responses (Fig. 3*C*). Similar results were obtained with T cells from PD-L1$^{-/-}$ mice with WT PD-1 expression (data not shown).

We used a combination of anti-TCR and the agonistic anti-PD-1 mAb to determine the effect of TCR/PD-1 coligation on immature WT thymocytes in vitro. Strikingly, coligation of TCR and PD-1 mAb impairs maturation of immature thymocytes (Fig. 4, *A* and *B*). Twenty to 40% of thymocytes treated with anti-TCR mAb alone up-regulate CD69 (Fig. 4*A*). CD69 expression is indistinguishable on untreated control thymocytes and thymocytes cultured with anti-TCR plus 50 μg/ml anti-PD-1 mAb. PD-1 ligation also inhibits TCR-induced CD4 and CD8 coreceptor down-regulation (Fig. 4*B*). Inhibition of phenotypic maturation is dependent on Ab concentration, with complete inhibition occurring with 50 μg/ml PD-1 agonistic Ab (data not shown). It should be noted that this experiment was performed with WT thymocytes with normal PD-1 expression, providing further evidence that the level of PD-1 expression induced on DP thymocytes is sufficient to transmit a physiological signal. These data indicate that PD-1 ligation opposes TCR-induced phenotypic maturation of DP thymocytes.

PD-1 was initially shown to be up-regulated on thymocytes undergoing cell death after anti-CD3 cross-linking (3). To directly address whether PD-1 ligation induces apoptosis, rather than inhibits phenotypic maturation of thymocytes, we determined whether TCR/PD-1 cocross-linking in vitro increased programmed cell death in developing thymocytes. The percentage of apoptotic cells was similar in DP cells treated with TCR-isotype and those treated with TCR-PD-1 mAb. Coincubation with anti-TCR and anti-CD28 mAbs has been shown to induce apoptosis in DP thymocytes (20), and was used as a positive control. We found no change in apoptosis induction nor accumulation of necrotic cells at 4 or 20 h after the initiation of culture in the presence of anti-PD-1 mAb (Fig. 4, *C* and *D*). Treatment with anti-TCR and anti-CD28 mAb induces significant increases in apoptotic cells at 4 h and necrotic cells at 20 h. Therefore, PD-1 cocross-linking inhibits thymocyte maturation, rather than inducing apoptosis of maturing thymocytes. These findings are consistent with our in vivo data

andsuggest that PD-1 ligation impairs TCR-mediated positive selection.

PD-1 ligation on developing thymocytes inhibits ERK phosphorylation and bcl-2 up-regulation

Recent studies have shown that PD-1 inhibits TCR signaling in peripheral T cells (10). Because our data indicate that TCR/PD-1 cocross-linking impairs positive selection, we examined the consequences of PD-1 ligation on events downstream of TCR signaling that are essential to thymocyte development. We analyzed two key events in positive selection, bcl-2 induction and ERK phosphorylation. Bcl-2 expression is enhanced in developing thymocytes after positive selection (27) and promotes survival of positively selected thymocytes. Although bcl-2 is not a causal event in positive selection, its up-regulation is tightly correlated with positive selection events. Cross-linking of the TCR on developing $CD4^+CD8^+$ thymocytes induces phosphorylation of ERK, which is critical for thymocyte maturation. Strong, transient ERK phosphorylation results in negative selection, while sustained ERK phosphorylation leads to positive selection (28).

We found that TCR/PD-1 cocross-linking inhibits both bcl-2 up-regulation and ERK phosphorylation in developing thymocytes. TCR cross-linking of DP thymocytes induces up-regulation of bcl-2 protein (Fig. 5*A*). However, when TCR and PD-1 are coligated, bcl-2 induction is impaired (bcl-2 MFI: TCR/IgG1, 28.5 ± 0.2 vs TCR/PD-1, 21.2 ± 0.2, $p < 0.0001$; bcl-2 untreated 17.7 ± 0.1). Intracellular staining with a phospho-ERK-specific Ab demonstrated that sustained TCR-induced ERK phosphorylation is inhibited by cocross-linking with PD-1 agonistic Ab (Fig. 5*B*). Phospho-ERK-positive DP^{dull} thymocytes are significantly decreased in thymocytes treated with anti-PD-1 mAb (phospho-ERK^+: TCR/IgG1, 65.4 ± 0.6% vs TCR/PD-1, 55.5 ± 2.1%, $p = 0.01$). Thus, PD-1 ligation impairs bcl-2 induction and ERK phosphorylation, thereby reducing TCR-induced positive selection.

PD-1 signaling negates thymocyte positive selection signals delivered by CD2 and CD28

Previous work has shown that both CD2 (20) and CD28 (29) cross-linking enhance TCR-driven thymocyte maturation in in vitro thymocyte selection assays. Costimulation provided by CD2 or CD28 can enhance thymocyte maturation, as indicated by increased CD69 up-regulation and enhanced down-regulation of CD4/CD8 coreceptor expression (20). This costimulation is associated with ERK-dependent positive selection and increased bcl-2 up-regulation (20). We postulated that PD-1 might dominantly inhibit positive costimulation delivered by CD2 or CD28. To understand the hierarchy of costimulatory signals in thymocyte positive selection, we studied the consequences of cocross-linking PD-1 along with CD2 or CD28 on TCR-mediated thymocyte maturation.

Immature DP thymocytes were cultured as described above, and assessed for CD69 up-regulation and CD4/CD8 coreceptor down-regulation. TCR ligation plus CD2 or CD28 cross-linking significantly increased thymocyte maturation over TCR treatment alone. However, cocross-linking of PD-1 with either CD2 or CD28 was sufficient to entirely negate the effect of CD2 or CD28 on CD69 up-regulation (Fig. 5*C*). As expected, both CD2 and CD28 enhanced TCR-mediated bcl-2 up-regulation. The addition of anti-PD-1 mAb to TCR/CD2- or TCR/CD28-treated cultures significantly decreased bcl-2 expression (Fig. 5*D*, bcl-2 MFI: TCR/IgG1, 16.3 ± 1.9; TCR/CD2, 24.1 ± 1 vs TCR/CD2/PD-1, 13.7 ± 1.3, $p = 0.003$; TCR/CD28, 18.6 ± 0.7 vs TCR/CD28/PD-1, 12.8 ± 1.2, $p = 0.01$). PD-1 ligation also significantly reduced ERK phosphorylation in TCR/CD2 (TCR/CD2 phospho-ERK^+, 68.5 ± 2.4% vs TCR/CD2/PD-1, 53.1 ± 4%, $p = 0.03$)- and TCR/CD28-treated (TCR/CD28 phospho-ERK^+, 64.9 ± 1.8% vs TCR/CD28/PD-1, 56.1 ± 0.4%, $p < 0.01$) DP^{dull} thymocytes (Fig. 5*E*). Thus, PD-1 cross-linking dominantly inhibits positive selection outcomes, even in the presence of additional costimulatory signals.

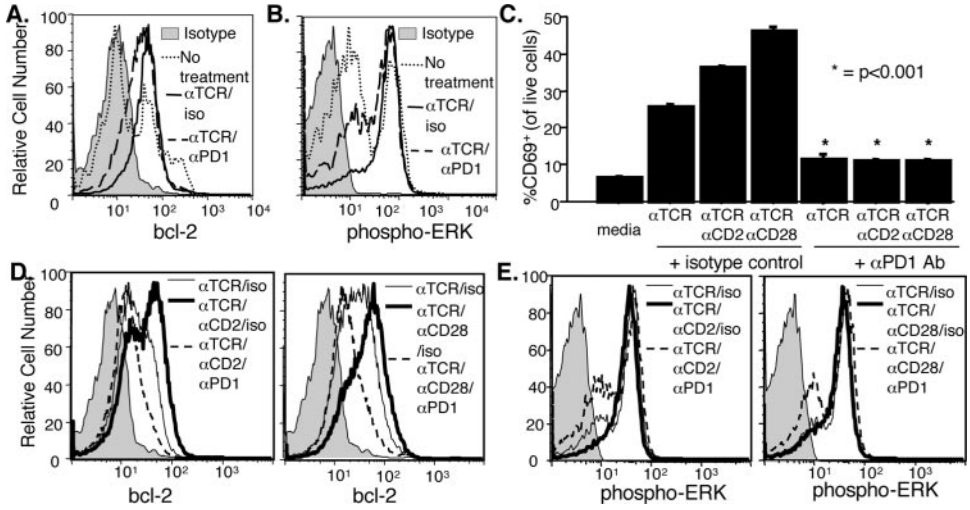

FIGURE 5. Cocross-linking of PD-1 and TCR inhibits up-regulation of bcl-2 and phosphorylation of ERK in developing thymocytes, even in the presence of costimulation. Downstream mediators of positive selection are impaired in developing thymocytes treated with PD-1 agonistic mAb. Immature DP thymocytes were isolated from WT mice and cultured as in Fig. 3. After 18 h, thymocytes were stained for developmental markers and intracellular proteins. *A*, Up-regulation of bcl-2 protein levels is inhibited in DP thymocytes treated with anti-TCR and anti-PD-1 mAb. Isotype control is shown as a shaded histogram. *B*, DP thymocytes that have undergone selection (DP^{dull} cells, as in Fig. 4*B*) show a reduction in sustained phosphorylation of ERK after treatment with anti-TCR and anti-PD-1 mAb. *C*, CD69 up-regulation was increased when DP thymocytes were treated with TCR plus CD2 or CD28 cross-linking mAbs, but the increase was abrogated by coligation with anti-PD-1 mAb. *D*, CD2 or CD28 costimulation enhanced TCR-induced up-regulation of bcl-2. The increase in bcl-2 observed in thymocytes treated with TCR plus CD2 or CD28 cross-linking mAb was inhibited by the addition of PD-1 mAb. *E*, ERK phosphorylation is impaired in DP^{dull} thymocytes treated with anti-PD-1 mAb plus anti-TCR and anti-CD2 or anti-CD28. Data are representative of three independent experiments.

These data suggest that PD-1 may act proximally to TCR signaling, whereas other costimulatory molecules most likely have a more distal effect on TCR-mediated signaling.

Loss of PD-L1, but not PD-L2, increases DP and SP cell numbers

PD-L1 and PD-L2 have nonoverlapping expression patterns in the thymus, suggesting that they may have distinct roles in thymocyte development. To address this question, we first evaluated the number and percentage of thymic subsets in WT, PD-L1$^{-/-}$, and PD-L2$^{-/-}$ mice (Fig. 6). We found no difference in the percentage of thymocyte subsets (Fig. 6*A*), but a significant increase in total thymocytes in PD-L1$^{-/-}$ mice as compared with WT controls (total cell number: WT mice, $128 \pm 47 \times 10^6$ vs PD-L1$^{-/-}$ mice, $176 \pm 57 \times 10^6$, $p = 0.032$). In PD-L1$^{-/-}$ mice, there are significantly more DP and SP4 thymocytes ($p < 0.04$), but not DN or SP8 thymocytes (Fig. 6, *B* and *C*, and data not shown). In contrast, there was no difference between PD-L2$^{-/-}$ and WT total cell numbers or cell subsets. The increase in DP and SP4 thymocytes in PD-L1$^{-/-}$ mice suggests that PD-L1 has a unique and nonredundant role in thymocyte maturation.

Because overexpression of PD-1 on DP thymocytes in PD-1B T^c mice results in defective positive selection with normal expression of PD-L1 and PD-L2 (Fig. 1), we examined the effect of PD-1 overexpression on positive selection in the absence of PD-L1 or PD-L2. We crossed PD-1B T^c mice with PD-L1$^{-/-}$ and PD-L2$^{-/-}$ mice. PD-1B T^c PD-L1$^{-/-}$ and PD-1B T^c PD-L2$^{-/-}$ mice were analyzed for thymocyte numbers. As shown in Fig. 6*A*, PD-1B T^c PD-L1$^{-/-}$ mice have similar thymocyte subset percentages to WT animals. In contrast, the positive selection defects in PD-1B T^c mice are preserved in PD-1B T^c PD-L2$^{-/-}$ mice. PD-1B T^c PD-L1$^{-/-}$ mice have an increased number of both DP and SP4 thymocytes in comparison with WT (Fig. 6, *B* and *C*). The percentage of CD69$^+$ DP thymocytes is similarly decreased in both PD-1B T^c and PD-1B T^c PD-L2$^{-/-}$ mice, but not in PD-1B T^c PD-L1$^{-/-}$ mice (Fig. 6*D*). These data demonstrate that PD-L1 mediates decreased positive selection in thymocytes overexpressing PD-1. The increased numbers of DP and SP4 thymocytes in PD-L1$^{-/-}$ mice suggest that PD-L1 also plays a similar role in positive selection when PD-1 is expressed at normal levels.

PD-1 overexpression decreases positive selection, and loss of PD-L1 increases positive selection in a TCR tg model

To further probe the effects of PD-1 and PD-L1 on thymocyte selection in an Ag-dependent system, we bred PD-1 B T^c and PD-L1$^{-/-}$ mice to H-Y TCR tg$^+$ mice. H-Y TCR tg$^+$ mice express a TCR specific for the male H-Y Ag in the context of H-2D^b, which results in positive selection of SP8 H-Y tg$^+$ thymocytes in female mice and negative selection of H-Y tg$^+$ thymocytes in male mice (15). Previous studies have shown that female PD-1$^{-/-}$ H-Y TCR tg$^+$ mice have an expanded number of DP thymocytes, but normal numbers of mature TCR tg$^+$ SP8 thymocytes.

PD-1 overexpression alters thymocyte cell numbers after the DP stage of maturation. PD-1B T^c H-Y tg$^+$ female mice have normal numbers of DN and DP thymocytes, but only one-fifth the number of TCR tg$^+$ mature SP8 thymocytes found in WT H-Y tg$^+$ controls. Consistent with an alteration in positive selection, CD5 and CD69 expression on DP thymocytes from female PD-1B T^c H-Y tg$^+$ thymocytes is reduced (CD5 MFI: H-Y tg$^+$, 153 ± 6 vs PD-1B T^c H-Y tg$^+$, 107 ± 4, $p = 0.01$; percentage of CD69$^+$: H-Y tg$^+$, 28.2 ± 3.7 vs PD-1B T^c H-Y tg$^+$, 13.9 ± 1.5, $p = 0.04$), but there are a normal number of peripheral CD8 cells (Fig. 7*A*). Overexpression of PD-1 on DP thymocytes in male H-Y mice does not alter the generation of DP and SP8 thymocytes, and has no discernible effect on negative selection (Fig. 7*B*).

The loss of PD-L1 results in an expansion in the number of thymocytes observed in TCR tg$^+$ thymus, regardless of positive or negative selection conditions. We found a 2- to 3-fold increase in the number of thymocytes in PD-L1$^{-/-}$ H-Y TCR tg$^+$ thymi in comparison with WT controls. In comparison, non-tg PD-L1$^{-/-}$ thymus had an average 1.5-fold increase in cell numbers in comparison with WT controls. In female PD-L1$^{-/-}$ H-Y TCR tg$^+$ mice, which positively select H-Y TCR tg$^+$ thymocytes, there are significantly more DN, DP, and SP8 thymocytes. There are also more peripheral CD8$^+$ T cells. The increase in DN thymocytes in these mice complicates the interpretation of the data, as the increased precursor frequency, which was not observed in a non-TCR tg system, will increase the number of mature thymocytes. To eliminate the possibility that the increase in DN thymocytes was due to H-Y TCR$^+$ DN thymocytes, we excluded H-Y TCR$^+$ cells from our analysis and found that there is a 3-fold increase in H-Y

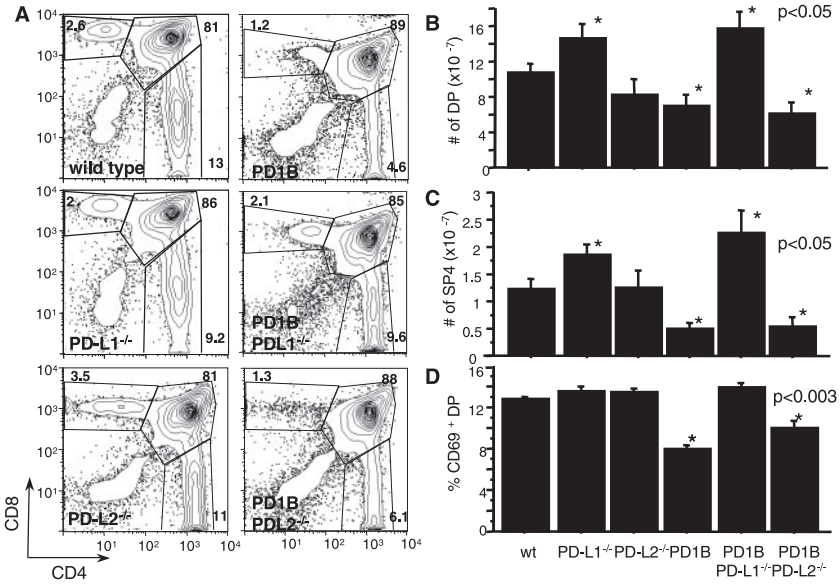

FIGURE 6. PD-1:PD-L1 interactions inhibit positive selection in vivo. PD-L1$^{-/-}$, PD-L2$^{-/-}$, and WT mice were evaluated for changes in thymocyte subsets. PD-1 B T^c mice were backcrossed onto a PD-L1$^{-/-}$ or PD-L2$^{-/-}$ background, and thymocyte numbers were calculated. *A*, CD4/CD8 expression on live thymocytes from a representative animal from each group is shown. *B*, DP thymocyte numbers are increased in PD-L1$^{-/-}$ ($n = 4$) and PD-1 B T^c PD-L1$^{-/-}$ ($n = 3$) mice. DP thymocyte numbers are decreased in PD-1 B T^c ($n = 8$) and PD-1 B T^c PD-L2$^{-/-}$ ($n = 6$) mice. *C*, SP4 thymocytes are reduced in PD-1 B T^c and PD-1 B T^c PD-L2$^{-/-}$ mice, but not in PD-1 B T^c PD-L1$^{-/-}$ mice. No significant difference in SP4 thymocytes was observed between WT ($n = 8$) and PD-L2$^{-/-}$ ($n = 6$) mice, but the number of SP4 thymocytes in PD-L1$^{-/-}$ was significantly increased. *D*, CD69 expression on DP thymocytes is reduced in PD-1 B T^c and PD-1 B T^c PD-L2$^{-/-}$ mice, but not in PD-1 B T^c PD-L1$^{-/-}$ mice. There was no significant difference in CD69 expression observed between WT and PD-L1$^{-/-}$ or PD-L2$^{-/-}$ animals.

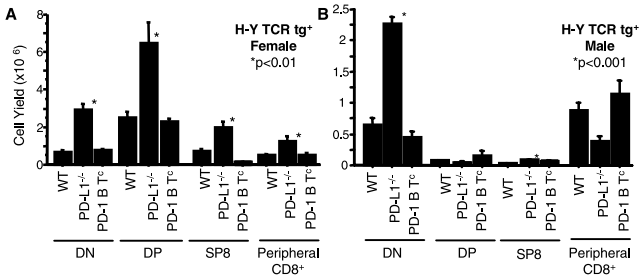

FIGURE 7. PD-1 overexpression decreases and PD-L1 loss increases cell numbers in an Ag-specific model of selection. PD-1 B T^c and PD-$L1^{-/-}$ animals were bred to the H-Y TCR tg^+ strain and evaluated for positive selection (females) and negative selection (males). *A*, Female PD-1 B T^c mice ($n = 3$) have decreased numbers of SP8 thymocytes. Female PD-$L1^{-/-}$ mice ($n = 5$) expressing the H-Y TCR tg have increased numbers of DN, DP, and SP8 thymocytes. PD-$L1^{-/-}$ H-Y TCR tg^+ mice also have an increased number of peripheral $CD8^+$ T cells. *B*, Male mice overexpressing PD-1 ($n = 2$) have no alteration in thymocyte subsets. PD-$L1^{-/-}$ H-Y TCR tg^+ ($n = 2$) mice have increased numbers of DN and SP8 thymocytes, but substantially fewer peripheral CD8 T cells.

TCR^- DN thymocytes in PD-$L1^{-/-}$ H-Y TCR tg^+ thymi in comparison with WT H-Y TCR tg^+ mice. There is also an expansion of both H-Y TCR^+ and H-Y TCR^- DN thymocytes in male PD-$L1^{-/-}$ H-Y TCR tg^+ mice. These data indicate that loss of PD-L1 expands the DN population in an Ag-specific system (Fig. 7*B*). The expansion in numbers of DP and SP8 thymocytes in comparison with WT H-Y TCR tg^+ animals supports the hypothesis that selection is altered. Overall, these in vivo studies confirm a role for PD-1:PD-L1 interactions in modulating positive selection outcomes in developing thymocytes.

Discussion

These data demonstrate that PD-1:PD-L1 interactions are critical to positive selection. Overexpression of PD-1 on DP thymocytes inhibits positive selection, consistent with a negative regulatory role for PD-1 in TCR signaling. Ligation of PD-1 by an anti-PD-1 agonist Ab inhibits positive selection of WT thymocytes, and inhibits bcl-2 expression and ERK phosphorylation. Inhibition through PD-1 can supersede positive costimulation through CD2 or CD28. In both PD-1-overexpressing thymus and PD-L1-deficient thymus, the loss of PD-L1 increases positive selection. Although PD-L2 is expressed in the thymus, it does not appear to be required for positive selection. These findings suggest that PD-1 ligation by PD-L1 during positive selection of DP thymocytes inhibits TCR-mediated signaling, thereby reducing the number of DP thymocytes that receive a TCR signal sufficient to induce maturation to the SP thymocyte stage.

Thymocyte selection depends upon the affinity and avidity of a particular TCR for endogenous peptide MHC (30). The modified affinity/avidity model posits that positive costimulation affects the fate of developing thymocytes (22). Consequently, a moderate affinity/avidity TCR-peptide-MHC interaction, which would normally fall within the threshold for positive selection, could be negatively selected if excessive positive costimulatory signals were delivered in combination with a TCR signal. By extension, a thymocyte expressing a TCR with the same moderate affinity/avidity interaction would fail to be positively selected if inhibitory costimulation was partnered with TCR signaling. In vitro studies showing changes in selection due to increased signaling through CD2 and CD28 support changing the affinity/avidity model to incorporate the demonstrated effects of both negative and positive costimulatory signals on thymocyte selection (20, 22, 29). Our data identify PD-1 as a negative regulator of positive selection capable of modulating thymocyte fate decisions. Furthermore, we demonstrate that PD-L1 is the ligand with which PD-1 interacts during positive selection.

After TCRα expression in the thymus, which occurs around the time of DP cell marker expression, maturing thymocytes undergo cell cycle arrest and are nonproliferative throughout the period of thymocyte selection (31). Our data show that PD-1 expression impairs maturation during β selection, in agreement with published studies showing that loss of PD-1 facilitates TCRβ selection at the DN to DP transition. However, we extend these findings by demonstrating that PD-1 overexpression impairs DP to SP maturation. This phenotype correlates directly with PD-1 overexpression before the SP stage, as PD-1A T^c, a PD-1 tg^+ line that does not express PD-1 on DP thymocytes, has no alteration in thymocyte cell numbers.

PD-1 signaling pathways have not been clearly defined, particularly in thymocytes and T cells. Our agonistic anti-PD-1 mAb shows that PD-1 negatively affects TCR signaling in thymocytes and mature T cells. Previously, we have demonstrated an association between PD-1 ligation and phosphorylation of SHP-2 (5). Direct interaction between PD-1 and SHP-1 and SHP-2 has been shown in T cells; PD-1 is constitutively associated with SHP-2, independent of PD-1 ligation (9). However, the role that SHP-2 plays in T cell activation remains unclear. Expression of a dominant-negative SHP-2 inhibits activation of ERK/MAPK and downstream Ras signaling in Jurkat T cells (32). We show in this study that PD-1 engagement on developing thymocytes results in a decrease in sustained ERK activation. It is possible that PD-1 association with SHP-2 does not result directly in signal transduction, but rather serves to inactivate or sequester SHP-2 from its appropriate signaling partners, resulting in inhibition of ERK phosphorylation. Alternatively, PD-1 may regulate TCR signaling through SHP-2 dephosphorylation of TCR-associated signaling molecules (33). Both of these hypotheses are compatible with the block we observe in TCR signal transduction, which is dominant to the effect that CD2 or CD28 exerts upon TCR signaling. Further work is needed to establish the downstream signals that mediate the effects of PD-1 on ERK phosphorylation and bcl-2 expression.

PD-L1 and PD-L2 both bind and signal through PD-1. Both PD-1 ligands are also expressed in the thymus. PD-L1 is broadly expressed in the thymic cortex, while PD-L2 expression is restricted to medullary stromal cells (13, 14). PD-L1 has been identified as a target of the winged-helix transcription factor (Whn), which is absent in athymic nude mice. The expression of PD-L1 on thymic epithelial cells in embryonic day 12.5 thymic rudiment suggests that PD-L1 expression may play a role in the earliest events in thymopoiesis (34). PD-$L1^{-/-}$ mice have grossly normal thymic development, as evidenced by their normal thymic structures, but as we demonstrate in this study, PD-$L1^{-/-}$ mice exhibit aberrant thymocyte maturation.

Our data indicate an essential and unique role for PD-L1 in mediating positive selection effects through PD-1 signaling. This is consistent with the cortical expression pattern of PD-L1, as positive selection occurs in the cortex, while negative selection occurs at the cortical-medullary junction (35). The changes in thymic selection in PD-$L1^{-/-}$ mice result in increased DP and SP4 cell numbers and changes in markers of thymocyte selection. Moreover, we observe an overall increase in thymocyte numbers in the PD-$L1^{-/-}$ mice. The increase in DN cells in both positive and negative selection conditions in H-Y tg^+ mice differs from an increase in negative selection, as described in 2C PD-$1^{-/-}$ $RAG^{-/-}$ mice (16). This may reflect a subtle role for PD-L2 in the 2C

system. Another possibility is that there are fundamental differences between selection in the 2C and H-Y systems, because an increase in DN cells was not observed in PD-$1^{-/-}$ H-Y tg^{+} mice (15), and we cannot rule out that the increase in this population is particular to the H-Y TCR tg system.

In summary, our data demonstrate that PD-1 engagement impairs TCR-induced signals that would normally result in positive selection. Our in vitro and in vivo data support a role for PD-1 as a negative regulator of TCR signaling in thymocytes. PD-1 signaling in thymocyte selection acts in opposition to TCR-transduced positive signaling, and positive costimulation through CD2 and CD28, by suppressing the up-regulation of bcl-2 and phosphorylation of ERK. The tg mice in which PD-1 is constitutively overexpressed on DP thymocytes display impaired positive selection and fail to up-regulate CD69. PD-1:PD-L1 interactions modulate positive selection in vivo. These findings indicate that PD-1:PD-L1 interactions regulate thymic selection at several stages during T cell development, during both TCRβ selection and positive selection.

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Disclosures

The authors have no financial conflict of interest.

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