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Absence of Programmed Death Receptor 1 Alters Thymic Development and Enhances Generation of CD4/CD8 Double-Negative TCR-Transgenic T Cells[1]

Christian Blank,* **Ian Brown,*** **Reinhard Marks,*** **Hiroyuki Nishimura,[†]** **Tasuku Honjo,[†] and Thomas F. Gajewski[2]***

Programmed death receptor 1 (PD-1) is expressed on thymocytes in addition to activated lymphocyte cells. Its ligation is thought to negatively regulate T cell activation, and PD-1$^{-/-}$ mice develop autoimmunity. To study the role of PD-1 on the development and function of a monoclonal CD8$^+$ T cell population, 2C TCR-transgenic/recombination-activating gene 2$^{-/-}$/PD-1$^{-/-}$ mice were generated. Unexpectedly, ~30% of peripheral T cells in these mice were CD4/CD8 double negative (DN). Although the DN cells were not activated by Ag-expressing APCs, they functioned normally in response to anti-CD3/anti-CD28. These cells had a naive surface phenotype and lacked expression of NK1.1, B220, and γδ TCR; and the majority did not up-regulate CD8αα expression upon activation, arguing that they are not predominantly diverted γδ-lineage cells. The thymus was studied in detail to infer the mechanism of generation of DN peripheral T cells. Total thymus cellularity was reduced in 2C TCR-transgenic/recombination-activating gene 2$^{-/-}$/PD-1$^{-/-}$ mice, and a relative increase in DN cells and decrease in double-positive (DP) cells were observed. Increased annexin V$^+$ cells among the DP population argued for augmented negative selection in PD-1$^{-/-}$ mice. In addition, an increased fraction of the DN thymocytes was HSA negative, suggesting that they had undergone positive selection. This possibility was supported by decreased emergence of DN PD-1$^{-/-}$ 2C cells in H-2^k bone marrow chimera recipients. Our results are consistent with a model in which absence of PD-1 leads to greater negative selection of strongly interacting DP cells as well as increased emergence of DN αβ peripheral T cells. *The Journal of Immunology,* 2003, 171: 4574–4581.

P rogrammed death receptor 1 (PD-1)[3] is a recently described receptor inducibly expressed on peripheral T and B lymphocytes upon activation (1, 2). *PD-1* gene-deficient mice develop lupus-like arthritis and glomerulonephritis, which is accelerated on the *lpr/lpr* background, and can develop autoimmune dilated cardiomyopathy (3, 4), suggesting a role as a negative regulatory receptor on lymphocytes. The cytoplasmatic region of PD-1 contains two tyrosines, one of which is located in an immunoreceptor tyrosine-based inhibitory motif (1, 5). Tyrosine phosphorylation of PD-1 recruits Src homology domain 2-containing tyrosine phosphatase 2 (6), which is thought to dephosphorylate key signaling molecules and down-regulate lymphocyte activation. Although PD-1 has homology to CD28, it lacks the MYPPPY motif, a sequence present in CTLA-4 and CD28 for binding to B7.1 and B7.2 (1). Two ligands have been identified,

B7-H1 (PD-L1) and B7-CD (PD-L2). Expression of these ligands at the mRNA level has been found constitutively in heart, lung, spleen, and kidney tissue and on thymocyte epithelium (7–9) and is inducible on dendritic cells, monocytes, and keratinocytes (7, 8). Up-regulation of PD-L1 on tumor cells has recently been proposed as a mechanism for tumor escape from immune destruction (10, 11).

Because PD-1 is expressed on thymocytes, and because PD-1 ligand mRNA has been found in thymic tissue, it is plausible that PD-1 plays a role in T cell development. Most current data support an avidity model of thymic selection, in which interaction between thymocytes at the double-positive (DP) stage with MHC-peptide complexes expressed on epithelial and hemopoietic cells induces TCR-dependent signaling, the intensity of which determines the fate of individual thymocytes. According to this model, weak signals result in delayed apoptosis (death by neglect), supraoptimal signals promote acute apoptosis (negative selection), and intermediate signals support survival and thus further maturation (positive selection) (12–15). Signal intensity is primarily determined by the TCR itself along with the CD4 or CD8 coreceptors (16–18), but in principle could be modified by additional receptors that influence threshold. In studies of thymocyte subsets, heat-stable Ag (HSA) has been widely used as a maturation marker, because it is expressed at high levels on immature double-negative (DN) thymocytes, at high-to intermediate levels on DP thymocytes, and at low levels on postselection single-positive (SP) thymocytes (19, 20). HSA expression is then absent from naive peripheral T cells.

Although the autoimmune propensity of PD-1-deficient mice could in part be due to a negative regulatory role of PD-1 on peripheral lymphocytes, it is conceivable that it could regulate thy-

mic development as well, perhaps allowing export of mature T cells that normally would not have survived thymic selection. It has been reported that PD-1$^{-/-}$ mice show altered TCR β selection, which in the context of a TCR transgene resulted in skewed thymic development in favor of endogenous rather than transgenic (Tg) TCRα gene expression (21). However, those experiments did not allow for examination of a fixed monoclonal population of T cells to define precisely the mechanism by which PD-1 alters thymic development. To study the role of PD-1 on the development and function of a monoclonal CD8$^+$ T cell population, 2C TCR-Tg/recombination-activating gene (RAG)2$^{-/-}$/PD-1$^{-/-}$ mice were generated. We provide evidence that absence of PD-1 both supports thymic development of DN T cells and increases negative selection at the DP stage, consistent with a role at controlling signaling threshold during development. Such changes were not observed in 2C/RAG2$^{-/-}$ mice lacking another negative regulatory receptor, CTLA-4. Our results suggest that alterations in thymic development could contribute to the autoimmune phenotype of PD-1-deficient mice.

Materials and Methods

Mice

2C/RAG2$^{-/-}$ or 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice (H-2^b) were maintained in a specific pathogen-free barrier facility at University of Chicago. The former have been described previously (22), and 2C/PD-1$^{-/-}$ mice have also been recently described (23). Animals were maintained and used in agreement with our Institutional Animal Care and Use Committee according to the National Institutes of Health guidelines for animal use.

Antibodies

Abs against the following molecules coupled to the indicated fluorochromes were purchased from BD PharMingen (San Diego, CA): CD4-PE, CD4-allophycocyanin, CD8α-FITC, CD8α-PE, CD8α-PerCP, CD8β-biotin, B220-PE, NK1.1-PE, CD24 (HSA)-PerCP, CD24-biotin, IL-2-PE Annexin V^{FITC}, and anti-trinitrophenyl isotype-control Abs. The 2C-TCR was stained using the mAb 1B2 (24), which was FITC coupled in our laboratory. Biotinylated anti-PD-1 was purchased from eBioscience (San Diego, CA), and streptavidin (SA)-conjugated PerCP was obtained from BD PharMingen (San Diego, CA). BrdU staining was performed using a kit from BD PharMingen.

Flow cytometry and sorting

Flow-cytometric analysis was performed as described previously (25) using FACScan and LSR (BD Biosciences, Mountain View, CA) flow cytometers and CellQuest software (BD Biosciences). Cell sorting was performed, staining cells with 1B2-FITC and CD8α-PE in 100 μl of complete medium using FACStar^{Plus} cell sorter (BD Biosciences). Flow cytometry figures were prepared using FlowJo software (Tree Star, San Carlos CA).

T cell purification

Spleens were harvested from 2C/RAG2$^{-/-}$ or 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice and prepared into single-cell suspensions. CD8$^+$ T cells were purified by negative selection bead separation system SpinSep according to the manufacturer's instructions (StemCell Technologies, Vancouver, BC, Canada). An aliquot of purified cells was stained with 1B2-FITC and CD8α-PE mAb for analysis by flow cytometry. T cell purity was routinely >95%.

Tumor cells and reagents

The P815.B71 mastocytoma cell line was generated previously and maintained as described in the presence of geneticin (1 mg/ml) (26). The hybridomas producing anti-CD3 mAb (145-2C11) and anti-CD28 mAb (PV1) were grown in our laboratory and purified by standard methods. PMA (50 ng/ml) and ionomycin (0.5 μg/ml) were purchased from Cellgro (Herndon, VA). For T cell stimulations, 96- or 24-well flat-bottom plates were coated with 1 μg/ml each mAb in 100 μl of sterile PBS overnight at 4°C, and washed twice with PBS before the addition of T cells.

Cytokine and proliferation assays

Purified or sorted T cells were incubated with mitomycin C-treated P815.B71 (each 50,000 cells), PMA plus ionomycin, or anti-CD3/anti-

CD28 mAbs. Supernatants were collected at 18 h, and the concentration of cytokines was detected by ELISA using Ab pairs as instructed by the manufacturer (BD PharMingen). Parallel plates were cultured for 48 h and analyzed for proliferation by pulsing with [^{3}H]thymidine (1 μCi) for the last 6 h of the culture. Cells were harvested, and radioactivity was counted as described (27) using a TopCount-NXT instrument (Packard Instrument, Meriden, CT).

CFSE staining

Purified T cells were washed twice with sterile PBS. Cells (1 × 10^6) were incubated for 5 min in 500 μl of 0.5 μM CFSE (Vybrant CFDS SE Cell Tracer kit; Molecular Probes, Eugene, OR), and staining was stopped by adding 5 ml of FCS. After washing the cells three times with complete medium, they were stimulated with mitomycin C-treated P815.B71 cells and incubated at 37°C. Flow-cytometric analysis was performed in combination with anti-CD8α-PE staining at the indicated time point.

Intracellular FACS staining

Purified naive T cells were stimulated for 6 h with either P815.B71, PMA plus ionomycin, or plate-bound anti-CD3/CD28 mAb. Brefeldin A (1 μg/ml) was added for the last 2 h of incubation. Cells were washed twice with PBS containing 2% FCS and 0.1% NaN$_3$. After staining with anti-CD8α-PE, cells were incubated in 100 μl of PBS containing paraformaldehyde (4%) for 10 min, washed with PBS and then with permeabilization buffer (PBS plus 2% FCS plus 0.1% NaN$_3$ plus 0.1% saponin), and stained for intracellular IL-2 using anti-IL-2-PE or anti-trinitrophenyl isotype mAb-PE (2 μg/ml) for 30 min at 4°C. Stained cells were washed and then analyzed by flow cytometry.

Bone marrow transplantation

Bone marrow was harvested from 2C/RAG2$^{-/-}$ or PD-1$^{-/-}$/2C/RAG2$^{-/-}$ mice and T cell depleted using anti-Thy1.2 mAb (AT83A). No T cells were detected by flow cytometry afterward. Bone marrow cells (2.5 × 10^6 per mouse) were transferred into irradiated (900 rad) H-2^b or H-2^k RAG2$^{-/-}$ mice. Six weeks later, the spleens were removed, and the overall numbers of 1B2$^+$CD8$^+$ and 1B2$^+$CD8$^-$ T cells per spleen were determined by flow cytometry.

Assay for CD8$\alpha\beta$ expression

Spleen cells were harvested from 2C/RAG2$^{-/-}$ or 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice and prepared into single-cell suspensions. CD8$^+$ T cells were depleted using anti-CD8 mAb (3.155) and low-tox guinea pig complement (CL4051; Cedarlane Laboratories, Hornby, Ontario, Canada). Subsequently, the 1B2$^+$CD8α^- population was further enriched by negative sorting using flow cytometry. As a control, CD8$^+$ SP T cells were also sorted. FACS analysis was performed. T cells were stimulated for 4 days with anti-CD3/CD28 beads and mouse IL-2 (20 U/ml), and analyzed by flow cytometry using FITC-1B2, PE-CD8α, and SA-PerCP-biotin-CD8β mAbs.

Statistical analysis

Differences in thymocyte and splenocyte populations were determined using the unpaired t test as calculated with MiniTab 13.31 statistical software (MiniTab, State College, PA).

Results

PD-1-deficient 2C/RAG2$^{-/-}$ mice show an increased proportion of peripheral DN T cells

To dissect the function of PD-1 on a monoclonal population of CD8$^+$ T cells, 2C TCR-Tg/RAG2$^{-/-}$/PD-1$^{-/-}$ mice were generated. Unexpectedly, increased percentages of CD4$^-$CD8$^-$ (DN) 1B2$^+$ T cells in young *PD-1* gene-deficient mice were observed (Fig. 1). When analyzed among a population of mice from independent litters, spleens from PD-1$^{-/-}$ mice contained a mean of over 31% DN 1B2$^+$ T cells compared with 5% in PD-1$^{+/+}$ controls (Table I). Further analysis by flow cytometry showed lack of expression of CD4, NK1.1, and B220, and high expression of CD62L (Fig. 2), indicating that this population of cells represented conventional, naive, DN T cells expressing the clonotypic TCR. We also compared the phenotype of T cells in mice deficient for
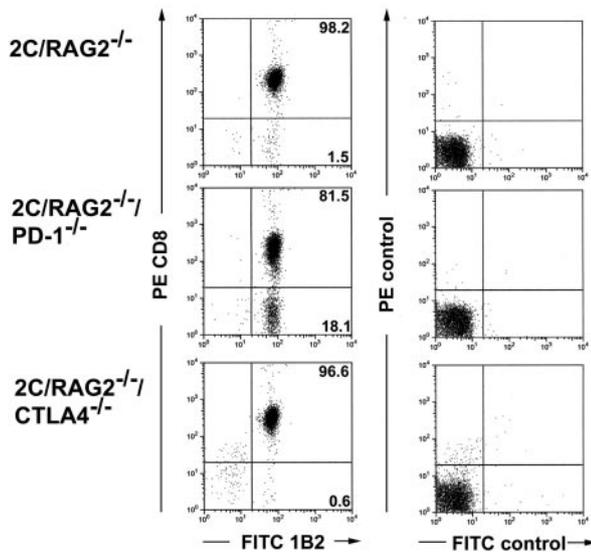
FIGURE 1. Flow-cytometric analysis of splenic T cells from 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice. Purified T cells from 2C/RAG2$^{-/-}$, 2C/RAG2$^{-/-}$/PD-1$^{-/-}$, and 2C/RAG2$^{-/-}$/CTLA-4$^{-/-}$ mice were isolated, stained with FITC anti-1B2 and PE anti-CD8, and analyzed by flow cytometry. Similar results were observed in at least five experiments.

FIGURE 2. Surface phenotype of CD4/CD8 DN 1B2$^+$ T cells from 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice. Spleen cells from 2C/RAG2$^{-/-}$ and 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice were stained and gated on 1B2$^+$CD8$^+$ cells (FITC anti-1B2; PerCP anti-CD8); PD-1$^{-/-}$ splenocytes were also gated on the 1B2$^+$CD8$^-$ population. Third color staining (PE) was performed with Abs against CD4, NK1.1, B220, and CD62L as indicated. Similar results were seen in two independent experiments.

another negative regulatory receptor, CTLA-4. In contrast to PD-1-deficient mice, no increase in DN T cells was seen in 2C/RAG2$^{-/-}$/CTLA-4$^{-/-}$ mice (Fig. 1), suggesting that this finding is relatively unique and not a consequence of eliminating the contribution of any inhibitory receptor.

Peripheral DN T cells from 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice respond to anti-CD3/anti-CD28 stimulation but not to Ag

We next inquired whether the peripheral DN T cells in 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice were functional. Splenic T cells were purified and stimulated with P815.B71 cells that express both Ag and B7-1, and intracellular flow-cytometric analysis was performed to assess IL-2 production by the CD8$^+$ and the CD8$^-$ populations. As shown in Fig. 3A, only the CD8$^+$ subpopulation produced detectable IL-2. The proliferative capacity of DN T cells also was examined. Using CFSE labeling, the DN population of peripheral T cells from 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice was observed not to divide in response to P815.B71 cells at 36 h (Fig. 3A). The CD8$^-$ cells were no longer present at 72 h, consistent with death due to lack of activation (data not shown).

To exclude a caveat that the DN T cells up-regulated CD8α during activation and shifted into the CD8$^+$ gate, we also sorted cells according to CD8 expression before stimulation. As shown in Fig. 3B, purified DN T cells from 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice produced minimal IL-2 compared with CD8$^+$ T cells in response

to stimulation with APCs. Similarly, DN T cells were unable to produce IL-4 or IL-10 upon Ag stimulation (data not shown). However, this nonfunctionality was bypassed when stimuli that are independent of CD8 engagement were used, namely with anti-CD3/anti-CD28 mAbs or PMA/ionomycin (Fig. 3B). DN T cells from 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice also proliferated poorly in response to P815.B71 cells, yet proliferated normally when stimulated with anti-CD3/anti-CD28 mAbs or with PMA/ionomycin (Fig. 3C). Collectively, these results indicate that the DN T cells from 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice do not respond to APCs, but exhibit normal function in response to stimuli that do not depend on CD8 interactions.

PD-1-deficient 2C/RAG2$^{-/-}$ mice show an increased proportion of DN thymocytes

To infer whether the peripheral DN T cells in PD-1-deficient 2C/RAG2$^{-/-}$ mice were thymically selected, thymocytes were enumerated and analyzed by flow cytometry. As shown in Fig. 4A, a marked relative increase in DN thymocytes and decrease in DP thymocytes were observed in 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice compared with age-matched 2C/RAG2$^{-/-}$ controls. The overall thymus cellularity was also statistically significantly reduced, and a significant decrease in the absolute number of DP thymocytes was calculated in PD-1-deficient mice (Table II). These thymic changes were not seen in 2C/RAG2$^{-/-}$/CTLA4$^{-/-}$ mice (Fig. 4A), consistent with the lack of DN T cells in the periphery of those mice. Despite the differences in the numbers of thymocytes, the cellularity of the spleen in 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice was normal (Table I).

The shift in thymocyte subsets in 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice suggested that PD-1 itself should be expressed in the thymus. As shown in Fig. 4B, flow-cytometric analysis revealed expression of PD-1 in DN thymocytes from 2C/RAG2$^{-/-}$ mice, with lower expression observed at the DP and SP stages. In C57BL/6 mice, a lower but significant percentage of PD-1$^+$ cells was seen. As a control, 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ thymuses also were examined and showed no detectable PD-1 expression in any subset. Thus, PD-1 is expressed at the appropriate times during thymocyte development to exert effects on thymic selection.

Altered thymic selection in 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice

There are several potential mechanisms that could explain the increased number of DN cells in 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ thymuses.

Table I. *Splenic 1B2$^+$ populations in 2C/RAG2$^{-/-}$ and 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice^a*

Genotype	Number of Mice	Spleen		
		Total cell number ($\times 10^6$)	1B2$^+$ CD8$^+$ (%) of 1B2$^+$ T cells	1B2$^+$ CD8$^-$ (%) of 1B2$^+$ T cells
+/+	8	69.5 ± 21.1	94.6	5.4
-/-	10	59.6 ± 33.1	68.1	31.9
		$p = 0.450$	$p = 0.018$	$p = 0.007$

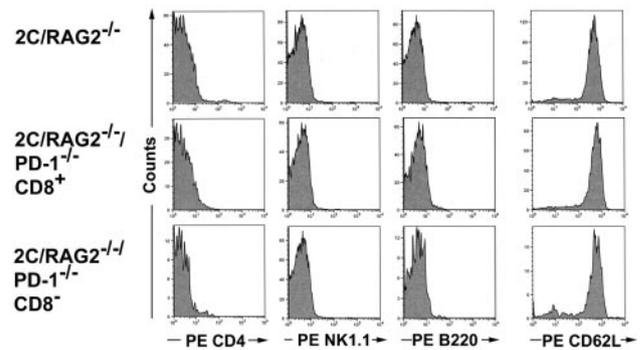^{a} The numbers are shown as mean ± SD. Percentages indicate subsets of the 1B2-positive population.
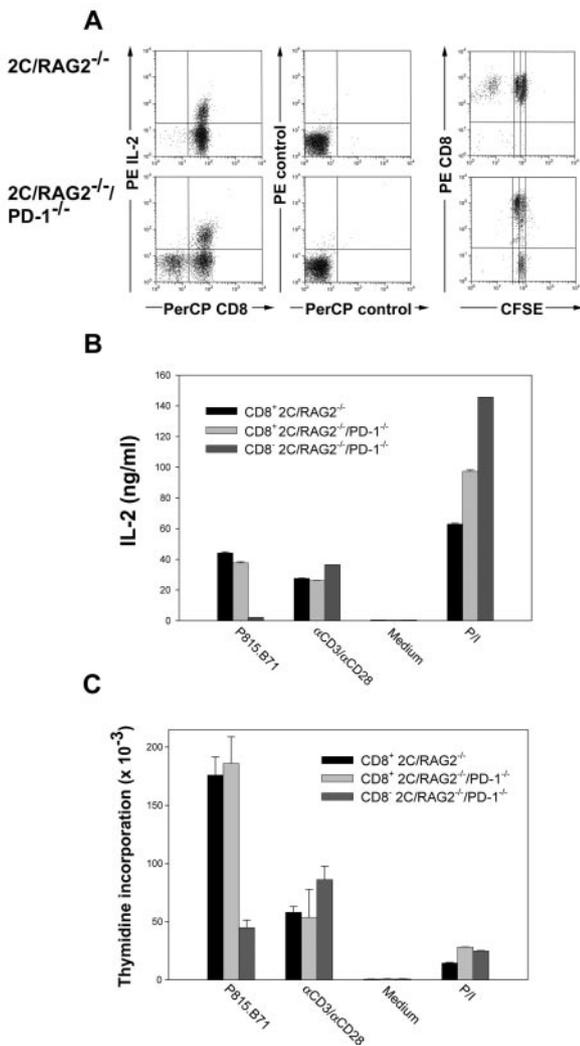
FIGURE 3. Functional analysis of CD4/CD8 DN 1B2$^+$ T cells from 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice. *A*, Intracellular IL-2 production and CFSE dilution. 2C/RAG2$^{-/-}$ and 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ T cells were stimulated with P815.B71 cells, stained with PerCP anti-CD8, permeabilized, and stained intracellularly with PE anti-IL-2 at 6 h. In parallel, T cells were stained with CFSE and analyzed 36 h after stimulation. *B*, IL-2 production by sorted cells. T cells were purified from the spleens of the indicated mice, stained with FITC 1B2 and PE anti-CD8 mAb, and sorted sterilely based on CD8 expression. The sorted cells were stimulated with either P815.B71 cells, anti-CD3/anti-CD28 mAbs, culture medium alone, or PMA plus ionomycin (P/I). Supernatants were collected 18 h later, and IL-2 content was measured by ELISA. *C*, Proliferation of sorted cells. The indicated sorted cells were stimulated with either P815.B71 cells, anti-CD3/anti-CD28 mAbs, culture medium alone, or PMA plus ionomycin (P/I). [^{3}H]Thymidine was added during the last 6 h of a 48-h culture. Similar results were observed in at least two experiments.

First, it was conceivable that this was secondary to an early developmental arrest. However, when thymocytes were gated on 1B2$^+$ cells, the increased fraction of DN cells in 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice was preserved (data not shown and Fig. 5*B*), arguing that a major proportion of these cells was at a later maturation stage. Second, increased negative selection at the DP stage could explain a relative increase of DN thymocytes (28–30). To address this directly, flow-cytometric analysis was performed to determine the proportion of dying cells by annexin V staining. Although no significant change in annexin V$^+$ cells was observed among the DN population in 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice (data not shown),
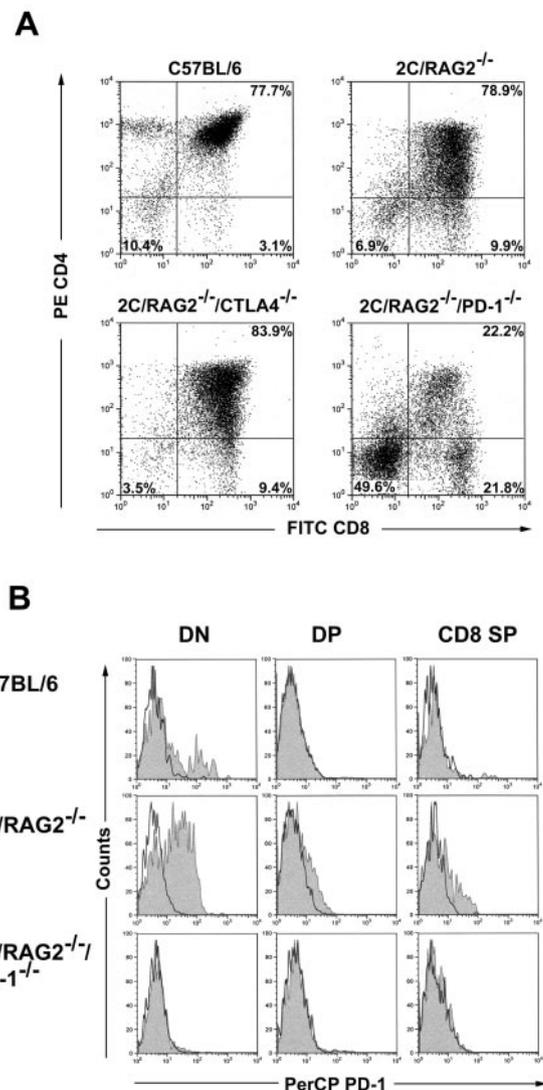

FIGURE 4. Flow-cytometric analysis of thymocytes from 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice. *A*, CD4 vs CD8 staining. Thymuses from the indicated mice were isolated and stained with FITC anti-CD8 and PE anti-CD4 mAbs. Flow-cytometric analysis was performed using a lymphocyte-size gate. *B*, PD-1 expression on thymocyte subpopulations. Thymuses from the indicated mice were stained with PE anti-CD8, allophycocyanin anti-CD4, and biotinylated anti-PD-1 mAbs followed by the addition of SA PerCP. The indicated CD4/CD8 thymus subpopulations were gated upon and analyzed for expression of PD-1. Similar results were seen in at least three experiments.

a substantially increased proportion of DP cells showed annexin V binding (Fig. 5*A*). When averaged over four independent pairs of mice, 23.4% of 1B2$^+$ DP thymocytes from 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice were annexin V$^+$ compared with 1.1% in 2C/RAG2$^{-/-}$ controls ($p = 0.007$). We conclude that the decreased proportion of DP thymocytes in PD-1-deficient mice is due to increased cell death, presumably as a consequence of negative selection.

Because DN T cells appeared to be exported to the periphery, it was conceivable that a third mechanism was operational, successful positive selection of DN thymocytes. Such a mechanism could theoretically occur if the absence of PD-1 increased signaling intensity and supported survival despite absence of CD8. HSA has been used to describe the maturation stage of thymocytes, because it is highly expressed on immature thymocytes in the DN stage and down-regulated during maturation, leading to intermediate expression at the DP stage and low/negative expression at the SP stage

Table II. *Thymocyte subpopulations in 2C/RAG2$^{-/-}$ and 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice^a*

Genotype	Number of Mice	Mean Age (wk)	Thymus						
			Cell number	DN (%)	DP (%)	SP CD8$^+$ (%)	Calculated total number DN	Calculated total number DP	Calculated total number SP CD8$^+$
+/+	7	7.00	103.7 ± 42.3	8.1 ± 4.0	66.8 ± 8.1	18.7 ± 9.5	8.0 ± 3.6	70.8 ± 35.4	18.9 ± 11.6
−/−	7	6.71	32.1 ± 15.2	35.9 ± 13.7	31.9 ± 15.7	23.6 ± 7.6	11.1 ± 7.0	10.9 ± 9.8	7.0 ± 2.5
			$p = 0.004$	$p = 0.001$	$p = 0.001$	$p = 0.310$	$p = 0.330$	$p = 0.005$	$p = 0.038$

^{a} All numbers are shown as mean ± SD. DN, CD4/CD8 DN thymocytes; DP, CD4/CD8 DP thymocytes; SP, CD8 SP thymocytes.

(19, 20). We confirmed this expression pattern in control C57BL/6 mice (Fig. 5*B*). However, an increased fraction of the DN cells in 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice was found to be HSA negative. When five pairs of mice were independently analyzed, ~5-fold greater HSA$^-$ DN thymocytes were observed in PD-1-deficient mice overall ($p = 0.001$). This result was preserved when thymocytes were gated on 1B2 (Fig. 5*B*). In addition, CD8 SP thymocytes were largely HSA negative in these mice, compared with the HSA-intermediate phenotype seen in 2C/RAG2$^{-/-}$ controls (Fig. 5*B*; 54 vs 10% HSA$^-$; $p = 0.001$). Peripheral T cells in both types of mouse were HSA negative, as expected (data not shown). Moreover, the proliferative rate of the DN cells was not increased in

PD-1-deficient thymuses as measured by in vivo BrdU labeling (data not shown). Collectively, these data provide evidence that a proportion of these DN thymocytes may have undergone positive selection in the thymuses of 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice with subsequent export to the periphery.

The majority of peripheral DN T cells from 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ is unlikely to represent diverted γδ-lineage cells

Although the DN T cells that emerged from 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice were not true γδ cells, because they did not express a γδ TCR by flow cytometry and were generated on a RAG2$^{-/-}$ background, it was nonetheless conceivable that they were redirected γδ lineage cells, as has been described for AND TCR-Tg mice (31). To test this possibility, DN 2C splenic T cells, obtained by anti-CD8α/complement depletion followed by flow-cytometric sorting, were stimulated with bead-bound anti-CD3/anti-CD28 mAb and 20 U/ml IL-2 for 4 days. These populations were all >95% pure before activation (data not shown). Similar to previous reports, a significant up-regulation of CD8α in the absence of CD8β (44.6%) was observed following stimulation of DN T cells from 2C/RAG2$^{-/-}$ mice, consistent with a significant proportion of these cells having properties of the γδ lineage. In contrast, only a minor fraction of the DN PD-1$^{-/-}$/2C/RAG2$^{-/-}$ T cells (12.8%) showed such up-regulation. More than 80% remained CD8α$^-$ CD8β$^-$ (Fig. 6). This result suggests that the majority of the DN peripheral T cells found in PD-1$^{-/-}$ 2C/RAG2$^{-/-}$ mice is unlikely to represent diverted γδ-lineage T cells.

To examine this issue further, and to determine whether the peripheral DN cells were generated in part through positive selection, the MHC dependence of their development was examined
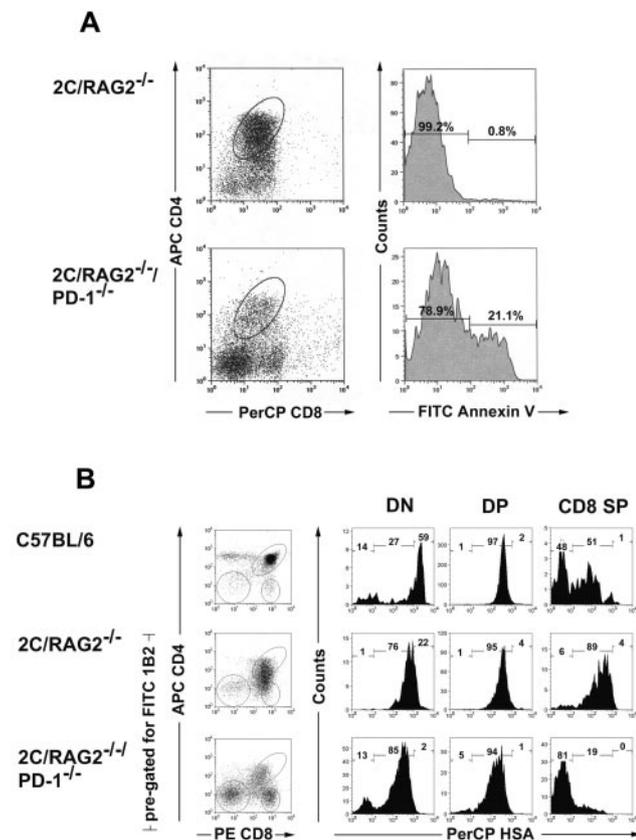

FIGURE 5. Evidence for altered thymic selection in 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice. *A*, Annexin V staining of DP cells. Thymuses from the indicated mice were stained with allophycocyanin anti-CD4, PerCP anti-CD8, and FITC annexin V. The CD4/CD8 DP population was gated on and analyzed for binding to annexin V. Similar results were obtained in three experiments. *B*, HSA expression on thymocyte subpopulations. The indicated thymuses were stained with FITC 1B2, PE anti-CD8, allophycocyanin anti-CD4, and PerCP-SA plus biotinylated anti-HSA Abs. Primary gating was on 1B2$^+$ thymocytes (except C67BL/6 mice), followed by analysis of the CD4 vs CD8 populations. The indicated subpopulations were gated and analyzed for expression of HSA.
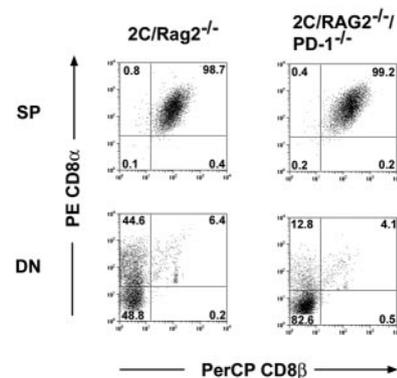
FIGURE 6. CD8α expression on stimulated DN or SP peripheral T cells. 1B2$^+$CD8$^-$ peripheral T cells from 2C/RAG2$^{-/-}$ and 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice were obtained by depletion of CD8a$^+$ cells and additional flow-cytometric sorting. 1B2$^+$CD8$^+$ T cells were obtained by sorting only. Purified cells were stimulated for 4 days using anti-CD3/CD28 beads and IL-2. Cells were harvested, and the beads were removed by Ficoll-Hypaque centrifugation. PE-CD8α and PerCP-CD8β expression was determined by flow cytometry. Similar results were observed in two experiments.

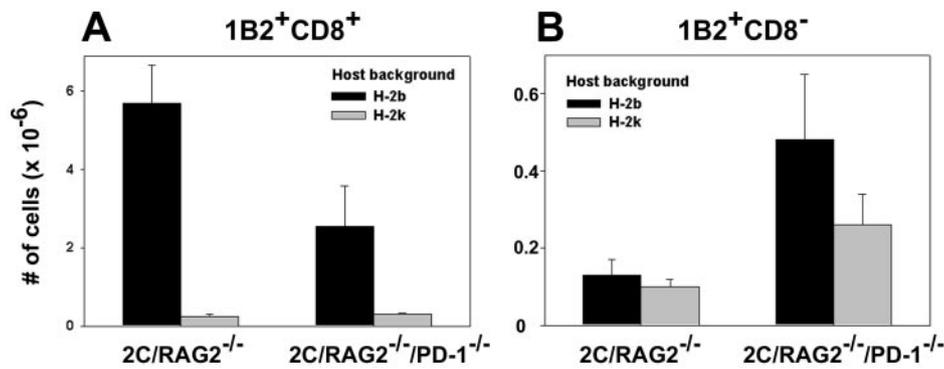**FIGURE 7.** Bone marrow transfer into MHC-mismatched hosts. T cell-depleted bone marrow from either 2C/RAG2$^{-/-}$ or 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice was transferred into three each of irradiated RAG2$^{-/-}$ mice of either the H-2^b or the H-2^k background. Six weeks later, cell numbers of 1B2$^+$CD8$^+$ and1B2$^+$CD8$^-$ T cells were calculated by staining spleen single-cell suspensions with FITC-1B2, PE-CD8α, and propidium iodide followed by analysis by flow cytometry. Numbers of live splenocytes were determined by trypan blue exclusion. Mean results and SDs of each of three mice per group are shown.

(32, 33). T-depleted bone marrow from 2C/RAG2$^{-/-}$ or PD-1$^{-/-}$/2C/RAG2$^{-/-}$ mice was transferred into irradiated syngeneic RAG2$^{-/-}$ H-2^b or RAG2$^{-/-}$ H-2^k mice that should support less positive selection of the 2C $\alpha\beta$ TCR (34, 35). As expected, the numbers of 1B2$^+$CD8$^+$ cells was substantially reduced in H-2^k recipients whether PD-1 was expressed or not, consistent with inferior positive selection of 2C cells in this MHC context (Fig. 7A). Moreover, an increased percentage of CD4/CD8 DN cells emerged in H-2^b mice that received bone marrow from PD-1-deficient 2C/RAG2$^{-/-}$ mice, reproducing the phenomenon seen in straight 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ animals. However, the number of DN T cells observed in H-2^k recipients of 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ bone marrow was reduced by ~50% (Fig. 7B). These results indicate that a significant proportion of the DN T cells arising in the absence of PD-1 depend on MHC for their development and likely arise through positive selection.

Discussion

2C TCR-Tg/RAG2$^{-/-}$/PD-1$^{-/-}$ mice were generated to dissect the role of PD-1 on the development and function of a monoclonal population of CD8$^+$ T cells. We observed the appearance of a large subpopulation of 1B2$^+$ CD4/CD8 DN cells in the periphery of these mice, constituting ~30% of the T cell pool. In the thymus, an increased proportion of DN thymocytes was found, many of which had an HSA-negative phenotype, suggesting that they may have undergone positive selection. In addition, increased death and decreased numbers of DP thymocytes were observed, consistent with increased negative selection as well. These results support a model in which absence of PD-1 increases the signal intensity of thymocytes interacting with APCs during development, allowing positive selection of weakly interacting DN thymocytes and greater negative selection of a subset of strongly interacting DP thymocytes.

Our results are at first glance inconsistent with those of a previous report that showed an overall increase in the thymic cellularity in 2C × PD-1$^{-/-}$ mice (21). However, our current study was performed on a RAG2$^{-/-}$ background, excluding the opportunity for developing thymocytes to be selected on endogenously rearranged alternative TCRs. Fixing the $\alpha\beta$ TCR allowed us to track a single monoclonal population of developing thymocytes. Thus, although the thymocyte flow cytometry profiles from the present and previous studies appear different, the implications of the results are compatible and indicate an important contribution of PD-1 to the regulation of thymic selection. Of note, only a subset of T cells in 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice showed a DN pheno-

type, arguing for some heterogeneity in the fate of thymocytes that developed in the absence of PD-1. This could be because of additional negative receptors that contribute to selection, or due to a stochastic process that enables some DN thymocytes to be positively selected and some DP thymocytes to escape negative selection. In addition, straight PD-1$^{-/-}$ mice did not show an increased number of DN thymocytes (data not shown), arguing that the ability to undergo normal TCR gene rearrangement prevents the emergence of this population. This observation suggests that the T cell repertoire might be different in wild-type vs PD-1$^{-/-}$ mice, a hypothesis that should be addressed in future studies.

Not all negative regulatory receptors appear to regulate signaling threshold during thymic development. In our study, CTLA4$^{-/-}$ 2C/RAG2$^{-/-}$ mice did not show an altered thymocyte phenotype and did not generate increased peripheral DN cells. In contrast, shifts in thymocyte subsets have been reported in CD5-deficient TCR-Tg mice (36) as well as in mice overexpressing the positive costimulatory molecules B7-1 (37) and HSA (38), the latter result suggesting that increasing costimulation can in some instances have a similar outcome. Interestingly, the ability of CD5 to perturb thymic development does not depend on its extracellular domain (39), suggesting that the effect is ligand independent. Although it is likely that the PD-1 effect is mediated through engagement by PD-L1 or PD-L2, this has yet to be demonstrated directly. It is plausible that perturbed thymic development contributes to the autoimmune propensity of PD-1$^{-/-}$ mice through alteration of the T cell repertoire.

Although the developmental lineage of the DN T cells that arose in 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice is not completely known, our data suggests that they are largely conventional $\alpha\beta$ T cells that were positively selected in the thymus and exported to the periphery. Previous studies have noted that TCR-Tg mice often display an abnormally high proportion of DN T cells that presumably is due to early expression of the TCR (40, 41). In contrast to those results, we did not observe DN T cells in 2C/RAG2$^{-/-}$ mice but only in the PD-1-deficient context. It has been argued that the DN $\alpha\beta$ T cells that emerge in TCR mice are diverted from a precommitted $\gamma\delta$ lineage (31). Although our experiments were done on a RAG2$^{-/-}$ background, thus precluding $\gamma\delta$ gene rearrangement, we additionally observed that only a minor fraction of the DN T cells acquired CD8$\alpha\alpha$ expression following activation. Moreover, the number of DN cells was reduced in chimeras having an MHC background that did not positively select the 2C TCR, suggesting that at least a proportion of the cells was positively selected. Although the possibility that a fraction of these DN cells represent

diverted $\gamma\delta$ lineage cells cannot be ruled out, these results argue that positively selected $\alpha\beta$ TCR-expressing cells that lack CD4 and CD8 represent a component of this population.

Although the peripheral DN T cells found in 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice did not respond to stimulation with APCs, they did produce IL-2 and proliferate in response to anti-CD3/anti-CD28 mAbs. This difference is most likely due to a requirement for CD8 coreceptor engagement for optimal activation by peptide/MHC complexes on APCs. The peripheral T cell phenotype observed in 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice has similarities to what has been reported with male anti-HY TCR-Tg mice that express Ag in the thymus (42, 43), but is distinct. In that system, no peripheral TCR-Tg T cells expressing normal CD8 levels were found in the periphery, but rather CD8^{low} cells were observed. In contrast, 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice display one population expressing normal CD8 and a second population lacking CD8. In addition, PD-1-deficient CD8-positive T cells show normal function to Ag stimulation, whereas the CD8^{low} T cells from the HY model were refractory to Ag presented by APCs. Thus, the peripheral phenotype in 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice is unlikely to be explained by increased negative selection alone.

Although regulatory T cell function has been found among conventional DN peripheral $\alpha\beta$ T cells (28, 44), we did not find that such T cells from 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice were inhibitory for T cell cytokine production or proliferation (C. Blank and T. Gajewski, unpublished observation). In addition, when nonfractionated 2C T cells were stimulated with P815.B71 cells, we found comparable CFSE dilution among the CD8^{+} cells from PD-1$^{-/-}$ mice and PD-1$^{+/+}$ mice despite the presence of DN T cells, arguing against a negative contribution of the DN T cells.

In summary, our results provide evidence that PD-1 can regulate signaling threshold during T cell development and thus influence thymic selection. Despite these thymic effects, the resulting peripheral CD8^{+} T cell population in 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice appears to respond normally to Ag, which will make it a convenient model to study a potential negative regulatory role of PD-1 on peripheral CD8^{+} T cell responses following adoptive transfer in vivo.

Acknowledgments

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