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PD-1 Regulates Self-Reactive CD8$^+$ T Cell Responses to Antigen in Lymph Nodes and Tissues[1]

Mary E. Keir,* Gordon J. Freeman,† and Arlene H. Sharpe[2]*

PD-1, an inhibitory receptor expressed on activated lymphocytes, regulates tolerance and autoimmunity. We tested the role of PD-1:PD-1 ligand (PD-L) interactions in cross-presentation and the generation and control of CD8$^+$ responses against self-Ag. Ag-naive PD-1$^{-/-}$ OVA-specific OT-I CD8$^+$ T cells exhibited exacerbated responses to cross-presented Ag in mice expressing soluble OVA under the control of the rat insulin promoter (RIP-ova^{high}). Following adoptive transfer into RIP-ova^{high} recipients, PD-1$^{-/-}$ OT-I T cells expanded in the pancreatic lymph node. In contrast to wild-type OT-I cells, PD-1$^{-/-}$ OT-I T cells secreted IFN-γ and migrated into the pancreas, ultimately causing diabetes. Loss of PD-1 affected CD8$^+$ cells intrinsically, and did not significantly alter the responses of wild-type OT-I T cells adoptively transferred into the same RIP-ova^{high} recipient mouse. PD-1:PD-L interactions also limited CD8$^+$ effector cells, and PD-L1 expression on parenchymal tissues protected against effector OT-I T cell attack. Finally, we found that the loss of PD-1 on effector OT-I cells lowers the threshold for Ag recognition in peripheral tissues. These findings indicate two checkpoints where PD-1 attenuates self-reactive T cell responses: presentation of self-Ag to naive self-reactive T cells by dendritic cells in the draining lymph node and reactivation of pathogenic self-reactive T cells in the target organ. ***The Journal of Immunology,*** **2007, 179: 5064–5070.**

The ability of the immune system to discern self- from nonself-Ags is dependent on both central and peripheral tolerance (1). In the periphery, dendritic cells (DCs)[3] can present endogenous self-Ags as well as take up exogenous soluble self-Ag and present it to CD8$^+$ T cells. The latter process, termed cross-presentation, can result in an abortive immune response that leads to the induction of tolerance through deletion or anergy of self-reactive T cells (2). Cross-tolerance has been well characterized using adoptive transfer models of Ag-specific CD8$^+$ T cells that are transferred into recipient mice that express a defined Ag in a peripheral tissue (3). In this paper, we use rat insulin (RIP) promoter ova^{high} transgenic animals which produce soluble OVA in their pancreatic β cells and can induce deletional tolerance in adoptively transferred OT-I CD8$^+$ T cells (4). Tolerance induction in the RIP-ova^{high} model is induced via cross-presentation of soluble pancreatic Ags by DCs in the pancreatic LN (5). Several factors control the induction of cross-tolerance by DCs. The amount of Ag being presented and the activation status of the DC are known to impact the outcome of cross-presentation of self-Ags. In addition, costimulatory molecules may regulate cross-tolerance, but the influence of negative and positive second signals is not well understood.

Costimulatory molecules contribute to Ag receptor signaling elicited by peptide-MHC ligation (6). The best-characterized pathway consists of B7-1 and B7-2, which bind to both the costimulatory receptor CD28 and the inhibitory receptor CTLA-4. Full T cell activation requires B7-CD28 interactions, and B7 up-regulation on DCs is critical to their function as APCs (7). More recently, a pathway consisting of PD-1 and its two ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC), has been described. PD-L1 and PD-L2 regulate signaling by DCs and modify Ag receptor signals (8). The influence that PD-1 exerts on the immune responses is only beginning to be understood (9). PD-1 is up-regulated upon activation by both CD4$^+$ and CD8$^+$ T cells, and is expressed by exhausted CD8$^+$ T cells exposed to chronic viral Ag. PD-L1 and PD-L2 differ in their expression patterns. PD-L1 is expressed on T and B cells, macrophages and dendritic cells, as well as parenchymal cells such as pancreatic β cells, vascular endothelium, syncytiotrophoblasts in the placenta, and corneal endothelial cells in the eye (6). PD-L2 is restricted in its expression to DCs and macrophages. PD-L1 is expressed on resting DCs and sharply induced upon activation, while PD-L2 is expressed primarily on activated DCs. Because both PD-L1 and PD-L2 are expressed on DCs, the primary cell type responsible for cross-tolerance (10), we examined the role of the PD-1 receptor and ligands in cross-tolerance.

We explored the function of PD-1 on CD8$^+$ T cells during cross-tolerance and priming to self-Ags using the RIP-ova model system (4). Adoptive transfer of OVA-specific (OT-1) CD8$^+$ T cells into transgenic mouse lines expressing high or low amounts of soluble OVA under the control of the rat insulin promoter allowed us to characterize the requirements for PD-1 on the CD8$^+$ T cell during priming, and determine whether PD-1 exerts a cell-intrinsic or −extrinsic effect on T cell responses. We tested the requirement for PD-L1 on parenchymal cells vs APCs in regulating CD8$^+$ T cells. Together, our findings reveal two checkpoints where PD-1:PD-L interactions are critical for peripheral CD8$^+$ T cell tolerance: 1) initial cross-presentation interactions between DCs and CD8$^+$ T cells and 2) control of effector responses in the

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[3] Abbreviations used in this paper: DC, dendritic cell; ES, embryonic stem; ILN, inguinal lymph node; PD-1, programmed cell death-1; PD-L, programmed cell death ligand; PLN, pancreatic lymph node; RIP, rat insulin promoter; WT, wild type.

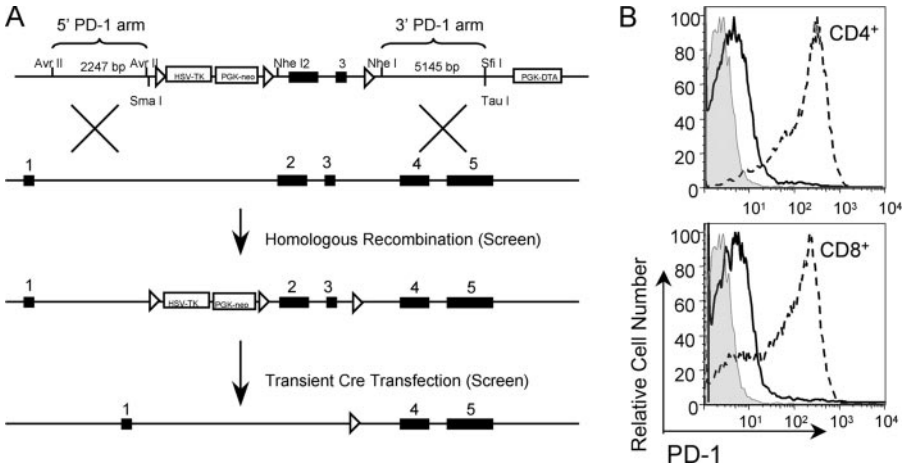

FIGURE 1. Generation of PD-1$^{-/-}$ mice. *A*, The targeting vector contains loxP sites on either side of a selection cassette containing neomycin (neo) under control of the phosphoglycerate kinase (PGK) promoter and thymidine kinase (TK) under control of the herpes simplex virus (HSV) promoter. The construct also contains diptheria toxin (DTA) under control of the PGK promoter, which allows selection against nonhomologous recombination events. The five exons of PD-1 are represented by filled rectangles. *B*, T cells were isolated from PD-1$^{-/-}$ mice and stimulated with plate-bound anti-CD3 and -CD28 for 48 h. Both CD4$^+$ (*top panel*) and CD8$^+$ (*bottom panel*) T cells from a WT mouse up-regulated PD-1 after stimulation, while T cells from PD-1$^{-/-}$ mice did not up-regulate PD-1. Unstained (shaded histogram), WT (dotted line), PD-1$^{-/-}$ (solid line).

tissues independently of priming events. These data indicate that PD-1 and its ligands regulate both the induction and maintenance of CD8$^+$ T cell tolerance.

Materials and Methods

Mice

PD-1$^{-/-}$ mice were constructed by targeting the second and third exon of PD-1 in C57BL/6 ES cells (Fig. 1*A*). This strategy resulted in the deletion of the ligand-binding and transmembrane domain of the PD-1 protein. The targeting vector was constructed from a backbone containing loxP sites on either side of a selection cassette containing neomycin (neo) under control of the phosphoglycerate kinase promoter and thymidine kinase under control of the herpes simplex virus promoter, kindly provided by Dr. David Kwiatkowski (Brigham and Women's Hospital). Exon 2 and 3 of PD-1 were cloned by PCR into the vector downstream of the selection cassette, while the flanking regions of the PD-1 gene were isolated and cloned from a bacterial artificial chromosome using standard techniques. Linearized vector DNA was electroporated into Bruce4 ES cells and the resulting neomycin-resistant ES cells were screened by PCR and southern blot for homologous recombination. Positive ES cells were transiently transfected with a cre plasmid and cells containing the neo/thymidine kinase selection cassette were selected against using 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouridine. Surviving ES cells were screened for the desired lox recombination and injected into pseudopregnant females to generate chimeras.

The offspring of chimeras were screened using the PD-1 deletion-specific primers, PD-1 primer 1 5′-ACAACACAGGGTAGGCATGTAGCA-3′, PD-1 primer 2 5′-TCCTGCCAAACCTTGTAGTCA-3′, and PD-1 primer 3 5′-gctagccaaccagaagtctaa-3′. PCRs were run at 94°C for 2 min, then 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 90 s, then 72°C for 5 min. Amplification of the knockout allele, a deletion of exon 2 and 3, with primer 1 and 2 yielded a 325 bp product, while the wild-type (WT) allele containing the floxed exon 2 and 3 with primer 1 and 3 yielded a 234 bp product. PD-1$^{+/-}$ offspring were mated to OT-I Thy1.1 mice (Jackson) and bred to homozygosity. RIP-ova^{high} and RIP-ova^{low} mice were obtained from Jackson Laboratories and bred in our facility. Mice were maintained and used according to institutional and National Institutes of Health guidelines in a pathogen-free facility. Harvard Medical School and Brigham and Women's Hospital are accredited by the American Association of Accreditation of Laboratory Animal Care.

Adoptive transfer of naive OT-I T cells

PD-1$^{-/-}$ OT-I Thy1.1$^{+/+}$, OT-I Thy1.1$^{+/+}$, and OT-I Thy1.1$^{+/-}$ mice were used as donors for adoptive transfers. The congenic marker Thy1.1, either expressed alone (Thy1.1$^{+/+}$) or as a heterozygote maker with Thy1.2 (Thy1.1$^{+/-}$), was used to differentiate adoptively transferred cells from endogenous WT cells (Thy1.2$^{+/+}$). Donor mice were sacrificed and lymphocytes were isolated from LN and spleens by smashing organs through 70 μm mesh. RBCs were lysed, and cells were washed twice before isolation of CD8$^+$ T cells using MACS beads. After isolation, cells were routinely >98% positive for CD8. Cells were labeled with CFSE as previously described (11), then 5×10^6 cells were adoptively transferred i.v. into RIP-ova^{high} or RIP-ova^{low} recipients. Recipient animals were either sacrificed at defined points after transfer or followed for 30 days and monitored daily for high urine glucose (Diastix; Bayer Pharmaceuticals). Positive glucosuria readings were confirmed by blood glucose measurement (Ascensia Elite; Bayer Pharmaceuticals).

Recipients were sacrificed and cells were isolated from the inguinal lymph node (ILN), pancreatic lymph node (PLN), and pancreas. The pancreas was teased apart, then digested for 20 min at 37°C in 400 U/ml Collagenase D in HBSS. Pancreas preps were then washed with 5 mM EDTA in HBSS and pipetted repeatedly, then filtered through 70 μm mesh to isolate lymphocytes. In some experiments, intracellular cytokine staining was done on freshly isolated lymphocytes as described previously (12). CFSE analysis was performed using the proliferation platform of FlowJo software (Treestar). For histologic evaluation, pancreata were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Adoptive transfer of activated OT-I T cells

OT-I or PD-1$^{-/-}$ OT-I CD8$^+$ T cells were isolated as described above. CD8$^+$ T cells were washed and counted, then incubated at a 1:10 ratio with irradiated APCs (3000 rads) from TCR$\alpha^{-/-}$ mice, plus SIINFEKL (500 ng/ml) and IL-2 (60 U/ml), which was obtained from the National Institutes of Health AIDS Reagent Repository. Cultures were split every second day, and CD8$^+$ T cells were re-isolated on day 5 using CD8 MACS beads as described above. CD8$^+$ cells were rinsed, counted, resuspended in PBS and 3.75×10^5 cells were transferred i.v into RIP-ova^{high} or RIP-ova^{low} recipients. Recipient animals were monitored daily for urine glucose as described above. After onset of diabetes, or at 30 days posttransfer, animals were sacrificed and pancreata were fixed in 10% formalin and processed for histologic evaluation.

Results

Generation of PD-1$^{-/-}$ mice

PD-1$^{-/-}$ mice were generated in our lab using a targeting strategy that eliminated exon 2 and 3 of the *pdcd1* gene, which encode the ligand binding and transmembrane domains of PD-1 (Fig. 1*A*). Phenotypic subsets in the PD-1$^{-/-}$ mouse were grossly normal, except for a small decrease in the B1 cell compartment and a modest increase in thymic, but not peripheral, Foxp3$^+$ regulatory T cell subsets (data not shown). Both CD4$^+$ and CD8$^+$ PD-1$^{-/-}$ T cells failed to up-regulate PD-1 upon stimulation with anti-CD3/

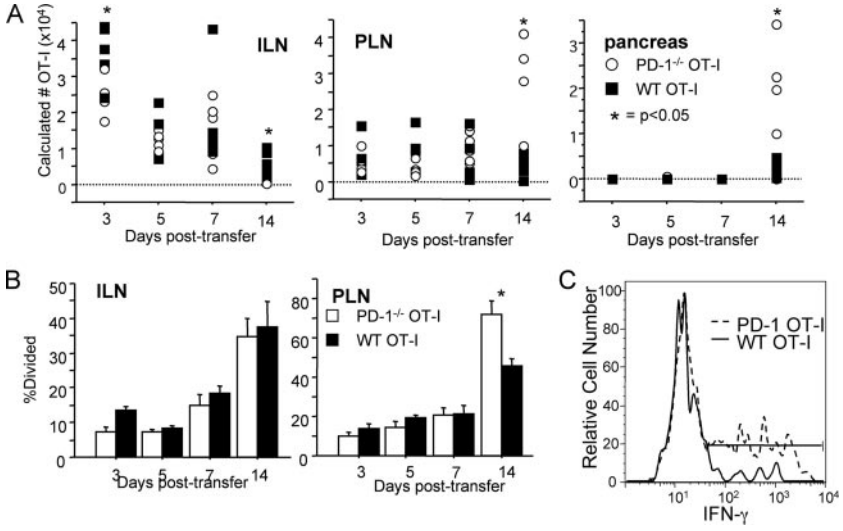

FIGURE 2. PD-1$^{-/-}$ OT-I CD8$^+$ T cells fail to be cross-tolerized in vivo. *A*, Mice that received WT or PD-1$^{-/-}$ OT-I T cells were sacrificed 3, 5, 7, or 14 days after adoptive transfer of CFSE labeled cells. The number of OT-I cells was determined by multiplying the cell yield by the percentage of CD8$^+$Thy1.1$^+$ cells. More PD-1$^{-/-}$ OT-I T cells were recovered from the PLN and pancreas. *B*, The percentage of OT-I CD8$^+$Thy1.1$^+$ cells that divided was determined by CFSE dilution. *C*, Intracellular cytokine staining of PD-1$^{-/-}$ OT-I T cells from the PLN of RIP-ova^{high} recipients showed increased IFN-γ production ex vivo in comparison to WT controls.

CD28 (Fig. 1*B*). Stimulation of PD-1$^{-/-}$ T cells with Con A or B cells with LPS also failed to result in PD-1 expression (data not shown). In contrast, PD-1 was up-regulated on T cells from WT littermates under these conditions.

PD-1$^{-/-}$ CD8$^+$ T cells are inappropriately activated by cross-presentation in vivo

To test the role of PD-1 in a model of in vivo cross-tolerance, we compared expansion, function, and persistence of OT-I and PD-1$^{-/-}$ OT-I T cells following adoptive transfer into RIP-ova^{high} recipients. Low-level proliferation of WT and PD-1$^{-/-}$ OT-I T cells was observed via CFSE dilution in the PLN and inguinal LN (ILN) at early timepoints (Fig. 2, *A* and *B*). This low-level proliferation has been previously reported to lead to deletion or anergy of OT-I T cells in RIP-ova^{high} recipients (4). There was an increase in the CFSE^{low} population among PD-1$^{-/-}$ OT-I T cells in comparison to WT controls. This began to be evident in PLN and pancreas around 7 days after transfer (Fig. 2*A*), and reached statistical significance at day 14 (Fig. 2, *A* and *B*). Earlier time-points showed similar division, as monitored by CFSE dilution, between the groups (Fig. 2*B*). The increased proliferation correlated with an infiltration of PD-1$^{-/-}$ OT-I T cells into the pancreatic islets as early as day 5 and 7 in matched samples (evident by histological analysis in 1 of 3 mice at day 5 and 3 of 3 mice at day 7). Because WT OT-I T cells undergo deletional tolerance after Ag exposure and proliferation, these data indicate that tolerance cannot be induced in PD-1$^{-/-}$ T cells by cross-presented peripheral self-Ag.

Increased numbers of PD-1$^{-/-}$ OT-I T cells persisted in both the pancreas and the PLN 2 weeks after transfer (Fig. 2*A*) and there was a substantial increase in IFN-γ production per cell by PD-1$^{-/-}$ OT-I T cells in the PLN and pancreas, but not earlier (Fig. 2*C* and data not shown). Although we could detect WT OT-I T cells in RIP-ova^{high} recipients 2–3 wk after transfer, the number of cells in recipients receiving PD-1$^{-/-}$ OT-I T cells was generally much higher than that found in RIP-ova^{high} mice that received WT OT-I T cells (5% vs 0.01% in pancreas and 1.7% vs 0.4% in PLN). Together, these data demonstrate that PD-1$^{-/-}$ OT-I T cells are not tolerized by cross-presentation in vivo, and fail to undergo normal deletional tolerance or anergy induction.

Adoptive transfer of PD-1$^{-/-}$ OT-I CD8$^+$ T cells into RIP-ova^{high} recipients induced diabetes within 1 to 3 weeks (Fig. 3). In contrast, WT OT-I CD8$^+$ T cells did not induce diabetes in RIP-ova^{high} recipients, in agreement with published reports (4). Histological analyses of the pancreas revealed severe insulitis in recipients of PD-1$^{-/-}$ OT-I T cells, while no peri-insulitis or insulitis was found in recipients of WT OT-I T cells at any timepoints (day 3, 5, 7, or 30 days) after adoptive transfer (Fig. 3, *B* and *C*; data not shown). Peri-ductal infiltrates and vasculitis within the pancreas were also commonly observed in recipients of PD-1$^{-/-}$ OT-I T cells (data not shown).

Increased response of PD-1$^{-/-}$ OT-I T cells is cell-intrinsic

The role of PD-1 ligation on T cells may be direct, through modulation of Ag receptor signaling, or indirect, through interactions with another cell type that expresses PD-L1 such as a regulatory T cell. To address this possibility, we adoptively transferred equal numbers of WT OT-I Thy1.1$^{+/-}$Thy1.2$^{+/-}$ T cells and PD-1$^{-/-}$ OT-I Thy1.1$^{+/+}$Thy1.2$^{-/-}$ T cells into RIP-ova^{high} Thy1.1$^{-/-}$ Thy1.2$^{+/+}$ recipients. We then used the differential expression of Thy1.1 and Thy1.2 on adoptively transferred cells to assess their proliferation and migration into tissues. Upon reisolation, we analyzed CD8$^+$Thy1.1$^+$ transferred cells and then further

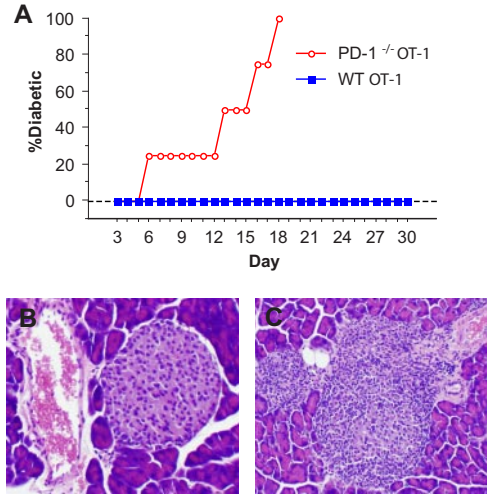

FIGURE 3. PD-1$^{-/-}$ OT-I CD8$^+$ T cells induce diabetes in RIP-ova^{high} recipients. *A*, Five million WT or PD-1$^{-/-}$ OT-I T cells were adoptively transferred into RIP-ova^{high} recipients and monitored for diabetes for 30 days. All of the recipients that received PD-1$^{-/-}$ OT-I T cells became diabetic. Representative histology for RIP-ova^{high} mice that received WT OT-I and were sacrificed 30 days after transfer (*B*) and diabetic PD-1$^{-/-}$ OT-I (*C*) CD8$^+$ T cells is shown.

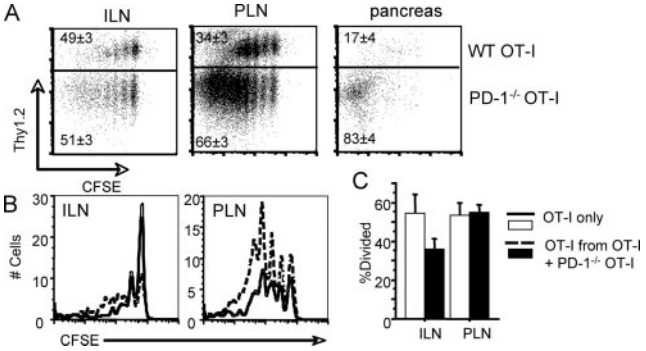

FIGURE 4. Enhanced proliferation of PD-1$^{-/-}$ OT-I T cells is cell intrinsic. An equal number (2.5×10^6) of PD-1$^{-/-}$ OT-I Thy1.1$^{+/+}$ and WT OT-I Thy1.1$^{+/-}$Thy1.2$^{+/-}$ CD8$^+$ T cells were labeled with CFSE and cotransferred into RIP-ova^{high} recipients. *A*, 14 days after transfer, transferred cells (both Thy1.1-positive, with WT also expressing Thy1.2) were evaluated for cellular division. Ten mice adoptively transferred with PD-1$^{-/-}$OT-I plus WT OT-I T cells were averaged for the percentage of Thy1.1$^+$Thy1.2$^+$ (WT) and percentage of Thy1.1$^+$Thy1.2$^-$ (PD-1$^{-/-}$) cells in each organ. *B*, OT-I T cell proliferation from mice that received WT OT-I T cells alone (solid line) or mice that received both WT OT-I T cells and PD-1$^{-/-}$ OT-I T cells (dotted line) is shown. *C*, The percentage of OT-I T cells that underwent division from seven mice adoptively transferred with WT OT-I T cells only (solid bar) and ten mice adoptively transferred with both OT-I and PD-1$^{-/-}$ OT-I T cells (open bar).

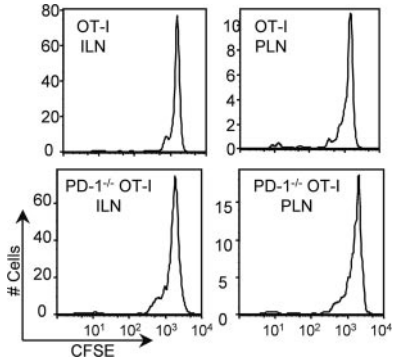

FIGURE 5. Naive PD-1$^{-/-}$ OT-I CD8$^+$ T cells do not proliferate in RIP-ova^{low} recipients. Five million CFSE-labeled WT or PD-1$^{-/-}$ OT-I Thy1.1$^+$ CD8$^+$ T cells were adoptively transferred into RIP-ova^{low} recipients. After 6 days, inguinal and pancreatic LN were harvested and Thy1.1$^+$ cells were monitored for CFSE dilution. No recipients of OT-I or PD-1$^{-/-}$ OT-I T cells became diabetic or showed signs of T cell infiltration into the pancreas.

subdivided this population into PD-1$^{-/-}$ OT-I (Thy1.2$^-$) and WT OT-I (Thy1.2$^+$) populations.

The lack of PD-1 on adoptively transferred T cells enabled them to proliferate more readily to cross-presented Ag in a cell-intrinsic manner (Fig. 4*A*). The percent of WT OT-I T cells and PD-1$^{-/-}$ OT-I T cells was equivalent in the ILN, where no Ag is cross-presented, but there were striking increases in the percentage of PD-1$^{-/-}$ OT-I T cells in both the PLN and pancreas, where Ag is available (Fig. 4*A*). The relative increase in PD-1$^{-/-}$ OT-I T cells in the pancreas and PLN 14 days after transfer, despite an initial 1:1 proportion of PD-1$^{-/-}$ OT-I T cells to OT-I T cells and the maintenance of a 1:1 ratio in the ILN, indicates that PD-1$^{-/-}$ OT-I T cells are responding specifically to Ag. This may reflect a lack of deletional tolerance in PD-1$^{-/-}$ OT-I T cells in response to cross-tolerance or increased division of these cells.

Proliferation of OT-I T cells was similar whether or not PD-1$^{-/-}$ OT-I T cells were present (Fig. 4, *B* and *C*). When the proliferation of OT-I T cells from mice that had received only OT-I T cells was compared with the proliferation of OT-I T cells from mice that had received both OT-I and PD-1$^{-/-}$ OT-I T cells, a similar dilution of CFSE was observed in both the ILN and PLN. The similarity in the number of cells that have divided suggests that the presence of PD-1$^{-/-}$ OT-I T cells does not affect other Ag-specific cells.

PD-1$^{-/-}$ effector CD8$^+$ OT-I cells remain ignorant of β cells with low Ag density

To test whether PD-1$^{-/-}$ OT-I T cells remain ignorant of low level Ag presentation in the tissues, we compared the consequences of transferring PD-1$^{-/-}$ OT-I and WT OT-I T cells into RIP-ova^{low} mice. RIP-ova^{low} mice express <1/30 the amount of OVA secreted by RIP-ova^{high} mice, and the level of Ag secretion is sufficiently low that there is little to no cross-presentation in the pancreatic LN (13, 14). Neither PD-1$^{-/-}$ OT-I nor OT-I T cells proliferated in the PLN of RIP-ova^{low} mice (Fig. 5). Diabetes induction did not occur in RIP-ova^{low} adoptive transfer recipients of either PD-1$^{-/-}$ OT-I or OT-I T cells followed for 30 days (data not shown). These data indicate that PD-1$^{-/-}$ T cells remain ignorant of low-level self-Ag presentation in the periphery and require Ag in the draining lymph node for activation.

PD-1$^{-/-}$ effector CD8$^+$ OT-I cells kill β cells with low Ag density

The capacity of in vitro activated OT-I effector cells to induce diabetes in RIP-ova recipients permits examination of secondary Ag encounter in peripheral tissues (13), because normal cross-tolerance and priming events are by-passed. After 5 days of activation in vitro, CD44 up-regulation and CD62L down-regulation were similar on PD-1$^{-/-}$ OT-I and WT OT-I T cells (Fig. 6*A*). We compared the ability of activated PD-1$^{-/-}$ OT-I T cells to induce disease in RIP-ova^{low} mice, reasoning that the low Ag density may

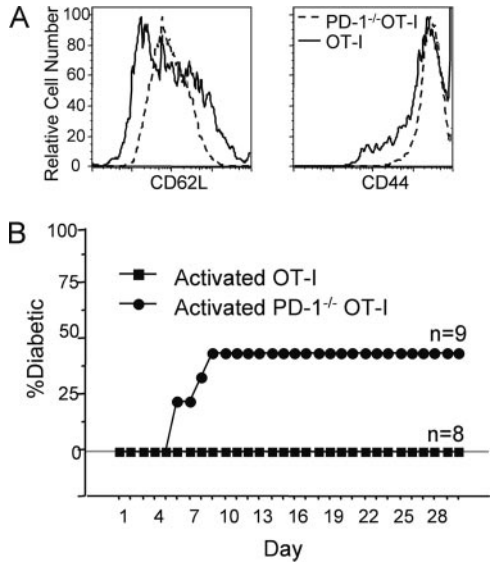

FIGURE 6. Activated PD-1$^{-/-}$ OT-I CD8$^+$ T cells induce diabetes in RIP-ova^{low} recipients. *A*, PD-1$^{-/-}$ OT-I or OT-I T cells up-regulate CD44 and down-regulate CD62L. CD8$^+$ T cells were activated in vitro for 5 days then isolated, and expression of activation markers was evaluated by flow cytometry. *B*, Adoptive transfer of in vitro activated PD-1$^{-/-}$ OT-I T cells into RIP-ova^{low} recipients induces diabetes. Four of nine recipients became diabetic after adoptive transfer of 3.75×10^5 activated PD-1$^{-/-}$ OT-I T cells, whereas no recipients of 3.75×10^5 activated OT-I T cells became diabetic.

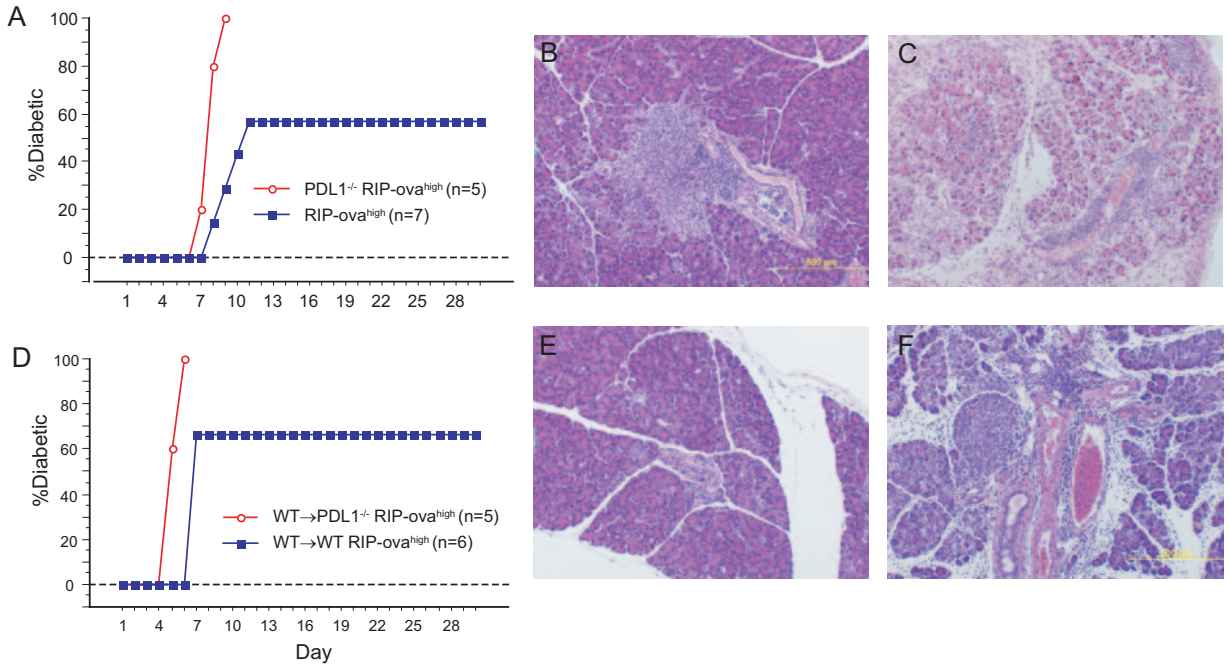

FIGURE 7. Activated OT-I T cells induce rapid onset of diabetes in PD-L1$^{-/-}$ RIP-ova^{high} mice. *A*, OT-I T cells were activated in vitro and then adoptively transferred into RIP-ova^{high} or PD-L1$^{-/-}$ RIP-ova^{high} recipients. Diabetes was monitored daily by urine glucose readings and confirmed by blood glucose. PD-L1$^{-/-}$ recipients developed diabetes significantly faster than WT recipients. Mice were sacrificed after diabetes onset, and the pancreas was removed and evaluated histologically. Representative histology is shown for WT RIP-ova^{high} (*B*) and PD-L1$^{-/-}$RIP-ova^{high} (*C*) recipients. *D*, PD-L1$^{-/-}$ RIP-ova^{high} and RIP-ova^{high} mice were lethally irradiated and given WT CD45.1 BM. After reconstitution, activated OT-I T cells were adoptively transferred into the BM chimeras as in *A*. WT→PD-L1$^{-/-}$RIP-ova^{high} recipients became diabetic more rapidly than WT controls. Representative histology is shown for WT→WT RIP-ova^{high} (*E*) and WT→PD-L1$^{-/-}$RIP-ova^{high} (*F*) recipients. Bars on images are equivalent to 500 μm.

reveal further dependence on PD-1 costimulation for reactivation to peripheral self-Ags expressed at low densities.

RIP-ova^{low} recipients of activated PD-1$^{-/-}$ OT-I T cells developed diabetes, while no diabetes was observed in recipients of WT OT-I T cells. RIP-ova^{low} animals given 3.75×10^5 activated cells were followed until they became diabetic or for 30 days. Four of 9 recipients of PD-1$^{-/-}$ OT-I T cells developed diabetes (Fig. 6*B*), and marked peri-insulitis and insulitis was found in 2 of the nondiabetic recipients sacrificed 30 days posttransfer. None of the recipients of WT OT-I T cells displayed diabetes or infiltration of the pancreas upon histological evaluation. These results demonstrate that PD-1 can alter the threshold of Ag density required for self-reactive T cells to recognize peripheral target cells.

PD-L1 expression on parenchymal tissue protects against OT-I mediated tissue destruction

RIP-ova^{high} recipients develop diabetes when given a sufficient dose of in vitro activated OT-I CD8$^+$ T cells. These cells can infiltrate the pancreas and destroy Ag-bearing β cells without further presentation by DCs. Because we have previously shown that PD-L1 on parenchymal cells can be protective against self-reactive CD4$^+$ T cells in the NOD mouse (12), we investigated whether PD-L1 on bone marrow or nonbone marrow-derived cells regulated responses of WT OT-I T cells in RIP-ova^{high} mice. These results led us to test whether PD-L1$^{-/-}$ RIP-ova^{high} recipients had rapid onset of diabetes following adoptive transfer of in vitro activated OT-I CD8$^+$ effector T cells. To determine whether PD-1:PD-L1 interactions are important in controlling effector responses in vivo, we first compared the consequences of transfer of activated WT OT-I T cells into WT vs PD-L1$^{-/-}$ RIP-ova^{high} recipients.

PD-L1$^{-/-}$ RIP-ova^{high} animals developed diabetes more rapidly than WT control recipients ($p < 0.05$). A suboptimal dose of activated OT-I T cells (3.75×10^5 cells) caused diabetes in 60% of RIP-ova^{high} recipients followed for 30 days posttransfer, while all PD-L1$^{-/-}$ RIP-ova^{high} recipients developed diabetes following transfer of this same number of activated OT-I cells (Fig. 7*A*). PD-L1$^{-/-}$ recipient animals developed diabetes earlier and exhibited more severe inflammation and tissue damage, as evidenced by total destruction of islets and intense vasculitis (Fig. 7, *B* and *C*).

We next constructed WT→PD-L1$^{-/-}$ RIP-ova^{high} bone marrow chimeras to address where PD-L1 exerts its effects. By constructing BM chimeras, we limited PD-L1 deficiency to radioresistant parenchymal cells, such as β cells within the islet and vascular endothelium. Donor chimerism in the APC compartment, measured by CD11b, CD11c, and B220 staining, was routinely >99%, while T cell chimerism was >90%. WT→PD-L1$^{-/-}$ RIP-ova^{high} and WT→RIP-ova^{high} bone marrow chimeras were given activated OT-I T cells 6 weeks after reconstitution. WT→PD-L1$^{-/-}$ RIP-ova^{high} mice developed diabetes more rapidly than WT→RIP-ova^{high} controls ($p < 0.001$; Fig. 7*D*). The percentage of WT→RIP-ova^{high} bone marrow chimeras that developed diabetes during the 30 days of follow-up was around 60%, similar to the proportion that developed diabetes in previous experiments using intact RIP-ova^{high} recipients. These findings show that PD-L1 on parenchymal cells regulates self-reactive CD8$^+$ T cells in immunocompetent mice.

Discussion

Our studies reveal a critical role for the PD-1:PD-L pathway in negatively regulating self-reactive CD8$^+$ T cell responses during the effector response when pathogenic T cells can produce tissue damage. PD-1$^{-/-}$ OT-I CD8$^+$ T cells responded to cross-presented Ag in vivo, and exhibited robust responses with increased cell numbers and cytokine production as compared with WT controls after 14 days. In RIP-ova^{high} recipients, PD-1$^{-/-}$ OT-I cells

expanded and migrated to the pancreas, leading to the development of diabetes. In contrast, WT OT-I T cells proliferated and were largely deleted by 2 weeks after transfer. PD-$1^{-/-}$ OT-I T cell have a cell-intrinsic alteration in their response to Ag, because there was a minimal impact on naive OT-I T cells coadoptively transferred into RIP-ova^{high} recipients. PD-L1 plays a critical role on parenchymal cells by inhibiting previously activated OT-I T cells upon Ag re-encounter in peripheral tissues. Together, these findings demonstrate that PD-1 is involved in protecting peripheral tissues from effector cell responses.

The ability of PD-1 to inhibit T cell proliferation and responses likely depends on both the quantity of Ag presented and the prominence of PD-L on the Ag-presenting cell. Previous work has shown that PD-$1^{-/-}$ T cells are hyperresponsive to DCs engineered to express Ag endogenously on MHC class I, rather than by cross presentation (15). Ab blockade of PD-1 or PD-L1 to cross-presented Ag also increases T cell responses (16). Our results are consistent with studies performed with Ab blockade, which found that both PD-1 and PD-L1 maintain tolerance to tissue Ags using the RIP-mOVA model system, which express membrane bound OVA under the RIP promoter. However, our work further demonstrates an important role for PD-1 in regulating the threshold of Ag density required for self-reactive effector cells to kill targets as well as for parenchymal expression of PD-L1. Surprisingly, although PD-$1^{-/-}$ T cells exhibit expansion and increased cytokine responses, these responses are delayed. The loss of PD-1 during initial priming is likely to be a factor in the eventual increase in the response, as increased cytokine production and expansion are found in the draining lymph node. The increased response of PD-$1^{-/-}$ T cells to both endogenous and exogenous presentation of Ag suggest that PD-L are important for the negative regulation of $CD8^+$ T cell priming by DC. The alteration in priming in the absence of PD-1 may also modify cell migration, a hypothesis that is supported by an increased infiltration of cells into the pancreas even at early time-points.

The regulation of $CD8^+$ responses to viral infection is also dependent on PD-1 (17). PD-1 is highly expressed on exhausted virus-specific $CD8^+$ T cells that have suppressed cytokine and proliferative responses to their Ag in both LCMV (18) and HIV infection (19–21). Treatment with anti-PD-L1 functions to release the suppression on these exhausted cells and promotes robust antiviral responses, leading to considerable reduction in viral load. There are parallels between exhausted $CD8^+$ cells and OT-I T cells adoptively transferred into RIP-ova^{high} animals. In the RIP-ova model, our data demonstrate that loss of PD-1 results in strong and sustained self-Ag responses. Both exhausted virus-specific cells and self-reactive WT OT-I T cells have weak responses to Ag, with low cytokine responses and proliferation. The reversal of this phenotype by PD-1 elimination or blockade in both cases suggests a common mechanism whereby sustained engagement of PD-1 allows $CD8^+$ T cells to persist, at least for a time, but remain largely nonresponsive. This has important therapeutic implications, giving impetus to immunotherapies that blockade this pathway to boost anti-viral and anti-tumor responses and approaches that engage this inhibitory pathway to inhibit pathogenic immune responses during graft rejection or autoimmune disease. Analysis of genes that may be more readily inhibited by PD-1 may provide mechanistic insights that will be valuable from a fundamental and therapeutic perspective.

Our findings demonstrate that PD-1 is particularly important during Ag re-encounters in the peripheral tissues. We found that previously activated PD-$1^{-/-}$ $CD8^+$ T cells responded more robustly to peripheral Ag in RIP-ova^{high} mice and RIP-ova^{low} mice (data not shown and Fig. 6*B*). PD-$L1^{-/-}$ RIP-ova^{high} recipients also developed diabetes more rapidly than WT RIP-ova^{high} recipients, and this phenotype could not be rescued by provision of WT APCs and $CD4^+$ T cells. PD-$L1^{-/-}$ RIP-ova^{high} recipients of naive WT OT-I T cells do not become diabetic, but do have increased numbers of OT-I T cells in their pancreas, particularly around ductal areas near the islet (data not shown). These findings show that self-reactive T cells are controlled in tissues and suggest that PD-L1 controls even naive WT OT-I T cells that have undergone priming in the PLN. $CD4^+$ T cells provide help to $CD8^+$ T cells, and adoptive transfer of activated OT-II $CD4^+$ T cells facilitates diabetes development in RIP-ova^{high} recipients given limiting numbers of OT-I T cells. Hyperactivation or inappropriate $CD4^+$ T cell help may similarly affect diabetes progression, and $CD4^+$ T cell help allows smaller numbers of OT-I T cells to induce disease (22). We show that WT $CD4^+$ T cells do not alter the induction of diabetes by naive or activated PD-$1^{-/-}$ OT-I T cells, indicating an intrinsic role for PD-1 on OT-I T cells. Our work argues against a direct role for PD-1 ligation of PD-L1 on regulatory T cells in controlling self-reactive cells in peripheral tissues.

The increase in PD-$1^{-/-}$ OT-I cell numbers at late time points in the RIP-ova^{high} model system points to a defect in proliferation or persistence in T cells responding to Ag. Previous reports have implicated PD-L1 in apoptosis induction (23, 24) and observed an association between PD-1 expression and apoptosis in vivo (21). PD-$1^{-/-}$ OT-I T cells are not less susceptible to activation induced cell death in vitro (data not shown), but the delayed increase in cell number in the RIP-ova^{high} system suggests that PD-1 controls T cell persistence. PD-1 could alter initial Ag receptor signaling in such a way that cell migration or secondary responses are dysregulated. Alternatively, PD-1 could play a more important role in secondary Ag encounter, and lead to increased expansion, or less deletion, of self-reactive T cells in tissues. A conditional deletion of PD-1 would allow these questions to be addressed.

In summary, these studies demonstrate that both PD-1 and PD-L1 are important for peripheral tolerance, beginning with initial activation events of self-reactive T cells by DCs. PD-L1 is also critical to pathogenic self-reactive T cell responses in target tissues. Our results suggest that there is a requirement for repetitive PD-1 ligation (both on DCs and on peripheral tissues) for maintenance of peripheral tolerance. Surveillance of peripheral tissues by "tolerant" $CD8^+$ T cells depends upon PD-L1 to maintain surviving Ag-specific T cells in a nonresponsive state (25). The correlation of high PD-L1 expression with poor prognosis in patients with several types of cancer suggests that PD-1 may be central to immune evasion by malignant cells (26, 27). Our data suggest that PD-1 will also impair responses of tumor Ag specific cells. Indeed, provision of PD-1 deficient cells to tumor-bearing mice promotes tumor clearance (28). The relative role of PD-1 in regulating naive and effector T cell responses is not yet clear. Understanding how PD-1 modifies Ag receptor signaling in both naive and effector T cells will provide insights into how to optimally manipulate this pathway to restore tolerance or overcome PD-1-mediated inhibition of exhausted T cells.

Disclosures

The authors have no financial conflict of interest.

References

1. Walker, L. S., and A. K. Abbas. 2002. The enemy within: keeping self-reactive T cells at bay in the periphery. *Nat. Rev. Immunol.* 2: 11–19.
2. Rock, K. L., and L. Shen. 2005. Cross-presentation: underlying mechanisms and role in immune surveillance. *Immunol. Rev.* 207: 166–183.
3. Heath, W. R., G. T. Belz, G. M. Behrens, C. M. Smith, S. P. Forehan, I. A. Parish, G. M. Davey, N. S. Wilson, F. R. Carbone, and J. A. Villadangos. 2004. Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunol. Rev.* 199: 9–26.

4. Kurts, C., R. M. Sutherland, G. Davey, M. Li, A. M. Lew, E. Blanas, F. R. Carbone, J. F. Miller, and W. R. Heath. 1999. CD8 T cell ignorance or tolerance to islet antigens depends on antigen dose. *Proc. Natl. Acad. Sci. USA* 96: 12703–12707.
5. Miller, J. F., C. Kurts, J. Allison, H. Kosaka, F. Carbone, and W. R. Heath. 1998. Induction of peripheral $CD8^+$ T-cell tolerance by cross-presentation of self antigens. *Immunol. Rev.* 165: 267–277.
6. Greenwald, R. J., G. J. Freeman, and A. H. Sharpe. 2005. The B7 family revisited. *Annu. Rev. Immunol.* 23: 515–548.
7. Borriello, F., M. P. Sethna, S. D. Boyd, A. N. Schweitzer, E. A. Tivol, D. Jacoby, T. B. Strom, E. M. Simpson, G. J. Freeman, and A. H. Sharpe. 1997. B7-1 and B7-2 have overlapping, critical roles in immunoglobulin class switching and germinal center formation. *Immunity* 6: 303–313.
8. Brown, J. A., D. M. Dorfman, F. R. Ma, E. L. Sullivan, O. Munoz, C. R. Wood, E. A. Greenfield, and G. J. Freeman. 2003. Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production. *J. Immunol.* 170: 1257–1266.
9. Okazaki, T., and T. Honjo. 2006. The PD-1-PD-L pathway in immunological tolerance. *Trends Immunol.* 27: 195–201.
10. Liang, S. C., Y. E. Latchman, J. E. Buhlmann, M. F. Tomczak, B. H. Horwitz, G. J. Freeman, and A. H. Sharpe. 2003. Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. *Eur. J. Immunol.* 33: 2706–2716.
11. Greenwald, R. J., V. A. Boussiotis, R. B. Lorsbach, A. K. Abbas, and A. H. Sharpe. 2001. CTLA-4 regulates induction of anergy in vivo. *Immunity* 14: 145–155.
12. Keir, M. E., S. C. Liang, I. Guleria, Y. E. Latchman, A. Qipo, L. A. Albacker, M. Koulmanda, G. J. Freeman, M. H. Sayegh, and A. H. Sharpe. 2006. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J. Exp. Med.* 203: 883–895.
13. Kurts, C., J. F. Miller, R. M. Subramaniam, F. R. Carbone, and W. R. Heath. 1998. Major histocompatibility complex class I-restricted cross-presentation is biased towards high dose antigens and those released during cellular destruction. *J. Exp. Med.* 188: 409–414.
14. Li, M., G. M. Davey, R. M. Sutherland, C. Kurts, A. M. Lew, C. Hirst, F. R. Carbone, and W. R. Heath. 2001. Cell-associated ovalbumin is cross-presented much more efficiently than soluble ovalbumin in vivo. *J. Immunol.* 166: 6099–6103.
15. Probst, H. C., K. McCoy, T. Okazaki, T. Honjo, and M. van den Broek. 2005. Resting dendritic cells induce peripheral $CD8^+$ T cell tolerance through PD-1 and CTLA-4. *Nat. Immunol.* 6: 280–286.
16. Martin-Orozco, N., Y. H. Wang, H. Yagita, and C. Dong. 2006. Cutting edge: programmed death (PD) ligand-1/PD-1 interaction is required for $CD8^+$ T cell tolerance to tissue antigens. *J. Immunol.* 177: 8291–8295.
17. Sharpe, A. H., E. J. Wherry, R. Ahmed, and G. J. Freeman. 2007. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat. Immunol.* 8: 239–245.
18. Barber, D. L., E. J. Wherry, D. Masopust, B. Zhu, J. P. Allison, A. H. Sharpe, G. J. Freeman, and R. Ahmed. 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 439: 682–687.
19. Day, C. L., D. E. Kaufmann, P. Kiepiela, J. A. Brown, E. S. Moodley, S. Reddy, E. W. Mackey, J. D. Miller, A. J. Leslie, C. DePierres, et al. 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 443: 350–354.
20. Trautmann, L., L. Janbazian, N. Chomont, E. A. Said, S. Gimmig, B. Bessette, M. R. Boulassel, E. Delwart, H. Sepulveda, R. S. Balderas, et al. 2006. Upregulation of PD-1 expression on HIV-specific $CD8^+$ T cells leads to reversible immune dysfunction. *Nat. Med.* 12: 1198–1202.
21. Petrovas, C., J. P. Casazza, J. M. Brenchley, D. A. Price, E. Gostick, W. C. Adams, M. L. Precopio, T. Schacker, M. Roederer, D. C. Douek, and R. A. Koup. 2006. PD-1 is a regulator of virus-specific $CD8^+$ T cell survival in HIV infection. *J. Exp. Med.* 203: 2281–2292.
22. Behrens, G. M., M. Li, G. M. Davey, J. Allison, R. A. Flavell, F. R. Carbone, and W. R. Heath. 2004. Helper requirements for generation of effector CTL to islet β cell antigens. *J. Immunol.* 172: 5420–5426.
23. Dong, H., S. E. Strome, D. R. Salomao, H. Tamura, F. Hirano, D. B. Flies, P. C. Roche, J. Lu, G. Zhu, K. Tamada, et al. 2002. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat. Med.* 8: 793–800.
24. Muhlbauer, M., M. Fleck, C. Schutz, T. Weiss, M. Froh, C. Blank, J. Scholmerich, and C. Hellerbrand. 2006. PD-L1 is induced in hepatocytes by viral infection and by interferon-α and -γ and mediates T cell apoptosis. *J. Hepatol.* 45: 520–528.
25. Fife, B. T., I. Guleria, M. Gubbels Bupp, T. N. Eagar, Q. Tang, H. Bour-Jordan, H. Yagita, M. Azuma, M. H. Sayegh, and J. A. Bluestone. 2006. Insulin-induced remission in new-onset NOD mice is maintained by the PD-1-PD-L1 pathway. *J. Exp. Med.* 203: 2737–2747
26. Ohigashi, Y., M. Sho, Y. Yamada, Y. Tsurui, K. Hamada, N. Ikeda, T. Mizuno, R. Yoriki, H. Kashizuka, K. Yane, et al. 2005. Clinical significance of programmed death-1 ligand-1 and programmed death-1 ligand-2 expression in human esophageal cancer. *Clin. Cancer Res.* 11: 2947–2953.
27. Ghebeh, H., S. Mohammed, A. Al-Omair, A. Qattan, C. Lehe, G. Al-Qudaihi, N. Elkum, M. Alshabanah, S. Bin Amer, A. Tulbah, et al. 2006. The B7-H1 (PD-L1) T lymphocyte-inhibitory molecule is expressed in breast cancer patients with infiltrating ductal carcinoma: correlation with important high-risk prognostic factors. *Neoplasia* 8: 190–198.
28. Iwai, Y., S. Terawaki, and T. Honjo. 2005. PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells. *Int. Immunol.* 17: 133–144.