Tissue expression of PD-L1 mediates peripheral T cell tolerance

Mary E. Keir,[1] Spencer C. Liang,[1] Indira Guleria,[2] Yvette E. Latchman,[1] Andi Qipo,[4] Lee A. Albacker,[1] Maria Koulmanda,[4] Gordon J. Freeman,[3] Mohamed H. Sayegh,[2] and Arlene H. Sharpe[1]

[1]Department of Pathology and [2]Transplantation Research Center, Brigham and Women's Hospital and Children's Hospital Boston, and [3]Department of Medical Oncology, Dana Farber Cancer Institute, Harvard Medical School, Boston, MA 02115
[4]Islet Transplantation Research Laboratory WH 541, Department of Surgery, Massachusetts General Hospital, Boston, MA 02114

Programmed death 1 (PD-1), an inhibitory receptor expressed on activated lymphocytes, regulates tolerance and autoimmunity. PD-1 has two ligands: PD-1 ligand 1 (PD-L1), which is expressed broadly on hematopoietic and parenchymal cells, including pancreatic islet cells; and PD-L2, which is restricted to macrophages and dendritic cells. To investigate whether PD-L1 and PD-L2 have synergistic or unique roles in regulating T cell activation and tolerance, we generated mice lacking PD-L1 and PD-L2 (PD-L1/PD-L2$^{-/-}$ mice) and compared them to mice lacking either PD-L. PD-L1 and PD-L2 have overlapping functions in inhibiting interleukin-2 and interferon-γ production during T cell activation. However, PD-L1 has a unique and critical role in controlling self-reactive T cells in the pancreas. Our studies with bone marrow chimeras demonstrate that PD-L1/PD-L2 expression only on antigen-presenting cells is insufficient to prevent the early onset diabetes that develops in PD-L1/PD-L2$^{-/-}$ non-obese diabetic mice. PD-L1 expression in islets protects against immunopathology after transplantation of syngeneic islets into diabetic recipients. PD-L1 inhibits pathogenic self-reactive CD4^{+} T cell–mediated tissue destruction and effector cytokine production. These data provide evidence that PD-L1 expression on parenchymal cells rather than hematopoietic cells protects against autoimmune diabetes and point to a novel role for PD-1–PD-L1 interactions in mediating tissue tolerance.

CORRESPONDENCE
Arlene H. Sharpe:
asharpe@rics.bwh.harvard.edu

Abbreviations used: ES, embryonic stem; ILN, inguinal LN; NOD, non-obese diabetic; PD-1, programmed death 1; PD-L, PD-1 ligand; PLN, pancreatic LN; tg, transgenic.

T cell costimulatory pathways regulate T cell activation and tolerance (1–3). Costimulation provides a second signal to T cells in conjunction with signaling through TCR. The well-characterized costimulatory molecules B7-1 and B7-2 augment and sustain T cell responses through binding to the CD28 costimulatory receptor. B7-1 and B7-2 also bind CTLA-4, a second, higher affinity receptor that provides inhibitory signals to T cells and regulates self-reactive T cells (4, 5). The B7/CD28 superfamily has expanded to include other costimulatory and inhibitory receptors, including inducible costimulator (ICOS) and programmed death 1 (PD-1), which are inducibly expressed on the surface of T cells and provide unique secondary signals that shape the immune response (6, 7).

There is mounting evidence that PD-1 plays a crucial role in peripheral tolerance (8). PD-1 expression is induced upon the activation of peripheral T and B cells as well as monocytes. The functional significance of the PD-1 inhibitory signal is demonstrated by the phenotype of PD-1–deficient (PD-1$^{-/-}$) mice. PD-1$^{-/-}$ mice develop features of a lupus-like disease on the C57BL/6 background and a dilated cardiomyopathy on the BALB/c background. These findings suggest that PD-1 may inhibit T and/or B cell activation and is important in regulating tolerance.

PD-1 has two ligands with distinct expression patterns: PD-1 ligand 1 (PD-L1; B7-H1) and PD-L2 (B7-DC). PD-L1 is expressed on resting T cells, B cells, DCs, and macrophages and is further up-regulated upon activation. PD-L1 is also expressed on parenchymal cells, including vascular endothelial cells and pancreatic islet cells (9–12). In contrast, PD-L2 is inducibly expressed only on DCs and macrophages

S.C. Liang and I. Guleria contributed equally to this paper.
Y.E. Latchman's present address is Puget Sound Blood Center, Seattle, WA 98104.
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(13, 14). The distinct expression patterns of PD-L1 and PD-L2 suggest that their relative functions may depend on the tissue microenvironment. The expression of PD-L1 on nonhematopoietic cells is particularly intriguing because it suggests that PD-L1 may regulate potentially self-reactive T cell responses in target organs and/or control the extent of pathogenic effector T cell–mediated inflammatory responses within tissues.

Whether PD-L1 and PD-L2 have overlapping or distinct functions is under active investigation. Some studies have suggested that PD-L1 and PD-L2 inhibit T cell proliferation and cytokine production (13, 15), whereas others support a stimulatory role for the PD-Ls (14, 16). The phenotype of PD-L1$^{-/-}$ mice demonstrates that PD-L1 has a critical negative regulatory role in vivo in inhibiting the expansion of CD4$^+$ and CD8$^+$ IFN-γ^+–producing cells (15). To evaluate the obligatory functions of PD-L1 and PD-L2 in vivo, we generated mice deficient in both PD-L1 and PD-L2 (PD-L1/L2$^{-/-}$). Comparative analyses of PD-L1/L2$^{-/-}$ mice and mice lacking either PD-L1 or PD-L2 provide a means to ascertain unique as well as overlapping functions for these two PD-Ls. In this study, we compare the roles of PD-L1 and PD-L2 in regulating CD4$^+$ T cell activation and tolerance using mice lacking PD-L1 and/or PD-L2. To evaluate the functional significance of PD-L1 expression in the pancreas, we backcrossed mice lacking PD-L1 and/or PD-L2 onto the non-obese diabetic (NOD) background and analyzed the roles of these molecules in spontaneous T cell–mediated autoimmune diabetes. We evaluated the requirement for PD-L1 and PD-L2 expression on hematopoietic versus parenchymal cells by transferring prediabetic T cells into WT or PD-L1/PD-L2$^{-/-}$ NOD SCID mice or into BM chimeras that expressed PD-L1 and PD-L2 solely on cells of nonlymphoid hematopoietic origin. The function of PD-L1 on islets was probed in islet transplant experiments using WT versus PD-L1/PD-L2$^{-/-}$ syngeneic islet cells. Our studies demonstrate that PD-L1 and PD-L2 have overlapping roles in inhibiting CD4$^+$ T cell effector cytokine production in lymphoid tissues, particularly in limiting IFN-γ production. However, we find a unique role for PD-L1 in inhibiting self-reactive T cell responses and protecting the pancreas from T cell–mediated tissue damage. Thus, PD-L1 is a key mediator of tissue tolerance.

RESULTS

Generation and characterization of PD-L2– and PD-L1/PD-L2–deficient mice

To evaluate the essential functions of PD-L1 and PD-L2 in vivo, we generated mice lacking PD-L2 or both PD-L1 and PD-L2 (Fig. 1, A and B; and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20051776/DC1). Although mice lacking PD-L1 or PD-L2 have been described by our laboratory (15, 17) and others (18, 19), this is the first study of mice lacking both ligands. The PD-L2–targeting vector replaced the IgV exon of the PD-L2 gene with the hygromycin drug resistance gene (Fig. 1 A). Because mouse PD-L1 and

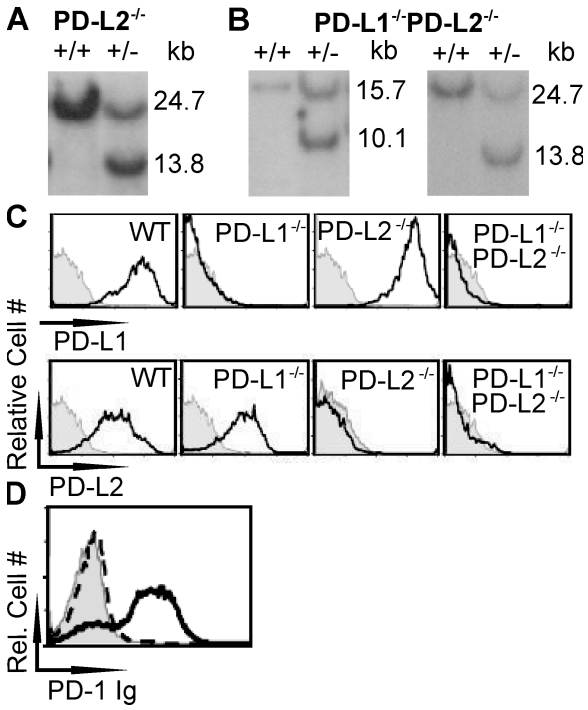

Figure 1. Generation of PD-L2$^{-/-}$ and PD-L1/PD-L2$^{-/-}$ mice. (A and B) Southern blot analyses performed on WT and PD-L2$^{+/-}$ ES cells (A) and PD-L1$^{+/-}$PD-L2$^{+/-}$ ES cells (B) are shown. For the PD-L1 locus, the 15.7-kb fragment represents the WT allele, and the 10.1-kb fragment represents the disrupted PD-L1 allele. For the PD-L2 locus, the 24.7-kb fragment represents the WT allele, and the 13.8-kb fragment represents the disrupted allele. (C) WT, PD-L1$^{-/-}$, PD-L2$^{-/-}$, and PD-L1/L2$^{-/-}$ BM-DCs were generated and evaluated for PD-L1 and PD-L2 expression (shown as a solid line; IgG isotype control, shaded histogram). (D) PD-1 Ig does not bind to PD-L1/L2$^{-/-}$ DCs. WT (solid line) and PD-L1/L2$^{-/-}$ (dashed line) BM-DCs were stained with mouse PD-1 Ig or control Ig, both with a human IgG1 Fc region, followed by anti–human IgG-PE. The Ig control is shown as a shaded histogram.

PD-L2 are closely linked on mouse chromosome 19, PD-L2 was targeted in PD-L1$^{+/-}$ embryonic stem (ES) cells (15) to generate a mouse strain lacking both PD-L1 and PD-L2 (Fig. 1 B). The absence of PD-L1 and/or PD-L2 expression was confirmed by evaluating PD-L1 and PD-L2 expression on BM-derived DCs (BM-DCs) from WT, PD-L1$^{-/-}$, PD-L2$^{-/-}$, and PD-L1/L2$^{-/-}$ mice (Fig. 1 C). PD-L1$^{-/-}$ BM-DCs did not express PD-L1 and had levels of PD-L2 similar to those of WT. PD-L2 was not detected on PD-L2$^{-/-}$ BM-DCs, whereas PD-L1 expression was comparable with WT. PD-L1/L2$^{-/-}$ DCs did not express PD-L1 or PD-L2. Mouse PD-1 Ig did not bind to PD-L1/L2$^{-/-}$ BM-DCs, suggesting that there are no additional PD-Ls on BM-DCs (Fig. 1 D).

Lymphocyte development and numbers in the PD-L2$^{-/-}$ and PD-L1/PD-L2$^{-/-}$ mice were comparable with WT. Flow cytometric analyses revealed similar percentages of thymocyte subsets in littermate control WT, PD-L1$^{-/-}$, PD-L2$^{-/-}$, and PD-L1/L2$^{-/-}$ mice (unpublished data). Analyses of spleen and LNs from all four strains revealed

comparable numbers of T cells ($CD4^+$ and $CD8^+$), Treg ($CD4^+$ $CD45RB^{lo}CD25^{hi}$), B cells ($CD19^+$), macrophages ($F4/80^+$), neutrophils ($CD11b^+$ $GR\text{-}1^+$), and DCs ($CD11c^+$) and no spontaneous activation of $CD4^+$ or $CD8^+$ T cells (no up-regulation of CD25 or CD69) at 8–12 wk of age (unpublished data).

Both PD-L1 and PD-L2 inhibit cytokine production during the activation of naive $CD4^+$ T cells

To begin to analyze the roles of PD-L1 and PD-L2 in regulating $CD4^+$ T cell activation and differentiation, we first isolated naive $CD4^+$ DO11.10 (DO11) T cells and stimulated them in vitro with BM-DCs from mice lacking PD-L1 and/or PD-L2. Because both PD-L1 and PD-L2 are expressed on BM-DCs, these studies enable a direct comparison of the two PD-Ls on the same cell type. For these studies, we used PD-$L1^{-/-}$, PD-$L2^{-/-}$, and PD-L1/PD-$L2^{-/-}$ mice that had been backcrossed to BALB/c for seven generations. BM-DCs lacking PD-L1 and/or PD-L2 expressed levels of MHC II, MHC I, CD80, and CD86 that were comparable with WT and produced similar levels of IL-12 in response to LPS, suggesting that there are no intrinsic defects in DC maturation or function in the absence of PD-L1 and PD-L2 (unpublished data). Among the four groups, no substantial differences were observed in DO11 T cell proliferation at day 3 or 5 of stimulation over a range of $OVA_{323–339}$ concentrations (unpublished data). However, IL-2 and IFN-γ production were modestly increased (20–50%) in the absence of PD-L1 or PD-L2 and markedly increased (300–400%) in the absence of both PD-L1 and PD-L2 (Fig. 2 A). Only a low level of IL-4 production was observed in these cultures. IL-4 production was comparable among WT, PD-$L1^{-/-}$, and PD-$L2^{-/-}$ BM-DCs but was reduced in PD-L1/PD-$L2^{-/-}$–stimulated cultures (unpublished data). These data suggest that PD-L1 and PD-L2 have overlapping roles in limiting IL-2 and IFN-γ production during naive $CD4^+$ T cell activation.

To compare the roles of PD-L1 and PD-L2 in regulating $CD4^+$ T cell responses in vivo where they are distinctly expressed, we used the DO11 adoptive transfer model (20). Naive DO11 $CD4^+$ cells were isolated and adoptively transferred into WT, PD-$L1^{-/-}$, PD-$L2^{-/-}$, or PD-L1/$L2^{-/-}$ recipients, which were immunized with OVA emulsified in IFA. Similar to our in vitro findings, DO11 T cell expansion did not differ substantially among the groups (unpublished data). Cytokine production was measured by ELISA after restimulation of DO11 T cells in vitro. DO11 T cells from PD-L1/$L2^{-/-}$ adoptive transfer recipients produced significantly increased levels of IL-2 and IFN-γ; IL-2 and IFN-γ production were modestly increased in the PD-$L1^{-/-}$ and PD-$L2^{-/-}$ groups (Fig. 2 B). Intracellular cytokine staining for IFN-γ at day 3 after in vitro stimulation confirmed these results (unpublished data). IL-4 was below the level of detection by ELISA, but intracellular cytokine staining showed that the $DO11^+$ $IL\text{-}4^+$ population was reduced by 56% in PD-$L1^{-/-}$ recipients, by 60% in PD-$L2^{-/-}$ recipients, and by 86% in PD-L1/$L2^{-/-}$ recipients as compared with WT

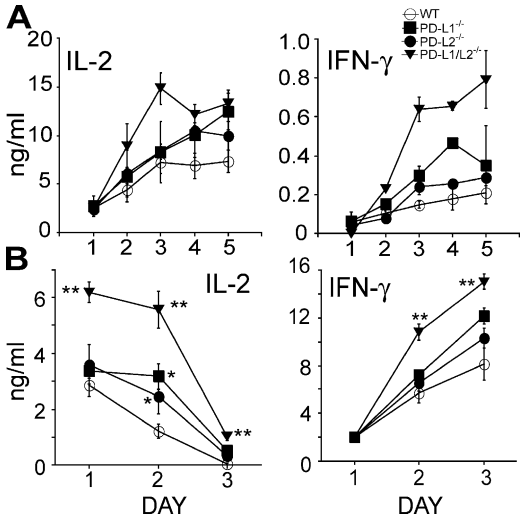

Figure 2. PD-L1 and PD-L2 have overlapping roles in limiting IL-2 and IFN-γ production during naive $CD4^+$ T cell activation. (A) Naive DO11 T cells were activated in vitro by WT, PD-$L1^{-/-}$, PD-$L2^{-/-}$, and PD-L1/$L2^{-/-}$ BM-DCs in the presence of 0–1 μg/ml $OVA_{323-339}$. To evaluate cytokine production, DO11 T cells were activated with 1 μg/ml $OVA_{323-339}$ for 5 d, and supernatants were collected every 24 h and assayed by ELISA. (B) For in vivo studies, DO11 T cells were purified and adoptively transferred into naive WT, PD-$L1^{-/-}$, PD-$L2^{-/-}$, or PD-L1/$L2^{-/-}$ hosts and, 24 h later, were immunized with 100 μg OVA emulsified in IFA. 5 d after immunization, draining LNs were harvested and stained for KJ126 using the clonotypic mAb and CD4, and 10^5 DO11 cells were restimulated as a whole LN culture with 1 μg/ml $OVA_{323-339}$. Supernatants were collected every 24 h and assayed for IL-2 and IFN-γ. *, $P < 0.05$; **, $P < 0.005$ by the unpaired *t* test. Data shown are representative of at least three experiments. Error bars represent SEM.

(unpublished data). These data indicate that PD-L1 and PD-L2 have overlapping roles in inhibiting proinflammatory cytokine production in vivo.

PD-L1/PD-$L2^{-/-}$ mice develop a rapid onset of autoimmune diabetes mellitus on the NOD background

The profound increase in proinflammatory cytokine production in mice lacking PD-L1 and PD-L2 led us to examine the functions of these PD-Ls on T cell responses in a disease setting. During the development of autoimmune diabetes in NOD mice, PD-L1 is expressed on the islets of Langerhans in the pancreas, vascular endothelium, and lymphoid cells as well as on APCs, whereas PD-L2 is expressed on activated APCs (21, 22). We chose to examine the functional significance of PD-L1 and PD-L2 in the NOD model of autoimmune diabetes to dissect the roles of PD-L1 and PD-L2 in controlling self-reactive T cells in the immune compartment and target tissue.

Because PD-L1 and PD-L2 have overlapping roles in controlling proinflammatory cytokine production in the DO11 model, we first crossed mice lacking both PD-L1 and PD-L2 onto the NOD background. PD-L1/PD-$L2^{-/-}$ mice were backcrossed four times onto the NOD background and fixed for homozygosity of the MHC II allele $I\text{-}A^{g7}$. The

loss of both PD-Ls resulted in the rapid onset of autoimmune disease. PD-L1/PD-L2$^{-/-}$ mice had a significantly earlier onset of hyperglycemia as compared with WT littermate controls ($P < 0.0001$), on average developing blood glucose readings of ≥250 mg/dL by 7.3 wk of age (Fig. 3 A). Interestingly, PD-L1/PD-L2$^{-/-}$ female and male mice developed diabetes with similar kinetics. On average, female mice became hyperglycemic at 6.6 wk, whereas male mice became diabetic at 7.7 wk of age. Autoimmune diabetes was completely penetrant in PD-L1/PD-L2$^{-/-}$ animals, whereas only one third of WT female littermates and one tenth of WT male littermates had developed autoimmune diabetes by 40 wk of age. The loss of sex-specific autoimmune effects, the increased incidence of disease, and the complete penetrance of autoimmune diabetes in PD-L1/PD-L2$^{-/-}$ NOD mice point to an essential role for the PD-Ls in controlling peripheral T cell tolerance. These results confirm data showing that PD-1$^{-/-}$ NOD mice (23) and NOD mice treated with PD-1– or PD-L1–blocking antibodies develop a rapid onset of diabetes (21).

PD-L1/PD-L2$^{-/-}$ NOD mice display the early onset of insulitis and increased numbers of IFN-γ–producing CD8^{+} T cells in the PLN

The striking increase in penetrance and onset of autoimmune diabetes in PD-L1/PD-L2$^{-/-}$ NOD mice raised the question of whether the pathology of autoimmune diabetes in the NOD mouse is substantially changed by the loss of PD-L1 and PD-L2. We compared the histology of WT and PD-L1/PD-L2$^{-/-}$ pancreata from mice between 4 and 7 wk of age to determine whether the loss of PD-L1 and PD-L2 led to any alterations in histopathology. Certain hallmarks of diabetes pathology are well known, including the development of peri-insulitis in prediabetic animals and the expansion of antigen-specific self-reactive T cells in the draining pancreatic LN (PLN; reference 1). WT NOD mice typically develop peri-insulitis by 3 to 4 wk of age. The progression to insulitis, where the islet is infiltrated and eventually destroyed by leukocytes, is severe in most mice by 10 wk of age but does not strictly correspond with diabetes incidence (24). Young PD-L1/PD-L2$^{-/-}$ NOD mice had significantly increased insulitis (48% of PD-L1/PD-L2$^{-/-}$ NOD vs. 0% of WT NOD; $P < 0.004$) and fewer normal islets (34% of PD-L1/PD-L2$^{-/-}$ NOD vs. 84% of WT NOD; $P < 0.002$; Fig. 3, B and C). The cellular composition of inflammatory infiltrates was similar in WT and PD-L1/PD-L2$^{-/-}$ NOD mice. The increased number of insulitic islets in PD-L1/PD-L2$^{-/-}$ NOD mice reflects an accelerated disease course.

Remodeling events that occur as part of pancreatic development in the mouse result in β cell antigen deposition around day 12–14 in the PLN. Self-reactive T cells can be activated by DCs bearing β cell antigen and migrate to and/or reactivate in the pancreatic islets (25). Therefore, the events that occur in the draining LN are critical to the onset of peri-insulitis and, ultimately, autoimmunity. In the PLN of young

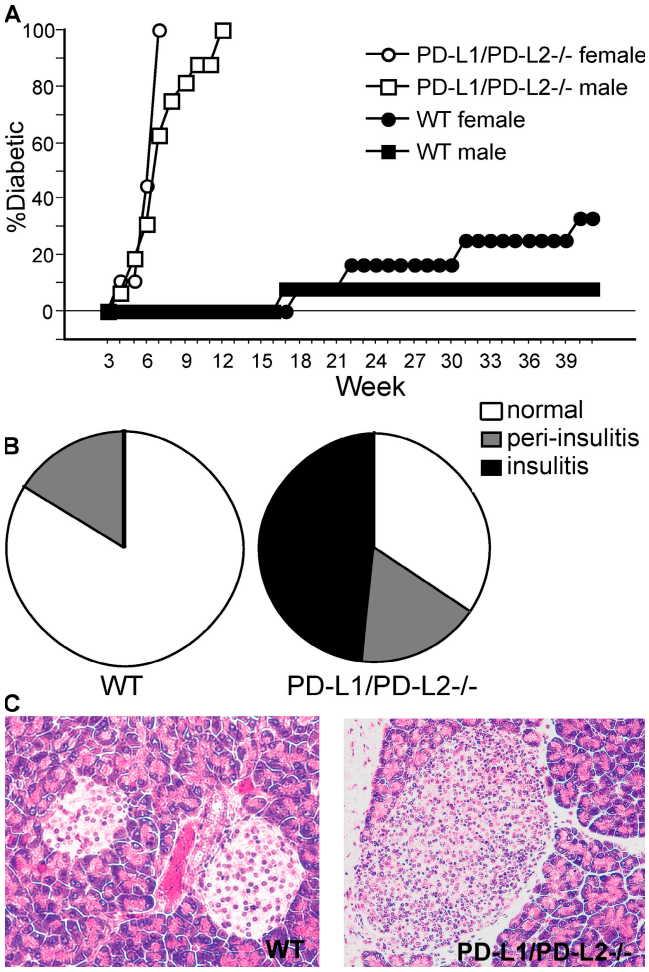

Figure 3. Loss of PD-L1 and PD-L2 results in the rapid onset of diabetes and the early onset of insulitis. (A) PD-L1/PD-L2$^{-/-}$ N4 mice on the autoimmune diabetes-prone NOD background were monitored for the development of hyperglycemia. Animals were scored as diabetic upon blood glucose readings of ≥250 mg/dL, and diabetes was confirmed by three consecutive positive reads. Both female PD-L1/PD-L2$^{-/-}$ NOD mice ($n = 9$) and male PD-L1/PD-L2$^{-/-}$ NOD mice ($n = 16$) develop autoimmune diabetes significantly ($P < 0.0001$; unpaired t test) faster than WT littermate controls (females, $n = 12$; males, $n = 13$). (B) PD-L1/PD-L2$^{-/-}$ and WT N5 NOD littermates were analyzed for early and late signs of immune cell infiltration of the pancreatic islets by histology. Pancreata from PD-L1/PD-L2$^{-/-}$ and WT NOD littermates ranging in age from 27 to 50 d were evaluated histologically. Islets were scored as normal, peri-insulitic, or insulitic, and the percentage of total islets in each category was averaged for each group. Three of nine PD-L1/PD-L2$^{-/-}$ mice were hyperglycemic, and all 10 WT animals were euglycemic at the time of killing. (C) Representative islets from WT (left; scored as normal) and PD-L1/PD-L2$^{-/-}$ (right; scored as insulitic) pancreas are shown. Islets pictured are from 50-d-old littermate females with normal blood glucose readings immediately before killing.

prediabetic and diabetic PD-L1/PD-L2$^{-/-}$ NOD mice, there was a significant expansion in cell numbers. There was no alteration in cell numbers in either the inguinal LN (ILN) or spleen, indicating that the expansion and/or increase in homing is specific for the PLN. There was a fourfold increase

in both $CD4^+$ and $CD8^+$ T cells in the PLN of prediabetic PD-L1/PD-$L2^{-/-}$ mice over that of WT NOD controls ($P \leq 0.0012$; Fig. 4 A). In diabetic mice, there was a twofold increase in both $CD4^+$ and $CD8^+$ T cells in PD-L1/PD-$L2^{-/-}$ mice compared with WT littermates ($P = 0.05$ for $CD4^+$ cells; Fig. 4 B). There was no significant change in activation markers (CD44 and CD62L) on either $CD4^+$ ($P = 0.16$) or $CD8^+$ ($P = 0.58$) populations (unpublished data). These data demonstrate that the loss of PD-L1 and PD-L2 leads to an early expansion and/or increased homing of T cells to the PLN, a key site of self-reactive T cell activation in the NOD mouse.

Increased T effector cell responses in PD-L1/PD-$L2^{-/-}$ NOD mice may contribute to the rapid onset of diabetes. Because the early destruction of pancreatic β cells by $CD8^+$ effector cells may contribute to antigen availability and progression to autoimmunity, young prediabetic PD-L1/PD-$L2^{-/-}$ NOD mice and age-matched nondiabetic WT littermate controls were evaluated for effector cytokine production in the ILN, PLN, pancreas, and spleen (Fig. 4 C and unpublished data). The percentages of $CD8^+$ T cells secreting either IFN-γ, TNF-α, or both of these proinflammatory cytokines in the PLN of prediabetic PD-L1/PD-$L2^{-/-}$ NOD mice was two- to threefold greater than in WT NOD littermates (Fig. 4 C). There also were more IFN-γ^+ $CD4^+$, TNF-α^+ $CD4^+$, and IFN-γ^+ TNF-α^+ cells in the PLN of prediabetic PD-L1/PD-$L2^{-/-}$ NOD mice. The substantial expansion of pathogenic effector T cells in the PLN of PD-L1/PD-$L2^{-/-}$ NOD mice likely contributes to the precipitated onset of diabetes in these mice.

PD-L1 and PD-L2 expression on nonlymphoid cells controls progression to autoimmune diabetes

The rapid onset of diabetes in PD-L1/PD-$L2^{-/-}$ NOD mice demonstrates that PD-L1 and PD-L2 expression inhibits the development of T cell–mediated spontaneous autoimmune diabetes in the NOD mouse. Because of the broad expression of the PD-Ls, there are several potential cell types that could engage PD-1 on T cells during the development of diabetes. To test whether the expression of PD-L1 and PD-L2 is important on lymphoid or nonlymphoid cells, we generated PD-L1/PD-$L2^{-/-}$ NOD SCID mice. These mice provide a means to dissect the role of PD-Ls in lymphoid versus nonlymphoid hematopoietic cells by serving as recipients of lymphoid cells from nondiabetic mice.

Autoimmune diabetes can be transferred from prediabetic NOD mice to NOD SCID recipients via unfractionated splenocytes. To begin to investigate the roles of PD-L1 and PD-L2 on nonlymphoid hematopoietic cells in the development of diabetes, 2×10^7 splenocytes from prediabetic WT NOD mice were adoptively transferred into WT or PD-L1/PD-$L2^{-/-}$ NOD SCID recipients. WT NOD SCID mice were markedly more resistant to diabetes induced by the transfer of splenocytes. PD-L1/PD-$L2^{-/-}$ NOD SCID mice progressed to diabetes at a mean of 6.5 wk after the adoptive transfer of WT splenocytes, whereas WT NOD SCID mice developed diabetes >18 wk after WT splenocyte transfer ($P < 0.0001$ by unpaired *t* test; Fig. 5 A).

B cells are not required for the adoptive transfer of diabetes, but there is evidence that antigen presentation by B cells contributes to the initiation of T cell–mediated diabetes (26, 27). To further probe the role of PD-L1 and PD-L2 on nonlymphoid cells in regulating protection against diabetes transfer, we examined whether T cells alone rapidly transfer diabetes to PD-L1/PD-$L2^{-/-}$ NOD SCID mice. 10 million purified WT NOD T cells, both $CD4^+$ and $CD8^+$ cells, were transferred into WT or PD-L1/PD-$L2^{-/-}$ NOD SCID mice. T cells rapidly transferred disease to PD-L1/PD-$L2^{-/-}$ NOD SCIDs with a mean onset of diabetes at 6 wk after transfer. In contrast, disease transfer to WT NOD SCIDs was delayed, with recipients remaining diabetes free >12 wk after adoptive transfer ($P = 0.0006$ by unpaired *t* test; Fig. 5 B). These results indicate that PD-L1/PD-L2 expression on nonlymphoid cells is sufficient to protect against diabetes.

Expression of PD-L1 and PD-L2 in hematopoietic cells is not sufficient to prevent the rapid onset of diabetes

We next examined whether PD-L1 and PD-L2 on nonlymphoid hematopoietic cells was required to inhibit potentially self-reactive T cells. It is possible that PD-L1 and PD-L2 expression on DCs or other APCs serves to inhibit autoreactive T cells at the time of priming in the PLN. Alternatively, the expression of PD-L1 within the pancreas might control tissue tolerance and prevent potentially self-reactive T cells from attacking target cells in the pancreas.

To evaluate the role of PD-L1 and PD-L2 on nonlymphoid hematopoietic cells, we generated chimeric mice that had PD-L1 and PD-L2 expression on nonlymphoid hematopoietic cells (such as DCs and NK cells) but not on parenchymal cells. Because of a DNA repair defect, NOD SCID mice have no B or T cells but have normal populations of APCs, myeloid cells, and effector cell types such as NK cells. BM chimeras were made by lethally irradiating PD-L1/PD-$L2^{-/-}$ NOD SCID mice and reconstituting them with either WT NOD SCID (chimera B) or PD-L1/PD-$L2^{-/-}$ NOD SCID (chimera A) BM. The resulting chimera B lack PD-L1/PD-L2 expression on all cell types except for nonlymphoid hematopoietic cells, which have WT levels of PD-L1/PD-L2. We found that chimera B animals had similar numbers of $CD11c^+$ DCs in comparison with chimera A animals (not depicted) and that the expression of PD-L1 and PD-L2 on $CD11c^+$ DCs was comparable with WT (Fig. 6, inset; and unpublished data). These chimeras were used as recipients for T cells from prediabetic NOD mice.

The adoptive transfer of T cells from nondiabetic WT NODs into BM chimeras A and B demonstrates that PD-L1 and PD-L2 expression on nonlymphoid hematopoietic cells does not protect from the rapid onset of diabetes. The onset of diabetes was statistically equivalent between the two groups, with a mean onset of 6.7 wk in BM chimera A and

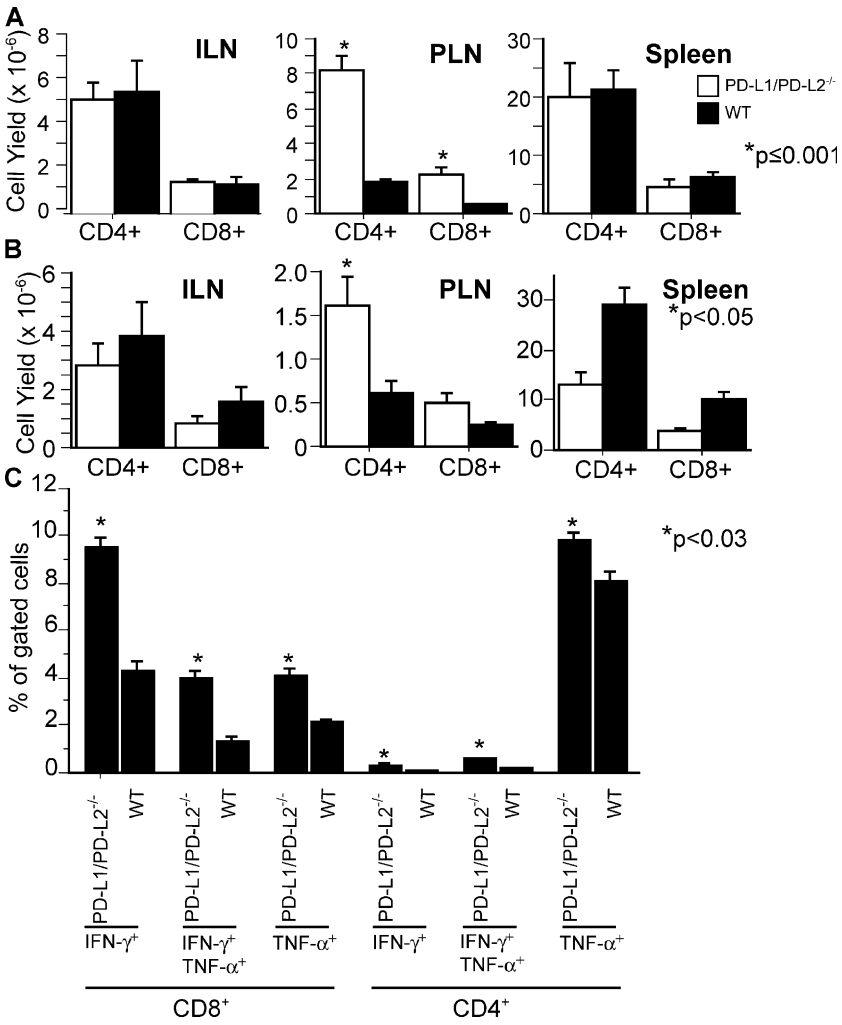

Figure 4. Increased T cell numbers and effector function in the PLN of PD-L1/PD-L2$^{-/-}$ NOD mice. Lymphocytes from the LNs or spleen of prediabetic and diabetic PD-L1/PD-L2$^{-/-}$ NOD mice and WT littermates were evaluated by flow cytometry and by restimulation in vitro. (A) Inguinal LNs (ILN) and pancreatic LNs (PLN), spleen, and pancreas were taken from prediabetic NOD N7 mice (typically between 3–5 wk of age and confirmed to be nondiabetic by blood glucose of ≤250 mg/dL). Lymphocyte subset numbers were calculated by multiplying the total cell yield from an organ by the percentage of CD4$^+$ or CD8$^+$ cells by flow cytometry. (B) Lymphoid organs from diabetic mice (typically between 5–8 wk of age for N4 PD-L1/PD-L2$^{-/-}$ and >13 wk for N4 WT; confirmed to be diabetic by a blood glucose reading of ≥250 mg/dL) were analyzed as in A. (C) The percentage of effector cytokine–producing CD4$^+$ and CD8$^+$ T cells in the PLN is increased in young prediabetic PD-L1/PD-L2$^{-/-}$ N7 mice ($n = 3$) in comparison with WT controls ($n = 4$; $P \leq 0.03$ by the unpaired t test). Error bars represent SEM. Data are representative of three independent experiments.

8.2 wk in BM chimera B ($P = 0.38$ by unpaired t test; Fig. 6). These findings demonstrate that the expression of PD-L1 and PD-L2 on DCs and monocytes is not sufficient to inhibit self-reactive T cells and suggest that the loss of PD-L1 expression within the pancreas causes accelerated diabetes onset in PD-L1/PD-L2$^{-/-}$ NOD mice.

To address the functional significance of PD-L1 expression on pancreatic islet cells, we compared the survival of islets purified from WT and PD-L1/PD-L2$^{-/-}$ NOD SCID donors after transfer into spontaneously diabetic WT or PD-L1/PD-L2$^{-/-}$ NOD mice that had been given insulin before islet transplant. We transplanted 800–1,000 under the kidney capsule and followed blood glucose levels in recipients as a means to monitor islet integrity indirectly via the capacity of the islets to produce insulin. When the transplant recipients became hyperglycemic, they were killed, and their islets were evaluated histologically. WT islets survived longer than PD-L1/PD-L2$^{-/-}$ islets. Histological analyses showed that PD-L1/PD-L2$^{-/-}$ islets were more rapidly destroyed as compared with WT controls (Table I). A more rapid tempo of immune-mediated destruction of PD-L1/PD-L2$^{-/-}$ islets in diabetic PD-L1/PD-L2$^{-/-}$ NOD recipients is suggested by the more rapid progression and resolution of inflammation and earlier scar formation at the transplant site. These data suggest that the expression of PD-L1 on islet cells protects against attack by autoreactive T cells.

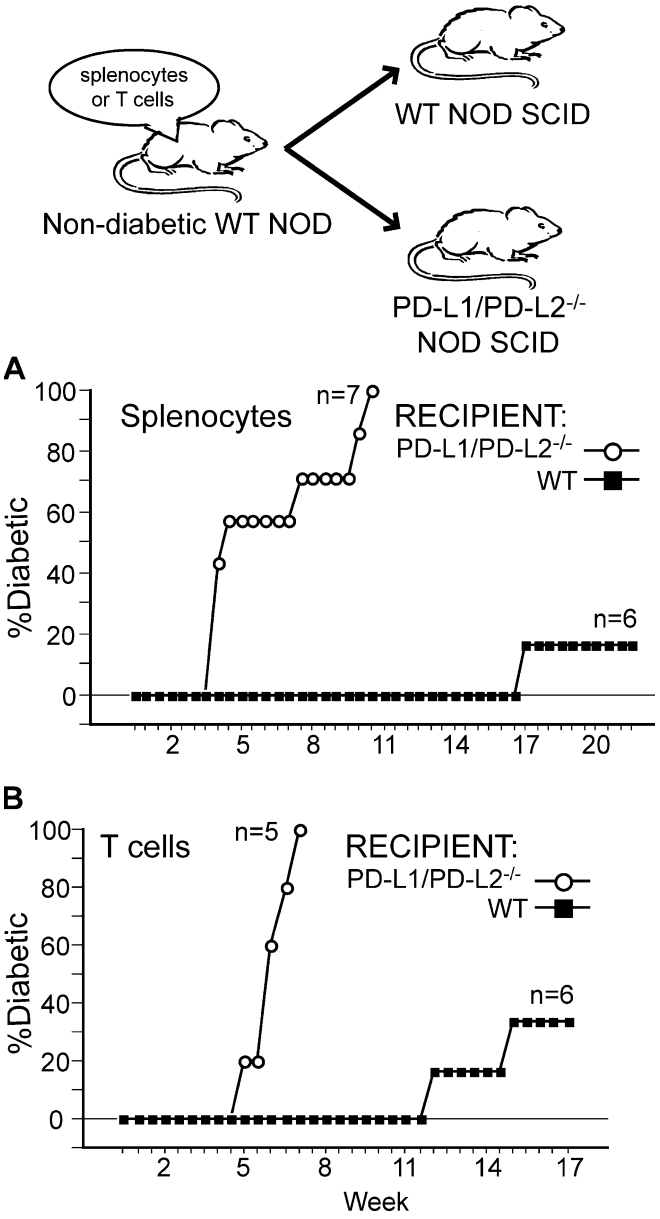

Figure 5. PD-L1/PD-L2 expression is required on nonlymphoid cells to inhibit the transfer of autoimmune diabetes. Cells isolated from nondiabetic WT NOD N5 mice were adoptively transferred into NOD SCID N5 mice that either expressed or lacked PD-L1 and PD-L2. (A) 20×10^6 WT splenocytes efficiently induce autoimmune diabetes in PD-L1/PD-L2$^{-/-}$ NOD SCID mice, whereas disease induction in WT NOD SCID mice is significantly delayed ($P < 0.0001$ by unpaired *t* test). (B) 10×10^6 WT T cells were transferred i.v. into PD-L1/PD-L2$^{-/-}$ or WT NOD SCID mice. PD-L1/PD-L2$^{-/-}$ NOD SCID animals had a significantly earlier onset of T cell–mediated autoimmune diabetes ($P = 0.0006$ by unpaired *t* test).

Table I. PD-L1 on islets protects against self-reactive T cells in diabetic mice

Group (islet donor→ diabetic recipient)	Days to rejection[a]	Infiltrate score[b]	Islet score[c]
PD-L1/PD-L2$^{-/-}$→WT	7	4	0
	5	4	0
	7	4	0
	2	4	0
	1	4	0
PD-L1/PD-L2$^{-/-}$→ PD-L1/PD-L2$^{-/-}$	1	1	0
	2	1	0
	1	1	0
	2	1	0
	8	1	0
WT→PD-L1/PD-L2$^{-/-}$	12	4	0
	11	4	1
	>57	1	3
	14	4	1
	8	4	0
WT→WT	2	3	2
	3	3	1
	8	4	1
	1	3	2
	26	3	2
	15	4	0

[a]Days to rejection signifies the first day at which a mouse had a blood glucose reading of ≥300 mg/dL.
[b]Graft infiltration was scored on a scale of 0–4: 0, no infiltration; 1, scattered infiltrate; 2, some infiltrate; 3, scattered islet cells with heavy infiltrate; 4, very heavy infiltration.
[c]Islet engraftment was scored on a scale of 0–4: 0, scar formation and no evidence of any islet tissue; 1, scattered islet cells with heavy infiltrate; 2, obvious foci of differentiated islet cells despite a heavy infiltrate; 3, well-differentiated graft with scattered infiltrate; 4, large differentiated graft with no infiltrate.

Expression of PD-L1 and PD-L2 can inhibit diabetogenic CD4$^+$ T cells

Diabetes can be transferred to NOD SCID mice by effector CD4$^+$ T cells from diabetic mice independently of CD8$^+$ T cells. The transfer of diabetogenic CD4$^+$ T cells into PD-L1/PD-L2$^{-/-}$ NOD SCID versus WT NOD SCID mice allowed us to test whether the expression of PD-L1 and PD-L2 can actively inhibit diabetes induction by pathogenic effector cells. Five million CD4$^+$ T cells from diabetic WT NOD mice (blood glucose of ≥250 mg/dL) were adoptively transferred into PD-L1/PD-L2$^{-/-}$ NOD SCID or WT NOD SCID mice. Recipient PD-L1/PD-L2$^{-/-}$ NOD SCID mice progressed to autoimmune diabetes more rapidly than WT NOD SCID recipients, with a mean onset of 5.3 wk in PD-L1/PD-L2$^{-/-}$ NOD SCID recipients as compared with 10.5 wk in WT NOD SCID recipients ($P = 0.005$ by unpaired *t* test; Fig. 7 A).

To further examine how PD-L1/PD-L2 expression regulates effector T cell responses, we used T cells isolated from pancreatic antigen-specific BDC2.5 TCR transgenic$^+$ (tg$^+$) NOD mice in our adoptive transfer system. PD-L1/PD-L2$^{-/-}$ NOD SCID recipients again developed diabetes more rapidly than WT NOD SCID recipients (11.2 vs. 14.8 d; $P < 0.02$ by unpaired *t* test; Fig. 7 B). We compared effector cytokine produced by BDC2.5 TCR tg$^+$ T cells in PD-L1/PD-L2$^{-/-}$ NOD SCID and WT NOD SCID recipients. 7 d after the transfer of 2×10^5 BDC2.5 TCR tg$^+$ CD4$^+$CD25$^-$CD62L$^+$ T cells, lymphocytes were isolated

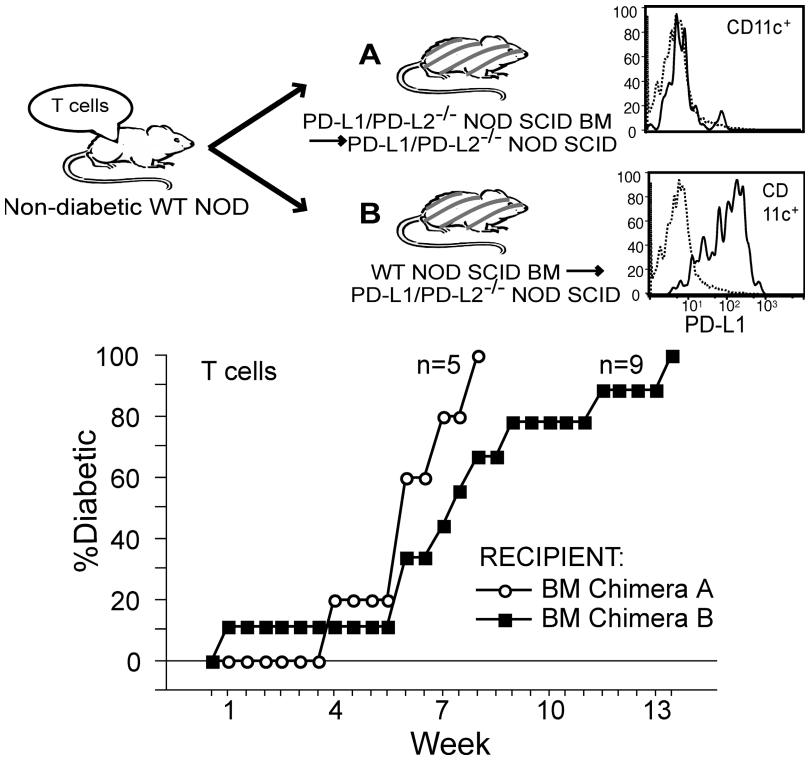

Figure 6. PD-L1/PD-L2 expression solely on nonlymphoid hematopoietic cells is not sufficient to prevent the rapid transfer of autoimmune diabetes. PD-L1/PD-L2$^{-/-}$ NOD SCID N5 mice were lethally irradiated and reconstituted with BM from WT NOD SCID (chimera B) or PD-L1/PD-L2$^{-/-}$ NOD SCID (chimera A) N5 animals. The resulting BM chimera B had no PD-L1/PD-L2 expression except on nonlymphoid hematopoietic cells such as CD11c^{+}CD3^{-}B220^{-} cells (inset; dotted line, isotype staining). WT NOD N5 T cells were adoptively transferred into BM chimeras A and B, which were monitored twice weekly for hyperglycemia. WT NOD T cells transferred autoimmune diabetes to BM chimeras A and B with similar kinetics ($P = 0.38$ by the unpaired *t* test).

from the ILN, PLN, and spleen, restimulated in vitro, and assayed for the production of IFN-γ and TNF-α. BDC2.5 TCR tg T cells isolated from PD-L1/PD-L2$^{-/-}$ NOD SCID recipients displayed a markedly increased production of proinflammatory cytokines, which is similar to our studies of DO11 T cells. Both IFN-γ and TNF-α were increased in the ILN and PLN (not depicted and Fig. 7 C). IFN-γ–producing T cells were doubled, and TNF-α–secreting cells were increased fivefold in LNs, whereas cells secreting both IFN-γ and TNF-α were increased eightfold in the PLN and 24-fold in the ILN. IFN-γ production was reduced in the spleen (unpublished data). Thus, effector cytokine production in LNs is enhanced in the absence of PD-L1 and PD-L2. Together, these studies demonstrate that diabetogenic effector CD4^{+} T cells can be significantly inhibited by PD-L1 and PD-L2 expression and illustrate the functional importance of PD-1 ligation on pathogenic T cells.

PD-L1$^{-/-}$ but not PD-L2$^{-/-}$ NOD mice develop a rapid onset of autoimmune diabetes

Our data indicate that PD-L1 plays a critical role in tissue tolerance by protecting islets from self-reactive T cells. To confirm the role that PD-L1 plays in autoimmune diabetes, we crossed mice lacking only PD-L1 or PD-L2 onto the NOD background. PD-L1$^{-/-}$ NOD mice displayed an indistinguishable phenotype from PD-L1/PD-L2$^{-/-}$ NOD mice, with an early onset of autoimmunity, loss of sex-specific delays in disease progression, and complete diabetes penetrance (Fig. 8 A). In contrast, PD-L2$^{-/-}$ NOD animals did not display a more rapid onset of diabetes in comparison with WT littermate controls (Fig. 8 B). The PD-L1/PD-L2$^{-/-}$ and PD-L1$^{-/-}$ strains were separately backcrossed onto the NOD background and provide independent confirmation of the importance of PD-L1 in autoimmune diabetes. These results are also consistent with studies showing that the administration of anti–PD-L1– but not anti–PD-L2–blocking antibodies induced a rapid onset of autoimmune diabetes. Thus, through multiple approaches, we find that PD-L1 expression on parenchymal cells is uniquely responsible for the increased onset and penetrance of autoimmune diabetes in PD-L1/PD-L2$^{-/-}$ NOD mice.

DISCUSSION

In this study, we present the initial characterization of mice lacking both PD-L1 and PD-L2 and compare them side by side with mice lacking either PD-L1 or PD-L2. Analyses of these three strains demonstrate important overlapping roles for PD-L1 and PD-L2 on APCs in regulating CD4^{+} T cell

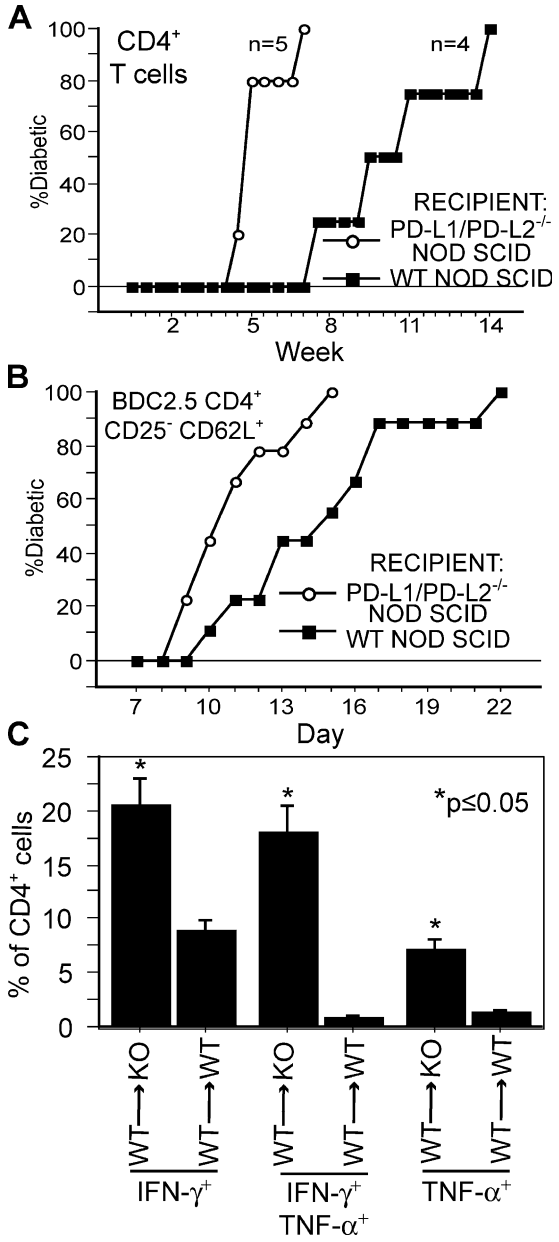

Figure 7. Diabetogenic T cells are inhibited by the expression of PD-L1/PD-L2 in recipient mice. (A) 5×10^6 $CD4^+$ T cells from diabetic WT NOD N5 mice were adoptively transferred into PD-L1/PD-$L2^{-/-}$ or WT NOD SCID N5 mice. The onset of diabetes was significantly delayed in WT NOD SCID mice ($P = 0.005$ by unpaired *t* test). (B) Sorted naive effector $CD4^+CD25^-CD62L^+$ cells from BDC2.5 TCR tg^+ NOD mice (2×10^5 cells) were adoptively transferred into PD-L1/PD-$L2^{-/-}$ or WT NOD SCID N5 mice. PD-L1/PD-$L2^{-/-}$ NOD SCID recipients had significantly earlier diabetes onset ($P = 0.02$ by unpaired *t* test). (C) BDC2.5 TCR tg^+ lymphocytes were reisolated from PD-L1/PD-$L2^{-/-}$ ($n = 4$) or WT NOD SCID ($n = 4$) recipients 7 d after adoptive transfer and restimulated in vitro. $CD4^+$ T cells were analyzed for the production of TNF-α and IFN-γ. Production of both effector cytokines was significantly increased in $CD4^+$ cells reisolated from PD-L1/PD-$L2^{-/-}$ NOD SCID recipients ($P \leq 0.05$ by the unpaired *t* test). The data are representative of three independent experiments. Error bars represent SEM. KO, knockout.

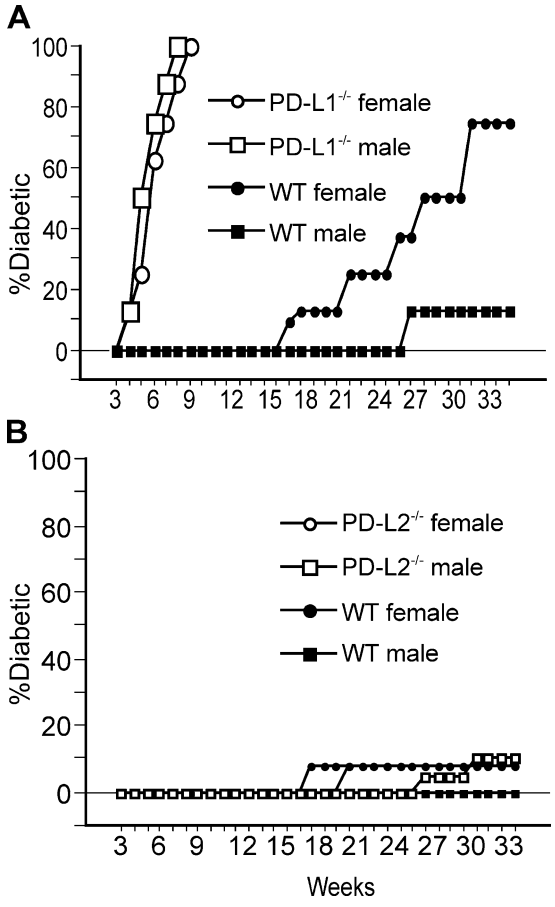

Figure 8. Loss of PD-L1 but not PD-L2 precipitates the rapid onset of autoimmune diabetes on the NOD background. PD-$L1^{-/-}$ and PD-$L2^{-/-}$ mice were crossed separately onto the autoimmune diabetes-prone NOD background. Animals were scored as diabetic as in Fig. 1. (A) PD-$L1^{-/-}$ NOD mice (equivalent of N4) develop a rapid onset diabetes in comparison with WT littermate controls. Both female and male PD-$L1^{-/-}$ NOD display a rapid onset of diabetes with complete penetrance. (B) PD-$L2^{-/-}$ NOD mice (equivalent of N4) do not develop a rapid onset of diabetes in comparison with WT littermate controls.

responses. We find that both PD-L1 and PD-L2 can function to limit IL-2 and IFN-γ production during the activation of naive $CD4^+$ T cells and that they have overlapping roles in controlling proinflammatory cytokine production during $CD4^+$ T cell activation. In contrast, PD-L1 has a unique role in controlling tissue tolerance. PD-L1/PD-$L2^{-/-}$ NOD mice of the N4 as well as the N7 and N10 backcross generations (unpublished data) have a substantially earlier onset of clinical diabetes, which is associated with earlier insulitis and increased numbers of effector T cells in the PLN. Our studies using BM chimeras and islet transplantation point to a novel role for PD-L1 in regulating potentially self-reactive T cells within the pancreas.

Several of the newer B7 family members, including PD-L1, ICOS ligand, B7-H3, and B7-H4(7), are expressed on nonhematopoietic cells, but the in vivo significance of such expression has been unclear. Our studies show for the first

time that the tissue-specific expression of PD-L1 is functionally important and has a key role in protecting against pathogenic self-reactive T cells in a target organ. Remarkably, BM chimeras that expressed PD-L1 and PD-L2 on hematopoietic cells but lacked PD-L1 on all other cell types developed early onset diabetes upon T cell transfer. These mice have a normal expression of PD-L1 on APCs as well as on nonlymphoid effector cells such as NK cells, eosinophils, and granulocytes. The rapid onset of autoimmune diabetes after the transfer of WT T cells from prediabetic mice into these BM chimeras demonstrates that the tissue expression of PD-L1 is necessary and sufficient to inhibit pathogenic T cell responses. In the pancreas, PD-L1 is constitutively expressed on pancreatic vascular endothelium at relatively high levels (10) and is up-regulated on pancreatic β cells during the development of diabetes (21, 22). The rapid destruction of islets lacking PD-L1 after transplant into diabetic mice supports that PD-L1 expression on islet cells is an important factor in controlling tolerance. The factors that govern tissue tolerance are not well understood. Our studies identify PD-L1 as a key mediator of T cell tolerance within tissues, protecting target organs from potentially pathogenic self-reactive T cells.

We find that PD-L1 is a critical negative regulator of self-reactive T cells during both the induction and effector phases of diabetes. Our studies indicate that PD-L1 directly inhibits self-reactive T cells but do not rule out the possibility that PD-L1 may also function through cell extrinsic mechanisms. It appears that the inhibitory signals delivered by PD-L1 retard the effector function of self-reactive T cells but do not permanently inhibit their responses. Therefore, PD-1–PD-L1 interactions may synergize with other inhibitory pathways to regulate the induction and/or maintenance of peripheral T cell tolerance. In particular, PD-1 and CTLA-4 appear to have complementary roles in the pathogenesis of diabetes. A study with anti–CTLA-4–neutralizing mAb supports a role for CTLA-4 in the very early stages of disease development because CTLA-4 blockade accelerates disease progression only if mAbs are administered before the onset of insulitis (28). In contrast, PD-1–PD-L1 interactions can inhibit both the initiation and progression of diabetes. Previous work has shown that treatment of 1–10-wk-old prediabetic NOD mice with PD-L1– or PD-1–blocking mAbs precipitates diabetes onset (21), which is in contrast to the narrow window in which anti–CTLA-4 mAb exerts its effect. In this study, we show that PD-L1 can regulate the initial activation of potentially pathogenic T cells as well as the responses of pathogenic effector T cells.

Studies of the tg overexpression of PD-L1 in pancreatic β cells have yielded discordant results. One group reported that tg overexpression of PD-L1 in the islets of C57BL/6 mice results in increased diabetes incidence and posit a costimulatory role for PD-L1 (29). A second group has found that tg overexpression of PD-L1 in the islets of NOD mice results in protection from autoimmune diabetes (Sytwu, H.-K., and C.-J. Wang. 2005. Federation of Clinical Immunology Societies. OR-91 [Abstr.]). It is difficult to reconcile these findings, but there is some evidence that genetic background is important for the function of the PD-1–PD-L1/PD-L2 pathway (8), and the disparity of these findings may represent a real biological effect of PD-L1 overexpression in different mouse strains. Expression of the BDC2.5 tg TCR on the C57BL/6 background results in an earlier onset of hyperglycemia than BDC2.5 tg^+ NOD mice (24) and may reflect the differences in $CD4^+CD25^+$ T_{reg} cell numbers on different genetic backgrounds (30). It is also possible that particular splice variants of PD-1 or PD-Ls may be favored in diabetes-resistant versus susceptible genetic backgrounds and influence disease progression, which is analogous to CTLA-4 (31). Alternatively, a second receptor that binds PD-L1 and PD-L2 has been proposed to be responsible for the costimulatory effect of these ligands but has not yet been identified (32).

Complementary approaches with receptor and ligand-deficient strains are important for understanding the functions of this immunoregulatory pathway. PD-$L1^{-/-}$, PD-$L2^{-/-}$, and PD-$L1/L2^{-/-}$ mice on the BALB/c background do not develop any signs of dilated cardiomyopathy or spontaneous mortality up to at least 8 mo of age (unpublished data), which is in contrast to PD-$1^{-/-}$ mice (33). These differing phenotypes might reflect epistatic interactions between flanking DNA in the targeted region and BALB/c DNA. It is important to note that the PD-$1^{-/-}$ mouse was made using an approach that eliminated the transmembrane domain but did not eliminate the ligand-binding domain, leaving some question as to whether the residual binding domain is functional in the PD-$1^{-/-}$ mouse. Because PD-1 mRNA is made, it is possible that a soluble form of PD-1 is synthesized in the PD-$1^{-/-}$ mouse strain. We have not found any evidence for additional PD-Ls. PD-$L1/L2^{-/-}$ DCs did not bind PD-1 Ig, but it is possible that additional PD-Ls exist on other cell types or are up-regulated on DCs by other stimuli. The deficits observed in PD-$L1/L2^{-/-}$ mice suggest that if additional PD-Ls exist, they either do not function to inhibit T cells or only function to regulate T cells in a restricted microenvironment.

These comparative analyses of PD-$L1^{-/-}$, PD-$L2^{-/-}$, and PD-$L1/L2^{-/-}$ mice demonstrate overlapping inhibitory functions for PD-L1 and PD-L2 on APCs and a unique role for PD-L1 in tissue tolerance. The broad expression of PD-L1 in hematopoietic and nonhematopoietic tissues and the more restricted but overlapping expression of PD-L2 in DCs and macrophages may explain, in part, how these PD-Ls can have overlapping as well as unique functions. Our data show conclusively that the loss of PD-L1 in NOD mice results in increased diabetes and indicate that PD-L1 expression in the target organ protects against the initiation and progression of autoimmune diabetes. In diabetes, the inhibitory function of PD-L1 within the pancreas predominates over inhibitory functions of PD-L1 and PD-L2 on APCs. PD-L1 and PD-L2 may have more subtle roles during the activation of self-reactive T cells in lymphoid organs, but the unique expression of PD-L1 in the pancreas, together with inhibitory signals delivered by PD-L1 to effector and naive T cells, may lead to the

predominant role for PD-L1 during autoimmune diabetes. Our findings give impetus to the development of therapeutic approaches that exploit the PD-1–PD-L1 pathway for controlling diabetes and/or islet transplant rejection.

MATERIALS AND METHODS

Mice. PD-L1$^{-/-}$ mice were generated as described previously (13). To generate PD-L2$^{-/-}$ mice, a PD-L2–targeting vector was electroporated into C57BL/6 ES cells. This targeting vector replaced the IgV exon of the PD-L2 gene with the hygromycin drug resistance gene. Homologous recombinants were identified by EcoRV digest and Southern blot analysis using a probe external to the genomic DNA used in the targeting vector. The probe was generated by PCR using the primers 5′-TGAAATGAGTGTCCTGACTG-3′ and 5′-TAACTGTGTTTTCTCTTACA-3′. EcoRV digest yields a 28.6-kb band in WT and a 13.8-kb band in the targeted allele. Three PD-L2$^{+/-}$ ES clones were microinjected into blastocysts and gave rise to germline transmission of the PD-L2 mutation. Genotyping of mice was performed by PCR using the following primers. Primers to detect the IgV region deleted in PD-L2$^{-/-}$ mice were 5′-AAGCTTTAACCCCCGTTACCTTGA-3′ and 5′-CCGCCTGGGACTACAAGTACCTG-3′. Sequences of the Hygro primers were 5′-AGACCTGCCTGAAACCGAAC-3′ and 5′-CAGTCAATGACCGCTGTTAT-3′. PCR products were 223 and 350 bp for PD-L2 and Hygro, respectively.

To generate PD-L1/L2$^{-/-}$ mice, PD-L1$^{+/-}$ C57BL/6 ES cells were retargeted with the PD-L2–targeting vector described in the previous paragraph. Hygromycin- and neomycin-resistant ES cells were selected, and homologous recombinants were identified by Southern blot analysis as described previously (15). C57BL/6 PD-L1$^{+/-}$PD-L2$^{+/-}$ ES cells were microinjected into blastocysts and gave rise to germline transmission of both the PD-L1 and PD-L2 mutations. Because the PD-L1$^{+/-}$ ES cell clone was heterozygous for the PD-L1 deletion, the PD-L2 homologous recombination event could have occurred on the same chromosome 19 as the PD-L1 mutation or on the sister chromatid. We used the neomycin resistance and hygromycin resistance genes as markers for individual recombination events. Analysis of offspring revealed two clones that gave rise to progeny in which the hygromycin resistance and neomycin resistance gene cosegregated, indicating that the PD-L1 and PD-L2 mutations were on the same chromosome.

For DC and DO11 adoptive transfer studies, PD-L1$^{-/-}$, PD-L2$^{-/-}$, and PD-L1/L2$^{-/-}$ mice were backcrossed onto the BALB/c background for at least seven generations. In contrast to the fatal cardiomyopathy that develops in 4–6-wk-old PD-1$^{-/-}$ mice backcrossed onto the BALB/c background for 10 generations, we found no change in survival among WT, PD-L1$^{-/-}$, PD-L2$^{-/-}$, and PD-L1/L2$^{-/-}$ mice that had been backcrossed onto the BALB/c background for at least seven generations and monitored for up to 8 mo of age. Histological analyses of hearts in 2-mo-old mice from the three PD-L–deficient BALB/c strains did not reveal cellular infiltration or dilated cardiomyopathy (unpublished data). Littermate WT and WT BALB/c from Taconic were used as controls.

PD-L1/PD-L2$^{-/-}$ mice were backcrossed to NOD/MrkTac mice (Taconic Farms). I-Ag7 was fixed by screening N3 breeders for I-Ag7. The backcross generation of the mice used in specific experiments (N4, N5, or N7) is indicated in the figure legends. WT littermates were used as controls for all experiments. Importantly, no Idd loci have been identified on chromosome 19, upon which both PD-L1 and PD-L2 reside. PD-L1$^{-/-}$ (34) mice were speed backcrossed to NOD/LtJ (Jackson ImmunoResearch Laboratories) and were confirmed to be homozygous for NOD Idd1, Idd2, Idd3, Idd4, Idd5a, Idd5b, Idd7, Idd8, Idd9, Idd10, Idd11, Idd12, Idd13, Idd14, and Idd15 loci. PD-L2$^{-/-}$ mice were speed backcrossed to NOD/MrkTac and were fixed at Idd1, Idd2, Idd3, Idd4, Idd9, Idd10, Idd11, Idd13, and Idd14 loci. Both strains were ∼93% NOD (N4) upon intercrossing. Mice were monitored weekly for high urine glucose (Diastix; Bayer Pharmaceuticals). Positive glucosuria readings were confirmed by blood glucose measurement (Ascensia Elite; Bayer Pharmaceuticals). Diabetes was confirmed by three consecutive blood glucose measurements of ≥250 mg/dL. Harvard Medical School and Brigham and Women's Hospital are accredited by the American Association of Accreditation of Laboratory Animal Care (AAALAC). Mice were maintained and used according to institutional and National Institutes of Health (NIH) guidelines in a pathogen-free facility. Islet transplant studies were performed at Massachusetts General Hospital (MGH), which is also accredited by AAALAC.

Mice were killed at 3, 4, or 6 wk of age or upon the onset of hyperglycemia. The ILN, PLN, pancreas, and spleen were removed and mashed through a 70-μm filter (Falcon). For histology, the pancreas was preserved in Bouin's fixative (Sigma-Aldrich), embedded in paraffin, and stained with hematoxylin and eosin. Islets were scored as peri-insulitic if mononuclear cells were outside the islet. Insulitic islets had varying degrees of mononuclear infiltrates invading the islets. Normal islets had no mononuclear cells surrounding or within the islets.

Flow cytometry. Cells were stained for 30 min on ice in staining buffer (2% BSA and 0.01% sodium azide) containing Fc block (eBioscience) and analyzed on a FACScalibur (Becton Dickinson). The following antibodies were used for staining: CD4 FITC, CD25 FITC, CD62L FITC, CD69 FITC, GR-1 FITC, KJ126 FITC, CD4 PE, CD11b PE, DX5 PE, CD44 PE, Vβ4 PE, IL-2 PE, IL-4 PE, CD3 PerCP-Cy5.5, CD4 PerCP-Cy5.5, CD8 PerCP-Cy5.5, B220 PerCP-Cy5.5, IFN-γ APC, CD25 APC (all from BD Biosciences), F4/80 FITC, CD8 APC (Caltag), PD-L2 FITC, PD-L1 PE, and PD-1 PE (eBioscience). Mouse PD-1 Ig was the gift of M. Collins (Wyeth Research, Cambridge, MA). Intracellular cytokine staining was performed as previously described (35).

In vitro T cell activation. BM was harvested from the tibia and femur, and red blood cells were lysed. Cells were cultured in 10 ng/ml GM-CSF and 1 ng/ml IL-4 (R&D Systems) for 7 d. DCs were purified using a CD11c isolation kit (Miltenyi Biotec). DO11 T cells were purified from DO11.10 TCR tg mice by a CD4$^+$ T cell isolation kit (Miltenyi Biotec), stained with KJ126, and 10^4 KJ126$^+$ DO11 cells were cultured 1:1 with purified DCs with 0.01–1 μg/ml $OVA_{323–339}$ (Analytical Biotechnology Services). Unless noted, all cell culture was performed in C10 (RPMI-1640, 10% FBS, 2 mM L-glutamine, 2 mM Hepes, 100 U/ml penicillin/streptavidin, and 55 μM β-mercaptoethanol; all were purchased from Invitrogen except FBS [Sigma-Aldrich]). Plates were pulsed with 1 μCi ^{3}H-Thymidine (PerkinElmer) for the last 6 h on day 3 or 5 of stimulation. Cytokines were assayed by culturing 10^5 KJ126$^+$DO11 cells with 10^5 DCs in a 24-well plate with 1 μg/ml OVA peptide. Supernatants were harvested every 24 h and assayed for cytokines by ELISA (eBioscience) as previously described (13). Detection limits were as follows: 10 pg/ml IL-2, 20 pg/ml IL-4, and 20 pg/ml IFN-γ.

DO11 adoptive transfer. Two million DO11 cells were purified and transferred i.v. via tail vein injection. On the following day, mice were immunized with 100 μg OVA (Sigma-Aldrich) emulsified in IFA (Sigma-Aldrich) in the hind footpads. Popliteal and ILNs were harvested on day 5 and stained for KJ126 and CD4. 10^5 DO11 cells from immunized mice were restimulated as a whole LN culture in 24-well plates with 1 μg/ml $OVA_{323–339}$. Supernatant was collected every 24 h and assayed for cytokines. Intracellular cytokine staining was performed on day 3.

NOD SCID adoptive transfer experiments. PD-L1/PD-L2$^{+/-}$ mice backcrossed to NOD four times were crossed to NOD/MrkBomTac-Prkdc^{scid} mice (Taconic) and intercrossed to generate WT NOD SCID and PD-L1/PD-L2$^{-/-}$ NOD SCID mice of the same N5 generation. WT NOD N5 mice were used as donors in adoptive transfer experiments. Donor mice were confirmed to be euglycemic (typically 100–120 mg/dL) or hyperglycemic (≥250 mg/dL) by blood glucose measurement. All recipient mice (both PD-L1/PD-L2$^{-/-}$ and WT NOD SCID) were given cells from pooled WT donors to minimize variability. For splenocyte adoptive transfer, spleens were removed and mashed through a 70-μm filter, the red blood cells were lysed in ammonium chloride lysis buffer, and the remaining lymphocytes

were washed twice in RPMI and counted. Splenocytes were resuspended to 20×10^7 cells/ml, and 100 μl was injected into the tail vein of a sex-matched WT NOD SCID or PD-L1/PD-L2$^{-/-}$ NOD SCID mouse.

For the adoptive transfer of T cells, the spleen, ILNs, and PLNs were taken from nondiabetic donor mice. CD4$^+$ and CD8$^+$ cells were isolated by positive selection using MACS (Miltenyi Biotec) beads and columns. Purity was assessed by FACS and was generally >98%. 10 million T cells (isolated CD4$^+$ and CD8$^+$ lymphocytes) were transferred by i.v. injection into WT NOD SCID, PD-L1/PD-L2$^{-/-}$ NOD SCID, or BM chimera recipients. For the adoptive transfer of diabetogenic CD4$^+$ T cells, the ILN, PLN, and spleen were harvested from diabetic WT mice. CD4$^+$ T cells were positively selected using CD4 beads and MACS columns. Five million CD4$^+$ T cells were adoptively transferred into WT NOD SCID or PD-L1/PD-L2$^{-/-}$ NOD SCID recipients. After transfer, recipient mice were monitored twice weekly for glucosuria, and positive readings were confirmed by blood glucose measurement. Upon the onset and confirmation of hyperglycemia, mice were killed, and the ILN, PLN, pancreas, and spleen were removed and analyzed by flow cytometry and histology.

For the adoptive transfer of BDC2.5 TCR tg$^+$ T cells, N7 donors were used. Lymphocytes were isolated from all LNs and spleen, and red blood cells were lysed and enriched for CD4 T cells by the depletion of MHC II and CD8 T cells before staining for CD62L, CD4, and CD25. Cells were sorted into a CD4$^+$CD25$^-$CD62L^{hi} fraction and adoptively transferred into WT NOD SCID or PD-L1/PD-L2$^{-/-}$ NOD SCID recipients. For these experiments, recipients were monitored daily after 7 d for the onset of diabetes.

Islet transplantation. NOD SCID mice and NOD SCID/PD-L1/PD-L2$^{-/-}$mice at 10–12 wk of age were used as donors for islet transplants. Islets were isolated using a modification of a method in which the pancreatic duct is distended with collagenase P (36). After purification on a Histopaque gradient (Histopaque^R-1077; Sigma-Aldrich), islets with diameters between 75 and 250 μm were hand picked and transplanted under the renal capsule. Each recipient received 800–1,000 islets.

Upon the onset of hyperglycemia (three consecutive blood glucose results of >300 mg/dl, according to an established protocol at MGH), the host pancreas and the grafted kidney were fixed in 10% formalin fixative. The kidney grafts were cross sectioned through the entire graft at 4-μm intervals, and alternate sections were stained with hematoxylin and eosin for routine assessment of the infiltrate. The remaining sections were stained for insulin, glucagon, and somatostatin cells. All specimens were coded and randomized, and blinded analysis of the sections was performed. Islet grafts and graft infiltration were scored on a scale of zero to four.

BM chimeras. BM was isolated from PD-L1/PD-L2$^{-/-}$ NOD SCID or WT NOD SCID donor animals, and cell clumps were removed. Cells were adjusted to 5×10^7 cells/ml in PBS. PD-L1/PD-L2$^{-/-}$ NOD SCID recipients were lethally irradiated with two doses of 150 rads given at least 2 h apart (37). Mice were i.v. injected immediately after the second radiation dose with 5×10^6 cells. Chimerism was confirmed by evaluating PD-L1 expression on splenic CD11c$^+$CD3$^-$ DCs. The numbers of DCs were calculated by multiplying CD11c$^+$ lineage-negative cells by the total cell yield. At least 8 wk after irradiation and BM reconstitution, BM chimeras were given 10^7 WT NOD T cells i.v. Mice were monitored twice weekly for the development of diabetes. Upon diabetes onset, mice were killed, and the spleen, PLN, and ILN were removed and analyzed by flow cytometry.

Online supplemental material. Fig. S1 shows the PD-L1– and PD-L2–targeting vectors and a strategy for generation of the PD-L2$^{-/-}$ and PD-L1/L2$^{-/-}$ mouse strains. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20051776/DC1.

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