Title

Corrigendum to ‘Mechanisms of PDL1-mediated regulation of autoimmune diabetes’ [Clin. Immunol. 125 (2007) 16–25]

Journal

Clinical Immunology, 150(1)

ISSN

1521-6616

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Publication Date

2014

DOI

10.1016/j.clim.2013.09.004

Peer reviewed

Mechanisms of PDL1-Mediated Regulation of Autoimmune Diabetes

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Running Title: PDL1 regulates autoimmune diabetes

Keywords: NOD, type 1 diabetes, programmed death ligand, negative costimulatory pathways

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The PD1-PDL1 pathway plays a critical role in regulating autoimmune diabetes as blockade or deficiency of PD-1 or PDL1 results in accelerated disease in NOD mice. We explored the cellular mechanisms involved in the regulation of these autoimmune responses by investigations involving various gene-deficient mice on the NOD background. Administration of blocking anti-PDL1 antibody to CD4+ T cell-deficient, CD8+ T cell-deficient and B cell-deficient mice demonstrated that PDL1-mediated regulation of autoreactive CD4+ and CD8+ T cells is critical for diabetes development. This concept was confirmed by adoptive transfer studies utilizing lymphocytes from BDC2.5 and 4.1 (CD4+) TCR transgenic mice and 8.3 (CD8+) TCR transgenic mice; efforts showing increased proliferation of both CD4+ and CD8+ T cells following PDL1 blockade *in vivo*. Furthermore, we observed that anti-PDL1-mediated acceleration is dependent upon events occurring in the pancreatic lymph nodes during early disease stages, but becomes independent of the pancreatic lymph nodes during later disease stages. These data provide strong evidence that PDL1 regulates autoimmune diabetes by limiting the expansion of CD4+ and CD8+ autoreactive T cells, and define the timing and locale of PDL1-mediated regulation of type 1 diabetes.

The immune system implements several mechanisms to prevent inappropriate T cell activation which can lead to autoimmunity [1]. An intricate balance between positive and negative costimulatory signals delivered to T cells following antigenic encounter is an important determinant for the fate of T cells and the immune response [2-4]. The inhibitory costimulatory molecule programmed death-1 (PD-1) and its ligands, PDL1 and PDL2, have been shown to play an important role in regulating T cell activation and peripheral tolerance [3, 5, 6]. While PDL2 expression appears to be limited to antigen-presenting cells [7, 8], PDL1 is expressed on resting T cells, B cells, dendritic cells (DC), and macrophages and is up regulated upon activation [9]. PDL1 is also expressed on parenchymal cells, including vascular endothelial cells and pancreatic islet cells [10-14]. PD-1 deficiency leads to lupus-like syndrome with glomerulonephritis and arthritis, or fatal dilated cardiomyopathy, depending on the genetic background of the animal [15, 16]. Studies with antibody blockade [17], as well as PDL1-deficient mice [18], confirm a critical role of the PD-1 pathway in regulating the immune response in experimental autoimmune encephalomyelitis (EAE). In the NOD mouse model of type 1 diabetes, we have previously shown that PD-1 interaction with PDL1 is critically important in the regulation of their autoimmune disease [14]. Consistent with these observations are recent reports by our group and others demonstrating that PD-1 or PDL1 deficiency accelerates the onset and frequency of type 1 diabetes in NOD mice, with strong Th1 polarization of islet infiltrating cells [19, 20]. Interestingly, PDL1 blockade resulted in acceleration of disease in all ages of NOD mice tested (i.e., neonatal to 10 week old mice). This finding correlated with increased numbers of IFN-γ-producing, glutamic acid decarboxylase (GAD)-reactive splenocytes and islet infiltration [14]. The effect of PDL1 blockade at early and late time points indicates that this molecule may be

involved in limiting both islet-reactive T cell immunity and β-cell death. Moreover, PDL1 is up-regulated on β-cells in inflamed islets obtained from NOD mice by 10 weeks of age [14]; a time when the pathogenesis of type 1 diabetes is independent of events in the pancreatic lymph nodes and more dependent upon events within the islets themselves [21]. Together, these studies suggest that lymphoid PDL1 expression is involved in controlling early pathogenic events in the natural history of type 1 diabetes, while islet-specific PDL1 limits late stage disease pathogenesis (i.e. β-cell death) within the target organ itself [20]. In this report, we investigated the relative role of various cell types in PDL1-mediated regulation of type 1 diabetes as well as the mechanism by which these cells contribute to this effect.

MATERIALS AND METHODS

Mice. Female NOD, NOD.*RAG*-deficient, CD4+ T cell-deficient NOD.CIITA, CD8+ T cell-deficient NOD.β2m and B cell-deficient NOD.μT mice were obtained from Jackson Laboratory. NOD.BDC2.5 TCR Tg mice were a kind gift of Dr. Christophe Benoist and Dr. Diane Mathis [22]. NOD.Thy1.1.BDC2.5 TCR Tg mice were used for adoptive transfer studies. All mice were cared for in accordance with institutional guidelines.

We made use of TCR transgenic mice to study the mechanisms underlying PDL1-mediated acceleration of type 1 diabetes. TCR-specific transgenic mice express the TCR-α and -β arrangements derived from a CD4+ or CD8+ β cell-autoreactive T cell clones. Specifically, NOD.BDC2.5 mice were derived from the I-A^{g7}-restricted beta cell-specific CD4+ T cell clone BDC2.5, 4.1-NOD mice from the I-A^{g7}-restricted beta cell-specific CD4+ T cell clone NY4.1, and 8.3-NOD mice from the H-$2K^{d}$-restricted beta cell-specific CD8+ T cell clone NY8.3 [22, 23].

Antibodies and Treatment Protocol. The anti–mouse PD-1 mAb (J43, hamster IgG), anti–mouse PDL1 mAb (MIH6, rat IgG2a), and anti–mouse PDL2 mAb (TY25, rat IgG2a) were generated as previously described [10, 14, 24]. The mAbs were manufactured and purified by BioExpress Inc. Hamster IgG (ICN Pharmaceuticals Inc.) and rat IgG (Sigma-Aldrich) served as controls. All mAbs were given intraperitoneally: 500μg on day 0, followed by 250 μg on days 2, 4, 6, 8, and 10 unless indicated otherwise. For *in vitro* studies anti-CD4 and anti-Vβ4, anti-CD8, anti-Vα8 and anti-Vβ2 mAb were purchased from BD–PharMingen.

Monitoring for Diabetes. The overt onset of type 1 diabetes was defined as a random (i.e., non-fasting) blood glucose reading of 250 mg/dL or greater for three consecutive days. Blood glucose

was measured by an Accu-Chek Advantage glucometer (Roche Diagnostics) three times a week until the mice became diabetic.

Adoptive transfer of TCR-specific cells. Splenocytes from NOD.Thy1.1.BDC2.5 TCR Tg mice, Thy1.1.4.1-NOD TCR Tg mice and Thy1.1.8.3-NOD TCR Tg (the later on *RAG*-deficient background) were used for adoptive transfer studies. For studies involving CD4+ TCR Tg T cells, anti-CD25 mAb (7D4) and rabbit complement were incubated with spleen and lymph node cells at 37 degrees for 1 hour to remove CD25+ cells. Remaining cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes), stained with anti-CD4 mAb, and the percentage of CD4+ T cells determined by FACS. One million CD4+ T cells were then injected retro-orbitally per recipient mouse. For CD8+ T cells, total splenocytes were enriched for CD8+ T cells by AutoMACS. $1x10^6$ of these cells were labeled with CFSE and injected retro-orbitally into pre-diabetic (6-8 week old) NOD female mice. The recipients received 500μg of either anti-PDL1 mAb or IgG Ab one day before transfer (day 0), and then 250μg on days 2 and 4. Pancreatic lymph nodes and inguinal lymph nodes were harvested on day 6 and the cells were acquired by flow cytometry for CFSE labeling.

Detection of apoptosis. Leukocytes recovered from pancreatic lymph nodes of adoptively transferred mice were surface stained for surface expression of clonotypic TCRs and resuspended in apoptosis buffer (BD Biosciences), according to the manufacturer's instructions, and incubated with 7-aminoactinomycin D (7-AAD) or 4', 6-diamidino-2-phenylindole (DAPI) and PE-conjugated Annexin V for 15 min at room temperature. The percentage of Annexin+7AAD- or Annexin+DAPI- cells was determined by flow cytometry.

Intracellular staining for Foxp3. Spleen cells or pancreatic lymph node cells were surface stained for CD4 and CD25, washed in cold flow cytometry staining buffer and incubated in fixation /permeabilization solution at 4°C for 1 hr in the dark. The cells were washed with permeabilization buffer, anti-mouse/rat Foxp3 (FJK-16s) antibody from ebiosciences added and incubated at 4°C for 30 minutes in the dark. The cells were washed twice with permeabilization buffer and analyzed by flow cytometry. Anti-CD4 and anti-CD25 were purchased from BD–PharMingen.

Pancreatic lymph node removal studies. Micro-surgery was performed on 3 week old and 11 week old female NOD mice to remove the pancreatic lymph nodes [21]. Control mice were treated similarly and exposed to the surgical manipulation, but pancreatic lymph nodes were left intact. Following surgery, the mice received either anti-PDL1 or IgG treatment. Mice were followed for the development of spontaneous diabetes.

RESULTS

Adaptive immune cell requirements for anti-PDL1-mediated diabetes acceleration. PDL1 is expressed at basal levels on resting B cells, T cells, and DC as well as on non-hematopoietic cells, and PD-1 is expressed on T cells, B cells, and NKT cells [5, 25]. Previous studies have demonstrated that B cells, as well as CD4+ and CD8+ T cells, are required for disease development in NOD mice [26-29]. In order to define the cellular components of PDL1-mediated regulation of type 1 diabetes, anti-PDL1 or control IgG was administered to B- and T cell-deficient NOD.*RAG*-deficient mice. B- and T cell-deficient NOD *RAG*-deficient mice failed to develop diabetes nor was diabetes induced by treatment with anti-PDL1 or control IgG.

We then treated CD4+ T cell-deficient (NOD.CIITA), CD8+ T cell-deficient (NOD.β2m), and B cell-deficient (NOD.μT) mice with anti-PDL1 or control antibody and monitored the mice for the development of type 1 diabetes. Similar to control IgG-treated mice, all NOD.CIITA and NOD.β2m recipients of anti-PDL1 antibody remained diabetes free throughout the study period, indicating that both CD4+ and CD8+ T cells are required for PDL1-mediated disease acceleration. While NOD.μT mice treated with control IgG were free of diabetes, 100% of NOD.μT mice receiving anti-PDL1 were diabetic within 30 days of treatment (Fig. 1). The incidence of diabetes in anti-PDL1 treated NOD.μT and wild-type (WT) mice was similar, although the kinetics of disease was slightly faster in WT NOD mice (Fig. 1). Taken collectively, these studies indicate that both CD4+ and CD8+ T cells are required for anti-PDL1-mediated acceleration of type 1 diabetes.

***In vivo* behavior of autoreactive CD4+ T cells in anti-PDL1 treated NOD mice.** To characterize the behavior of autoreactive CD4+ T cells during PDL1 blockade, we utilized

NOD.BDC2.5 TCR Tg mice which express a receptor specific for an unknown pancreatic islet cell antigen presented by the MHC class II I-A^{g7} molecule (the NOD allele). Disease appears very slowly in NOD.BDC2.5 TCR Tg mice; only 4.9% are diabetic before 12 weeks, and fewer than 20% become diabetic by 20 weeks of age with no sex bias apparent in disease incidence [30, 31]. The availability of a Vβ-specific mAb, anti-Vβ4, that marks more than 85% of the CD4+ T cells in heterozygotes, allows the transgenic T cells to be identified and tracked *in vivo* [32]. PDL1 blockade resulted in accelerated precipitation of diabetes in NOD.BDC 2.5 TCR Tg mice; approximately 75% (n=4, p =0.02) of mice developed diabetes within 3 weeks after initiation of treatment (Fig. 2A).

To characterize the effect of anti-PDL1 treatment on CD4+ T cells, NOD recipients were adoptively transferred with 1 x 10^6 CFSE-labeled islet reactive CD25- CD4+ T cells from NOD.Thy1.1.BDC2.5 TCR Tg mice and were treated with either anti-PDL1 or control IgG (Fig. 2B and C). Pancreatic lymph nodes and inguinal lymph nodes were harvested and mitotic events were calculated. As can be seen in Fig. 2B, although similar percentages of cells divided in both anti-PDL1 and control IgG treated groups, cells from control IgG treated animals underwent fewer mitotic events in pancreatic lymph nodes. As expected, few transferred CD25-CD4+ Thy1.1BDC2.5 TCR Tg T cells underwent cell division in the inguinal lymph nodes, a site that theoretically does not contain pancreatic antigens. The increased proliferation of CD25-CD4+Thy1.1+ BDC2.5 TCR Tg T cells in anti-PDL1 treated mice was not due to reduced apoptosis, since a similar percentage of transferred cells from both groups of mice exhibit phosphatidylserine on the surface, as judged by staining with Annexin V and DAPI (Fig. 2C). To ensure that the enhanced proliferation of transferred CD25-CD4+ Thy1.1+ BDC2.5 TCR Tg T cells in anti-PDL1 treated animals was not unique to a single TCR specificity, we also assessed

the behavior of CFSE labeled CD25-CD4+ Thy1.1+ 4.1 TCR Tg T cells adoptively transferred into NOD mice treated with either control IgG or anti-PDL1 antibody (Fig. 2D). Overall, CD25-CD4+ Thy1.1+ 4.1 TCR Tg T cells underwent fewer mitotic events than BDC2.5 TCR Tg T cells. Again although similar percentages of cells divided in both anti-PDL1 and control IgG treated groups, cells from control IgG treated animals underwent fewer mitotic events. As with the BDC2.5 TCR Tg T cells, very few 4.1 TCR Tg T cells underwent mitosis in the inguinal lymph node. Additionally, the increase in mitosis was not associated with a reduced susceptibility to apoptosis, since transferred cells demonstrated similar percentages of Annexin V+ 7AAD- cells (Fig. 2E). Taking a slightly different approach, we studied the absolute number of transgenic cells in anti-PDL1 treated mice by transferring splenocytes from NOD.BDC2.5 TCR Tg mice to NOD.SCID recipients. As can be seen in Fig. 3A, there was an expansion of Tg cells as shown by increase in absolute numbers in the anti-PDL1 treated as compared to control IgG treated group (n=4-7; P=0.026. This expansion was not due to reduced apoptosis as the number of cells undergoing apoptosis was not statistically significant between anti-PDL1 and control IgG treated group (Fig. 3B). These results suggest that PDL1 regulates expansion of autoreactive CD4+ T cells during diabetes.

***In vivo* behavior of autoreactive CD8+ T cells in anti-PDL1 treated NOD mice.** To characterize the behavior of autoreactive CD8+ T cells during PDL1 blockade, we utilized NOD.Thy1.1.8.3 TCR Tg mice. 8.3-NOD mice are derived from H-2K^d-restricted beta cell-specific CD8+ T cell clone NY8.3. We examined the effect of PDL1 blockade on the ability of adoptively transferred CFSE labeled CD8+ Thy1.1+ 8.3 TCR Tg+ T cells to proliferate in NOD mice (Fig. 4A). Similar to both CD4+ TCR Tg T cells tested above, CD8+ 8.3 TCR Tg+ T cells divided more frequently in mice that received anti-PDL1 antibody as compared to mice that

received control IgG. The observed increase in CFSE-diluted CD8+ T cells in anti-PDL1 treated mice was not due to a reduced rate of apoptosis, since the percentages of transferred cells that were Annexin V+ 7AAD- was similar between the two groups (Fig. 4B). These results suggest that PDL1 regulates the expansion of autoreactive CD8+ T cells thereby possibly controlling the extent of beta-cell destruction during diabetes.

The results obtained utilizing various gene-deficient and transgenic mice used in this study are shown in Table 1.

Effect of PDL1 blockade on regulatory T cell numbers. PD-1 is highly expressed on regulatory T cells (Tregs) [33] and very recently, PDL1 expression on vascular endothelium was shown to be critical for the immunoregulatory effect of CD4+CD25+Foxp3+ T cells [34]. We therefore studied if PDL1 blockade influences the frequency of Tregs in the pancreatic lymph nodes or spleen. As judged by FACS analysis, there was no significant difference in the percentage of CD4+CD25+Foxp3+ Tregs in the pancreatic lymph nodes or spleen of the anti-PDL1 mAb treated group versus control IgG treated group (Fig. 5).

Timing and locale of PDL1-mediated regulation of diabetes. We have previously shown that PDL1 blockade with anti-PDL1 mAb leads to the acceleration of diabetes in NOD mice of all ages, but disease kinetics increase most dramatically in pre-diabetic 10 week old mice with insulitis [14]. Additionally, PDL1 is up-regulated in inflamed islets from 10 week old NOD mice, a time when pathogenesis is dependent upon events within the islets rather than in the pancreatic lymph nodes [21]. Others have also shown that the removal of the pancreatic lymph nodes from 3-week-old NOD mice prevented diabetes. However, if the removal of lymph nodes is performed at 10 weeks of age, the course of diabetes was unaffected. These findings led to the hypothesis that blockade of PDL1 signaling in lymphoid tissues may be responsible for the

acceleration of diabetes during the early stages of disease progression, while blockade of PD-1/PDL1 within the islets may contribute to late-stage diabetes pathogenesis. We therefore removed the pancreatic lymph nodes in 3 and 11-week-old NOD mice, treated them with either anti-PDL1 or IgG control, and followed them for the development of diabetes (Fig. 6A, B). Excision of the pancreatic lymph nodes in 3-week-old mice dramatically reduced the incidence of diabetes precipitated by PDL1 blockade ($P = 0.02$). Approximately 67% of mice whose pancreatic lymph nodes were removed and treated with anti-PDL1 remained diabetes free throughout the duration of the study in contrast to only 14% of lymph node intact mice treated with anti-PDL1. In accordance with previous studies, 3 week old mice whose pancreatic lymph nodes were removed and treated with control IgG were protected from diabetes, while only 40% of sham manipulated NOD mice treated with control IgG remained diabetes free throughout the study period. Excision of the pancreatic lymph nodes in 11-week-old mice with more advanced insulitis was insufficient to prevent anti-PDL1-mediated disease acceleration. Approximately 77% of mice in which pancreatic lymph nodes were removed and all of the intact mice treated with anti-PDL1 became diabetic by day 11 after the initiation of treatment. In contrast, mice treated with control IgG developed diabetes more slowly and with reduced frequency than those treated with anti-PDL1. These results indicate that pancreatic lymph nodes, where priming of autoreactive T cells occurs, are required for PDL1-mediated regulation of autoreactive T cells during the priming phase of the disease pathogenesis. However, once the primed T cells have infiltrated the islets, pancreatic lymph nodes are no longer required for anti-PDL1-mediated diabetes acceleration, suggesting that PDL1 acts in the tissue during the late phase of disease to inhibit islet destruction.

DISCUSSION

We have previously shown that PDL1 blockade leads to acceleration of type 1 diabetes in NOD mice [14] and PDL1-deficient mice on NOD background develop rapid onset of disease [20]. These studies were aimed at elucidating the cellular mechanisms underlying PDL1-mediated immune regulatory function during autoimmune diabetes and the role of PDL1 expression in lymphoid tissue versus the islet in the context of different stages of disease progression.

First, we examined whether B cells were involved in PDL1-mediated inhibition of disease progression. PDL1 is known to be expressed on B cells and these cells are an important component in the development of autoimmune diabetes by virtue of their ability to act as the preferential antigen presenting cell population required for efficient expansion of diabetogenic CD4+ T cells [28]. B cell-deficient NOD mice are usually protected from the onset of type 1 diabetes [28, 35, 36]. Interestingly, when injected with anti-PDL1 antibody, these mice developed diabetes within 3-4 weeks after initiation of treatment (Fig. 1D) indicating that B cells are not required for PDL1 blockade-mediated disease acceleration. Previous studies with intact NOD mice, also did not reveal any changes in anti-insulin antibodies (a B cell-mediated effect) in anti-PDL1 versus control IgG treated group [14]. It is possible that in B cell-deficient mice, dendritic cells fully substitute for B cells as antigen presenting cells, since blockade of the inhibitory signal from PDL1 would be expected to enhance their potency to stimulate T cells.

Previous studies have established that both CD4+ and CD8+ T cells are required for precipitation of type 1 diabetes in NOD mice [26, 37-39]. Our data with CD4+ T cell and CD8+ T cell-deficient mice revealed that acceleration of diabetes by PDL1 blockade also requires both cell types. Upon ligation with its receptor, PDL1 has been reported to decrease TCR-mediated

proliferation and cytokine production by both CD4+ and CD8+ T cells [40, 41]. Our results with CD4+ BDC2.5 TCR Tg mice showed that there is enhanced proliferation of autoreactive CD4+ T cells in the pancreatic lymph nodes of anti-PDL1 treated mice. These results are consistent with those obtained in our recent study in transplantation [33] where using a novel alloreactive TCR-transgenic system, we showed that PDL1 blockade accelerated MHC class II-mismatched skin allograft rejection and enhanced expansion of alloreactive T cells. In addition, utilizing 8.3 CD8+ TCR Tg mice, we also show that PDL1 blockade results in increased proliferation of autoreactive CD8+ T cells. PD-1 was originally cloned from a T cell hybridoma undergoing apoptosis, and it was believed that PD-1 ligation by PDL1 resulted in apoptosis [42]. However, subsequent studies revealed that PD-1-PDL interactions lead to cell cycle arrest in G0-G1 rather than cell death [8]. Similarly, we find that in type 1 diabetes-prone NOD mice, apoptosis of autoreactive CD4+ and CD8+ T cells is not abrogated following anti-PDL1 mAb treatment. Although it is at present not clear in which conditions PDL1 blockade differentially leads to cell cycle arrest or apoptosis, our studies suggest that blockade of the PDL1 pathway in the autoimmune diabetes model does not result in reduced cell death.

Our results clearly indicate that PDL1 blockade leads to expansion of both autoreactive CD4+ as well as CD8+ T cells *in vivo*. Consistent with these findings is our recent report [20] showing that there is an increase in CD4+ and CD8+ T cells in the pancreatic lymph nodes of PDL1/PDL2 -deficient mice over that of wild-type NOD controls. There was no alteration in cell numbers in either the inguinal lymph node or spleen, indicating that the expansion and/or increase in homing is specific for the pancreatic lymph nodes which is a key site of self-reactive T cell activation in NOD mice. Also Latchman et al. [18] in their study in EAE have shown that CD4+ and CD8+ T cell responses were markedly enhanced in PDL1-deficient mice compared

with wild-type mice *in vitro* and *in vivo*. PDL1 blockade experiments carried out in the present study with autoreactive CD4+ TCR and CD8+ TCR Tg mice of defined specificities confirm the previously observed phenomenon of CD4+ and CD8+ T cell expansion in PDL1-deficient mice.

It has previously been reported that PD-1 mRNA is highly expressed in CD4+ Tregs, suggesting the involvement of PD-1 in the induction or function of regulatory cells [43-45]. More recently Schreiner et. al., have suggested a role for Tregs in PDL1 dependent immune inhibition [46]. In co-culture experiments with CD4+ cells containing the regulatory T-cell subset, neutralization of PDL1 had a more prominent effect than in CD4+ T cells depleted of CD25 positive cells, indicating that PDL1-dependent immune inhibition could in part be mediated by CD4+CD25+ Tregs [46]. Our data indicates that the number of Tregs are similar in the pancreatic lymph nodes and spleens of anti-PDL1 versus control IgG treated NOD mice (Fig. 4). Future studies will be required to address whether alteration of PD-1-PDL1 pathway affects specifically the function of Tregs in this system. However, our recent study using an adoptive BDC2.5 transfer model also suggests that PDL1 blockade does not limit regulatory T cell activity. Mice receiving BDC2.5 T effector cells with either IgG control or anti-PDL1 developed diabetes within 11 days post transfer into NOD.*RAG*- deficient mice, however, co-injection of Tregs with Teffector cells suppressed the induction of diabetes irrespective of the treatment. These results indicate that Tregs did not depend on PD-1-PDL1 interactions for tolerance induction and prevention of autoimmune diabetes [47]. This study also suggests that PDL1 blockade accelerates diabetes via a direct effect on pathogenic T cells [47].

Our study indicates that excision of the pancreatic lymph nodes in 3-week-old mice, but not in 11-week-old mice with more advanced insulitis, prevented anti-PDL1-mediated disease acceleration. This suggests that PDL1-mediated regulation of autoreactive T cells occurs in the

pancreatic lymph nodes during the priming phase of the disease pathogenesis. However, once the primed T cells have infiltrated the islets, pancreatic lymph nodes are no longer required for anti-PDL1-mediated diabetes acceleration, suggesting that PDL1 acts locally in the tissue during the late phase of disease to prevent the destruction of islet cells.

Taken together it appears that PDL1 blockade increases proliferation of CD4+ Th1 type cells which provides help for CD8+ T cells to traffic and accumulate in the pancreas leading to rapid destruction of islet cells. Indeed, autoreactive T cell priming in the pancreatic lymph nodes is required for PDL1-mediated regulation of autoimmune diabetes as in young (3 wk old) mice whose pancreatic lymph nodes were removed, PDL1 blockade did not precipitate diabetes. Our studies provide compelling evidence that PDL1 plays an important role in regulating the autoimmune responses that culminate in type 1 diabetes.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health grants P01-AI56299, P01-AI041521 and Juvenile Diabetes Research Foundation Center Grant on Immunological Tolerance in type 1 diabetes (to M.H. Sayegh), National Institutes of Health grant P01 AI-42288 (to M. Atkinson) and JDRF Islet Transplant Center # 4-2004-372 and U19 AI056388 grants (to J.A. Bluestone).

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FIGURE LEGENDS:

FIG. 1. B cells are not essential for anti-PDL1-mediated precipitation of diabetes. B cell-deficient NOD.μT mice were injected with anti-PDL1 mAb or control IgG at 10 weeks of age and were monitored for the development of diabetes. N=5-10 mice per group; $P<0.0001$ between NOD.μT anti-PDL1 versus control IgG treated group and $P=0.025$ between regular NOD anti-PDL1 versus control IgG treated group.

FIG. 2. NOD.BDC2.5 TCR Tg mice develop accelerated diabetes following PDL1 blockade and PDL1 blockade also induces antigen specific BDC2.5 CD4+ T and 4.1 CD4+ T cell proliferation. (A) 10 week old NOD.BDC2.5 TCR Tg mice were injected with anti-PDL1 or IgG control (500μg day 0; 250 μg days 2, 4, 6, 8 and 10) and were followed for their incidence of diabetes (n=4; $P=0.0221$). (B) NOD female mice were injected with anti-PDL1 or IgG control (500μg day 0; 250 μg days 2 and 4). One day following anti-PDL1 or IgG treatment, mice received $1x10^6$ CFSE labeled Thy1.1+$CD4^+CD25^-$ BDC2.5 T cells (B, C) or Thy1.1+$CD4^+CD25^-$ T cells from TCR transgenic 4.1 mice (D, E). Cells were harvested on day 6 and stained for Thy1.1, Annexin V, and either DAPI or 7AAD. (B). Representative CFSE histograms gated on Thy1.1+ cells obtained from the pancreatic or inguinal lymph nodes of anti-PDL1 or control rat IgG treated mice are shown. (C) The percentages of apoptotic cells (Annexin+DAPI-) for each group are plotted. Data are representative of two experiments. (D) Representative CFSE histograms gated on Thy1.1+ cells obtained from the pancreatic or inguinal lymph nodes of anti-PDL1 or control rat IgG treated mice are shown. (E) The percentage of apoptotic cells (Annexin+7AAD-) for each group are plotted. Data are representative of two experiments.

FIG. 3. PDL1 blockade results in expansion of transgenic CD4+ cells in a NOD.SCID adoptive transfer model. Cells from spleen and various lymph nodes were pooled from NOD.BDC2.5 TCR Tg mice and 5-6.0 x 10^7 cells were injected into NOD.SCID mice. The recipient mice were treated with anti-PDL1 mAb or rat IgG and the spleen and pancreatic lymph nodes were excised at day 5 and stained with labeled antibodies against Vβ4 (FITC), CD4 (APC), 7-AAD and annexin (PE). Expansion (A) and apoptosis (B) of transgenic cells was determined by flow cytometric analysis. Data is from n=4-7 mice per group; *P*=0.026 for expansion study.

FIG. 4. PDL1 blockade induces antigen specific 8.3 T cell proliferation. NOD female mice were injected with anti-PDL1 or IgG control (500μg day 0; 250 μg days 2 and 4). One day following anti-PDL1 or IgG treatment, mice received 1x10^6 CFSE labeled Thy1.1$^+$CD8$^+$ T cells from NOD.TCR Tg 8.3.*RAG*-deficient mice. Cells were harvested on day 6 and stained for Thy1.1, Annexin V, and 7AAD. (A). Representative CFSE histograms gated on Thy1.1+ cells obtained from the pancreatic or inguinal lymph nodes of anti-PDL1 or control rat IgG treated mice are shown. (B) The percentages of apoptotic cells (Annexin+DAPI-) for each group are plotted. Data are representative of three experiments with n=3-4 per group.

FIG. 5. Flow cytometry analysis of CD4+CD25+Foxp3+ Tregs in anti-PDL1-treated versus rat IgG treated controls. Ten-week-old NOD mice were injected with anti-PDL1 mAb or control rat IgG (500ug day 0; 250ug on days 2, 4, 6, 8, and 10; treatment was stopped when mice became hyperglycemic). Cells from the spleen and pancreatic lymph nodes were harvested and

stained with anti-CD4 and anti-CD25, followed by intracellular staining of Foxp3. (A) Mean percentages of CD25+Foxp3+ cells (gated on CD4+ T cells) in anti-PDL1 treated versus IgG-treated group (n=5 for each group). (B) Density plots shown are representative of the CD25 Foxp3 staining in pancreatic lymph nodes and spleen with gates on the double positive cells along with corresponding percentages.

FIG. 6. Priming of autoreactive T cells is required for PDL1-mediated regulation of autoreactive T cells. Microsurgery was performed on (A) 3 week old and (B) 11 week old NOD mice to remove the pancreatic lymph nodes. Control mice were treated similarly and exposed to the surgical manipulation, but pancreatic lymph nodes were left intact. Following surgery, the mice were treated with either anti-PDL1 or IgG and followed for the development of spontaneous diabetes. (A) Diabetes incidence from 3 week old mice whose pancreatic lymph nodes were removed and treated with anti-PDL1 (dark square, n=9), pancreatic lymph nodes removed treated with IgG (open squares, n=6), sham controls treated with anti-PDL1 (dark circles, n=6) and sham controls treated with IgG (opened circles, n=5). Three-week-old mice whose pancreatic lymph nodes were removed were significantly protected from developing diabetes even with the administration of anti-PDL1 ($P = 0.031$). (B) Shown is the diabetes incidence from 11 week old mice whose pancreatic lymph nodes have been removed and treated with anti-PDL1 (dark square, n=8), pancreatic lymph nodes removed treated with IgG (open squares, n=7), sham controls treated with anti-PDL1 (dark circles, n=9) and sham controls treated with control IgG (open circles, n=9).

Table 1. Various mouse strains used in the study to delineate the mechanism underlying PDL1 mediated regulation of type 1 diabetes.

Mouse Strain	**Intrinsic Defect/ Characteristics**	**Major Findings**
NOD.*RAG*-deficient	Both T and B cell deficient	Data suggests that either T or B or both cells are involved in PDL1 blockade mediated disease acceleration
NOD.CIITA	Deficient in CD4 T cells	Data suggests that CD4 T cells are required for PDL1 blockade mediated disease acceleration
NOD.β2m	Deficient in CD8 T cells	Data suggests that CD8 T cells are required for PDL1 blockade mediated disease acceleration
NOD.μT	B cell deficient	Data indicates that B cells are not required for PDL1 blockade-mediated disease acceleration
NOD.BDC2.5 TCR Tg	Derived from the I-A^{g7}-restricted beta cell-specific CD4+ T cell clone BDC2.5	Expansion of CD4 T cells in the pancreatic lymph nodes following PDL1 blockade
4.1-NOD	Derived from the I-A^{g7}-restricted beta cell-specific CD4+ T cell clone NY4.1	Confirming the expansion of CD4 T cells in the pancreatic lymph nodes following PDL1 blockade
8.3-NOD	Derived from the H-$2K^{d}$-restricted beta cell-specific CD8+ T cell clone NY8.3	Expansion of CD8 T cells in the pancreatic lymph nodes following PDL1 blockade

Figure 1

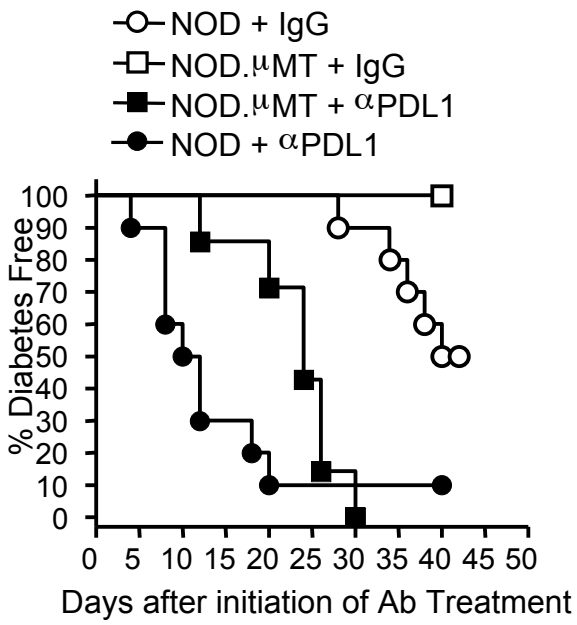

NOD + IgG
NOD.μMT + IgG
NOD.μMT + αPDL1
NOD + αPDL1
% Diabetes Free
100
90
80
70
60
50
40
30
20
10
0
0 5 10 15 20 25 30 35 40 45 50
Days after initiation of Ab Treatment

Figure 2

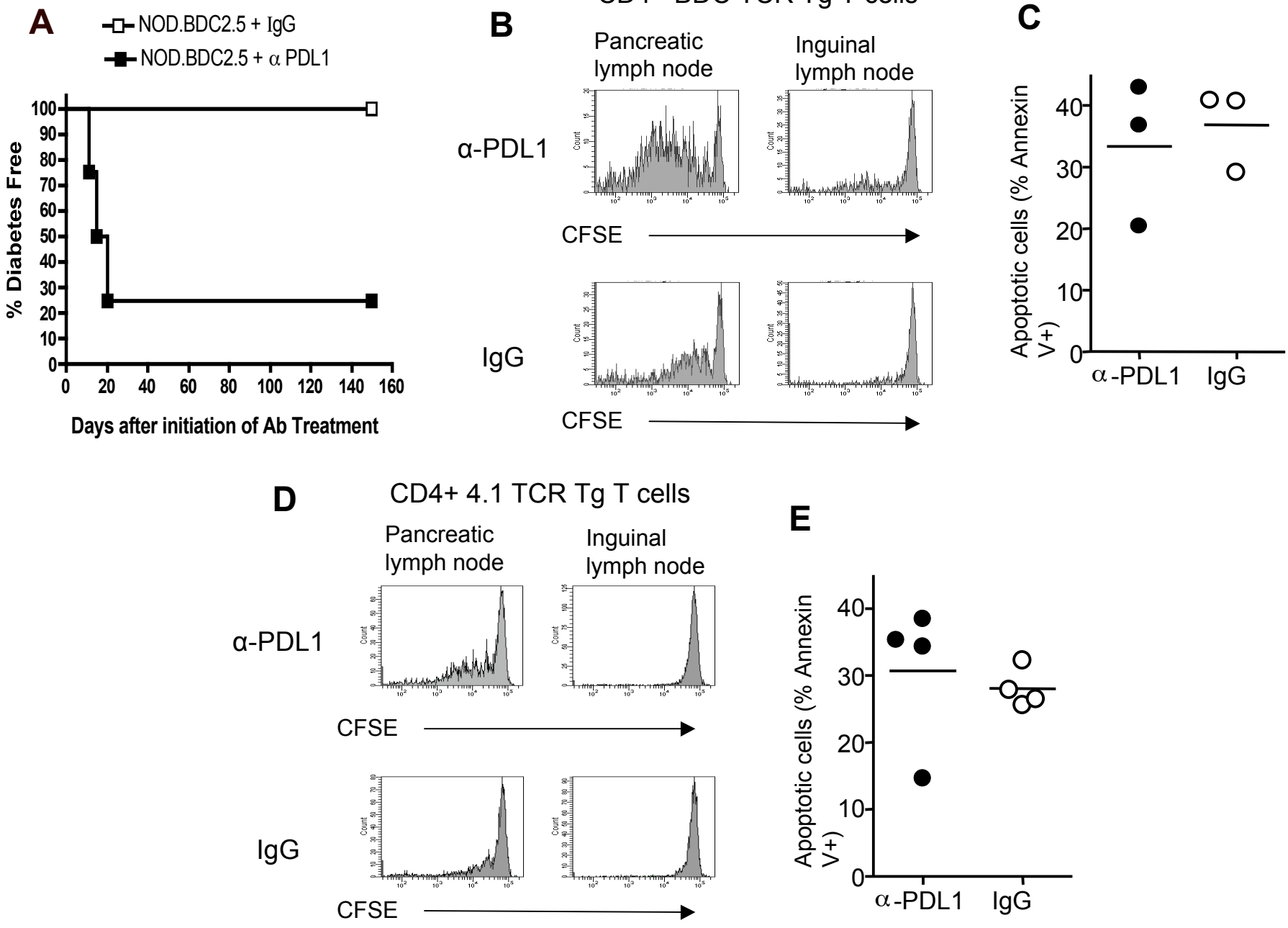

A
NOD.BDC2.5 + IgG
NOD.BDC2.5 + α PDL1
% Diabetes Free
Days after initiation of Ab Treatment
B
CD4+ BDC TCR Tg T cells
Pancreatic lymph node
Inguinal lymph node
α-PDL1
IgG
CFSE
C
Apoptotic cells (% Annexin V+)
α-PDL1
IgG
D
CD4+ 4.1 TCR Tg T cells
Pancreatic lymph node
Inguinal lymph node
α-PDL1
IgG
CFSE
E
Apoptotic cells (% Annexin V+)
α-PDL1
IgG

Figure 3

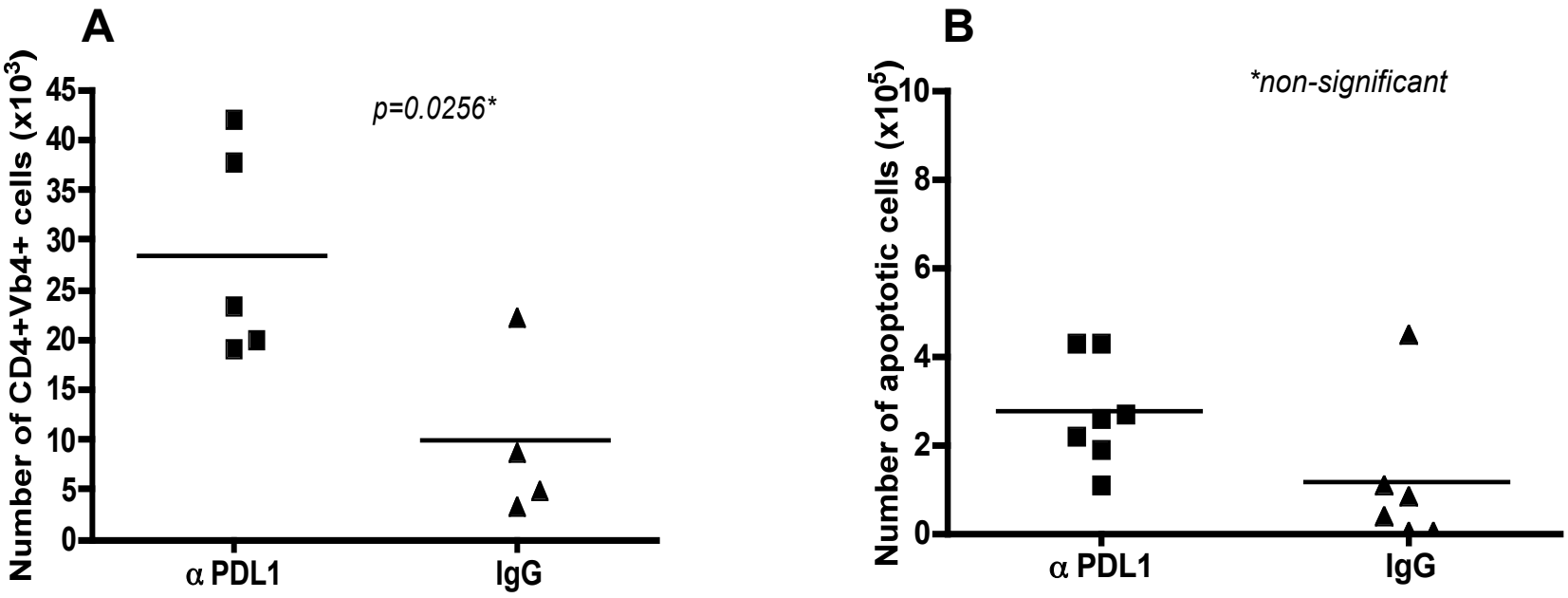

A
Number of CD4+Vb4+ cells (x10^3)
45
40
35
30
25
20
15
10
5
0
p=0.0256*
α PDL1
IgG
B
Number of apoptotic cells (x10^5)
10
8
6
4
2
0
*non-significant
α PDL1
IgG

Figure 4

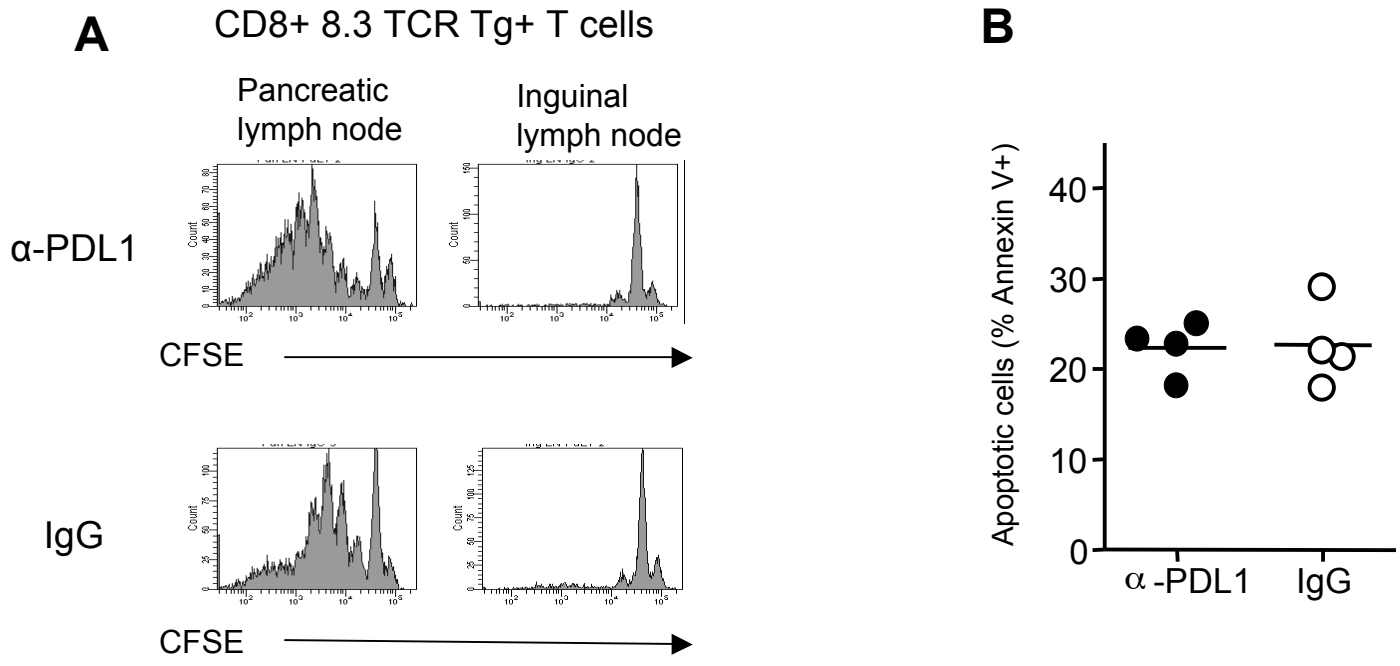

A
CD8+ 8.3 TCR Tg+ T cells
Pancreatic lymph node
Inguinal lymph node
α-PDL1
IgG
Count
CFSE
B
Apoptotic cells (% Annexin V+)
0
10
20
30
40
α -PDL1
IgG

Figure 5

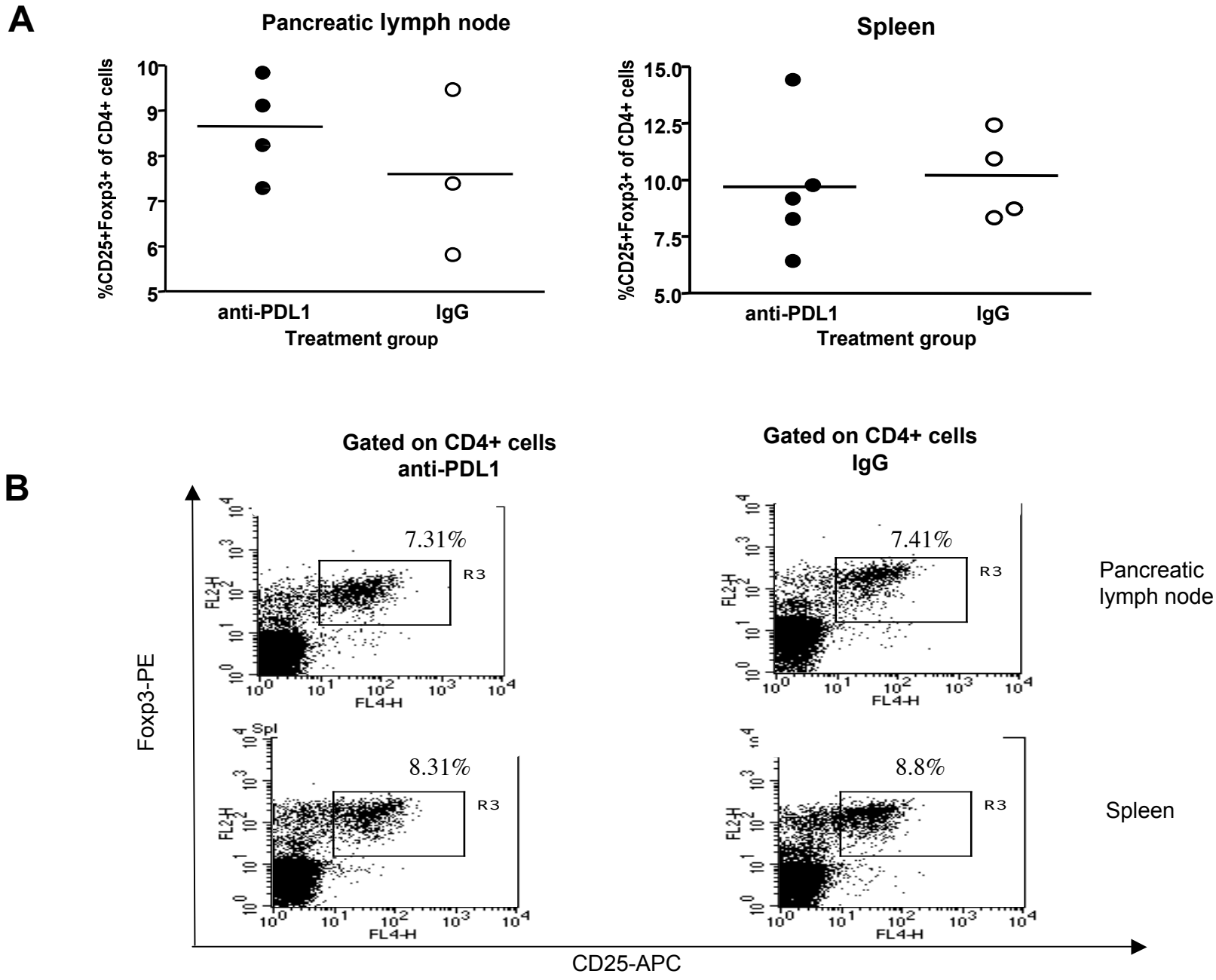
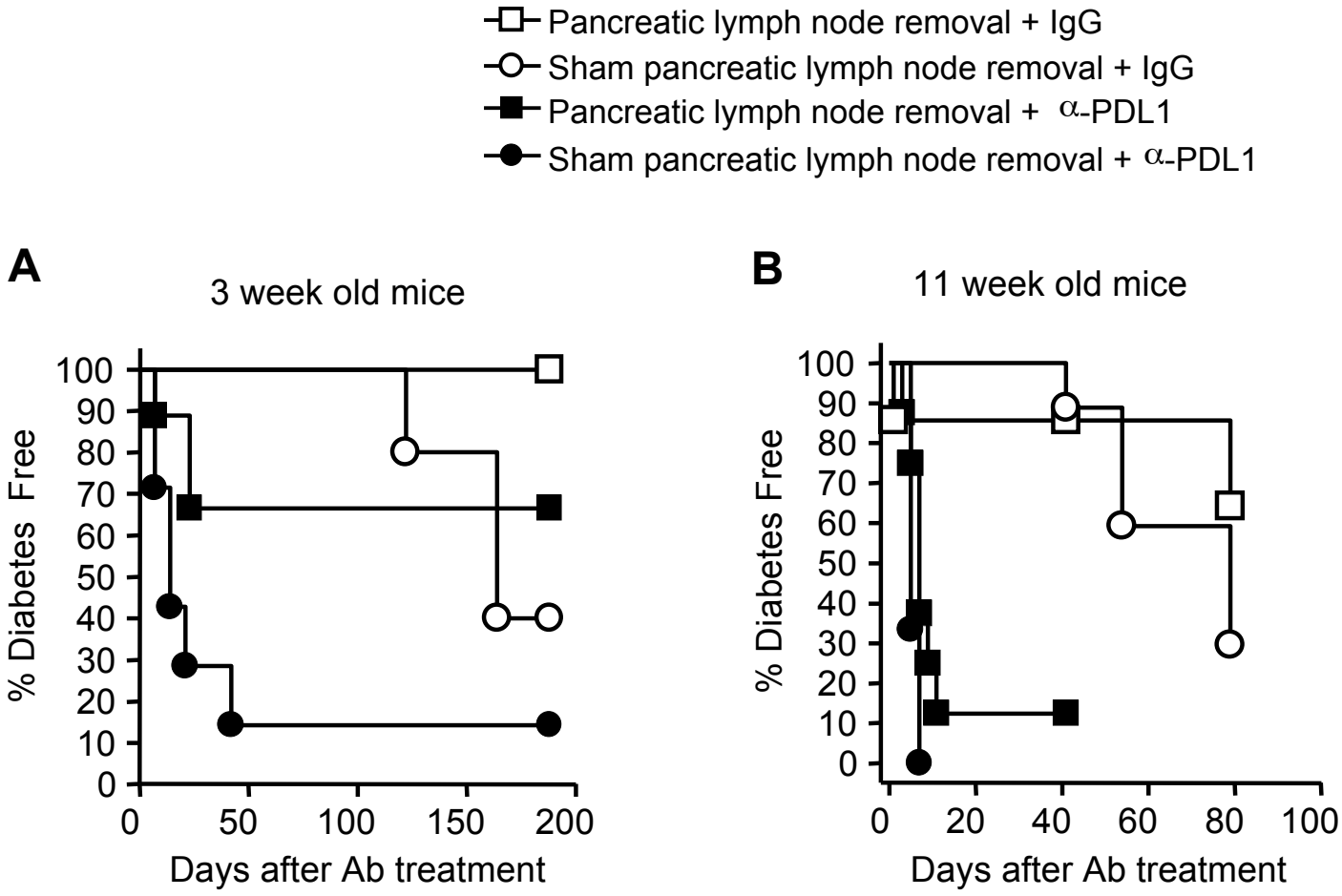

A
Pancreatic lymph node
%CD25+Foxp3+ of CD4+ cells
10
9
8
7
6
5
anti-PDL1
IgG
Treatment group
Spleen
%CD25+Foxp3+ of CD4+ cells
15.0
12.5
10.0
7.5
5.0
anti-PDL1
IgG
Treatment group
B
Gated on CD4+ cells
anti-PDL1
Gated on CD4+ cells
IgG
7.31%
R3
7.41%
R3
Pancreatic lymph node
Spl
8.31%
R3
8.8%
R3
Spleen
FL2-H
FL4-H
Foxp3-PE
CD25-APC

Figure 6


Pancreatic lymph node removal + IgG
Sham pancreatic lymph node removal + IgG
Pancreatic lymph node removal + α-PDL1
Sham pancreatic lymph node removal + α-PDL1
A
3 week old mice
% Diabetes Free
Days after Ab treatment
B
11 week old mice
% Diabetes Free
Days after Ab treatment