Cutting Edge

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Cutting Edge: Identification of Autoreactive CD4$^+$ and CD8$^+$ T Cell Subsets Resistant to PD-1 Pathway Blockade

Kristen E. Pauken,[*,†,1] Christine E. Nelson,[*,‡,1] Tijana Martinov,[*,†,1] Justin A. Spanier,[*,†] James R. Heffernan,[*,†] Nathanael L. Sahli,[*,†] Clare F. Quarnstrom,[*,‡] Kevin C. Osum,[*,†] Jason M. Schenkel,[*,‡] Marc K. Jenkins,[*,‡] Bruce R. Blazar,[*,§] Vaiva Vezys,[*,‡,2] and Brian T. Fife[*,†,2]

Programmed death-1 (PD-1) promotes T cell tolerance. Despite therapeutically targeting this pathway for chronic infections and tumors, little is known about how different T cell subsets are affected during blockade. We examined PD-1/PD ligand 1 (PD-L1) regulation of self-antigen–specific CD4 and CD8 T cells in autoimmune-susceptible models. PD-L1 blockade increased insulin-specific effector CD4 T cells in type 1 diabetes. However, anergic islet-specific CD4 T cells were resistant to PD-L1 blockade. Additionally, PD-L1 was critical for induction, but not maintenance, of CD8 T cell intestinal tolerance. PD-L1 blockade enhanced functionality of effector T cells, whereas established tolerant or anergic T cells were not dependent on PD-1/PD-L1 signaling to remain unresponsive. This highlights the existence of Ag-experienced T cell subsets that do not rely on PD-1/PD-L1 regulation. These findings illustrate how positive treatment outcomes and autoimmunity development during PD-1/PD-L1 inhibition are linked to the differentiation state of a T cell. *The Journal of Immunology*, **2015, 194: 3551–3555.**

The inhibitory receptor programmed death-1 (PD-1) interacts with PD ligand 1 (PD-L1) to regulate T cell function and autoimmunity (1–5). Prolonged, elevated PD-1 and PD-L1 expression occurs during chronic infections and cancer and leads to T cell exhaustion (6). PD-1 blockade can reinvigorate exhausted T cells, providing enhanced antiviral and antitumor responses (7, 8). These observations led to the development of PD-1 pathway blockers, which are anticipated to revolutionize cancer therapy.

While inhibitory blockade can be successful, not all patients had positive outcomes and some developed autoimmunity. These observations indicate differential susceptibility to PD-1/PD-L1 inhibitors. Recent reports have shown adverse events with anti–PD-1/PD-L1 in clinical trials for cancer, including vitiligo, colitis, hepatitis, thyroiditis, and type 1 diabetes (T1D) (9). The notable prevalence of these side effects strongly warrants further investigation into biomarkers to identify patients at risk prior to therapy. Therefore, we asked whether T cell activation or differentiation state impacted PD-1/PD-L1 dependence for effector function and loss of tolerance.

The goal of this study was to assess PD-1/PD-L1 regulation of self-antigen–specific CD4 and CD8 T cells to determine autoimmune risk with PD-1/PD-L1 inhibition. We used the NOD model of T1D to investigate CD4 T cells, given their requirement for disease. NOD mice deficient for PD-1 or PD-L1 develop accelerated T1D (1, 3), and selective loss of PD-1 on islet-reactive CD4 T cells enhances proliferation and pancreas infiltration (10). To investigate the role of PD-L1 in regulating mucosal CD8 T cell responses, we used the iFABP-OVA transgenic mouse, where transfer of naive OT-I CD8 T cells leads to Ag-specific tolerance (11, 12). Using these models, we re-evaluated the role of PD-1/PD-L1 during the induction and maintenance of T cell tolerance. Unexpectedly, PD-1/PD-L1 regulation of autoreactive T cells was dependent on the T cell differentiation state and timing of blockade relative to Ag encounter. Although PD-L1 blockade resulted in enhanced functionality of effector T cells, established anergic T cells were not sensitive to PD-L1 inhibition. These data have important clinical implications regarding the use of PD-L1 inhibitors, suggesting that productive antitumor response and patient autoimmune susceptibility are linked to T cell activation state at the time of treatment.

*Center for Immunology, University of Minnesota, Minneapolis, MN 55455; †Department of Medicine, University of Minnesota, Minneapolis, MN 55455; ‡Department of Microbiology, University of Minnesota, Minneapolis, MN 55455; and §Department of Pediatrics, University of Minnesota, Minneapolis, MN 55455.

[1]K.E.P., C.E.N., and T.M. contributed equally to this work.

[2]V.V. and B.T.F. contributed equally to this work.

ORCIDs: 0000-0001-8009-7655 (M.K.J.); 0000-0002-9608-9841 (B.R.B.); 0000-0002-2520-809X (V.V.); 0000-0001-9826-5637 (B.T.F.).

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Address correspondence and reprint requests to Dr. Brian T. Fife or Dr. Vaiva Vezys, Department of Medicine, Center for Immunology, University of Minnesota, 2101 6th Street SE, 3-144 WMBB, Minneapolis, MN 55455 (B.T.F.) or Department of Microbiology, Center for Immunology, University of Minnesota, 2101 6th Street SE, 2-180 WMBB, Minneapolis, MN 55455 (V.V.). E-mail addresses: bfife@umn.edu (B.T.F.) and vvezys@umn.edu (V.V.)

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Abbreviations used in this article: FR4, folate receptor 4; IEL, intraepithelial lymphocyte; LN, lymph node; pancLN, pancreatic LN; PD-1, programmed death-1; PD-L1, PD ligand 1; SLO, secondary lymphoid organ; SPL, spleen; T1D, type 1 diabetes.

Materials and Methods

Mice

Female mice were housed in specific-pathogen free facilities, and all experiments were Institutional Animal Care and Use Committee approved at the University of Minnesota. NOD mice were purchased from Taconic. OT-I, iFABP-OVA, B6.g7, NOD.PD-1$^{-/-}$, NOD.PD-L1$^{-/-}$, and NOD.BDC2.5 Thy1.1 mice were generated as described (10, 11).

Lymphocyte transfer, isolation, and detection

Seven thousand five hundred NOD.BDC2.5.Thy1.1$^+$ CD4 T cells from 4- to 6-wk-old donors were transferred into prediabetic NOD mice with or without CFSE labeling (10). Five hundred thousand naive OT-I CD8 T cells isolated from spleen (SPL) and lymph node (LN) were transferred i.v. to adult iFABP-OVA mice (11). Insulin-specific CD4 T cells were detected by double ins$B_{10–23r3}$:I-A^{g7} tetramer staining and enrichment (13). Intraepithelial lymphocytes (IEL) and SPL were isolated as described (11).

Flow cytometry

Surface staining was performed as described (13). Gating strategies were as follows: singlet$^+$, CD3$^+$lineage$^-$ (B220$^-$, CD11b$^-$, CD11c$^-$) CD4$^+$CD8α^-, ins$B_{10–23r3}$:I-A^{g7}–PE and –allophycocyanin double-positive (insulin-specific CD4$^+$ T cells); singlet$^+$, CD3$^+$lineage$^-$ (B220$^-$, CD11b$^-$, CD11c$^-$) CD4$^+$Thy1.1$^+$ (BDC2.5); singlet$^+$, CD45.1$^+$, CD8$^+$, K^b SIINFEKL$^+$ (OT-I) (11).

Histology

Islet inflammation was scored as follows: 0, no insulitis; 1, perinsulitis; 2, <25% of the islet is infiltrated; 3, <75% of the islet is infiltrated; 4, <25% of the islet mass is intact.

Administration of Abs

Anti–PD-L1 (M1H6 or 10F.9G2), PD-1 (J43), rat IgG2a, rat IgG2b, or hamster IgG was injected i.p. (10). For CD4 tolerance, mice received two to three doses (250 μg) as indicated in each figure, and 250 μg every other day for 10 d for T1D incidence. Glucose levels >250 mg/dl are diabetic. For CD8 tolerance induction, iFABP-OVA mice received 200 μg anti–PD-L1 i.p. (clone 10F.9G2) on the day of transfer and day +3. For CD8 tolerance maintenance, mice received 200 μg anti–PD-L1 starting at day 30 and doses every third day for 15 d.

Cytokine assays

IFN-γ was measured as described from BDC2.5 cells 4 h after 500 μg i.v. of acetylated P31 (1040-31) peptide (YVRPLWVRME) (Genemed Sythesis) or OT-I cells after 1 μg/ml SIINFEKL ex vivo for 4 h (New England Peptide) (10, 11).

Statistical analysis

Unpaired two-tailed Student *t* tests or Mann–Whitney tests with a 95% CI were performed using Prism 5 software (GraphPad Software). A p value <0.05 was considered statistically significant.

Results and Discussion

Loss of PD-1 or PD-L1 results in increased numbers of insulin-specific CD4 T cells in NOD mice

The PD-1/PD-L1 pathway is essential for tolerance and autoimmunity (1, 3, 14). We investigated the impact of genetic loss of PD-1 on endogenous insulin-specific CD4 T cells in diabetes-susceptible NOD mice. Using an ins$B_{10–23r3}$:I-A^{g7} tetramer (13, 15), we quantified insulin-specific CD4 T cells in the secondary lymphoid organs (SLO) and pancreas of NOD.PD-1$^{-/-}$ mice. We observed significantly more ins$B_{10–23r3}$:I-A^{g7} tetramer-binding cells (insulin-specific) in the pancreatic LN (pancLN) of prediabetic and diabetic NOD.PD-1$^{-/-}$ mice compared with WT control NOD (Fig. 1A) and PD-L1$^{-/-}$ mice, but not control $HEL_{11–25}$:I-A^{g7} tetramer binding cells (Supplemental Fig. 1A, 1B). There was not a significant change in the total number of CD4 T cells in PD-1 or PD-L1 knockout mice (Supplemental Fig. 1C). The number of insulin-specific CD4 T cells did not differ in either SLO or pancreas of prediabetic NOD and NOD.PD-1$^{-/-}$ mice, but was significantly higher in diabetic NOD.PD-1$^{-/-}$ mice (Fig. 1A). Histological analysis revealed that prediabetic NOD.PD-1$^{-/-}$ mice had more severe islet inflammation compared with age-matched nondiabetic NOD mice, and this increased with diabetes (Fig. 1B). These results indicate that PD-1 is a central regulator of autoreactive CD4 T cells in autoimmune-prone mice.

PD-L1 blockade fails to induce autoimmunity in diabetes-resistant B6.g7 mice

PD-1 loss is known to potentiate autoimmunity in susceptible mice (1); however, its role in regulating known self-antigen–specific T cells in genetically resistant hosts is unclear. We previously examined insulin-specific T cells in NOD and B6.g7 mice, because of the known genetic risk of MHC for T1D (16). We reported that both strains contained insulin-specific CD4 T cells. Insulin-specific CD4 T cells became activated in the pancLN and infiltrated the pancreas in NOD mice, but remained naive in B6.g7 mice (13). In this study, PD-1$^+$ insulin-specific T cells are increased in the SLO of NOD mice compared with B6.g7 mice (Fig. 2A, 2B). In B6.g7 mice, the frequency of insulin-specific PD-1$^+$ cells was low, consistent with a naive phenotype. Interestingly, in NOD mice, the highest frequency of PD-1$^+$ cells was in the pancreas (Fig. 2C). This result supports the idea that pancreatic cells are susceptible to PD-1 inhibition, consistent with previous work (10, 17).

Considering the differences in PD-1 expression, we next injected anti–PD-L1 to NOD and B6.g7 mice. NOD mice rapidly developed T1D (Fig. 2D) (2). However, despite the fact that B6.g7 mice carry the highest genetic risk factor for T1D (16), anti–PD-L1 did not induce T1D (Fig. 2D). We hypothesized that PD-L1 blockade in NOD mice, but not B6.g7 mice, would cause insulin-specific cells to increase due to PD-1 expression (Fig. 2A, 2B) (10). In NOD mice, CD44^{high} insulin-specific CD4 T cells were increased in the SPL and pancLN following anti–PD-L1 (Fig. 2E) and in the pancreas following anti–PD-1 but not anti–B7-1 blockade (Supplemental Fig. 1D). In B6.g7 mice, PD-L1 blockade failed to increase CD44^{high} cells in the SPL (Fig. 2E), but, unexpectedly, resulted

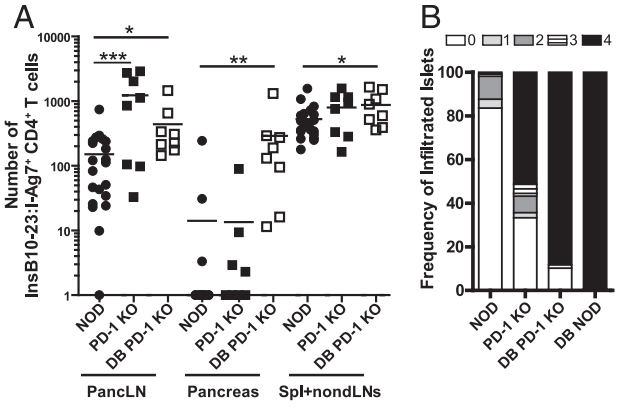

FIGURE 1. Loss of PD-1 increases insulin-specific CD4 T cells in NOD mice. (**A**) Insulin-specific (ins$B_{10–23r3}$:I-A^{g7} tetramer$^+$) CD4 T cells in the pancLN, pancreas, SPL, and nondraining LN of prediabetic WT (n = 21) and PD-1$^{-/-}$ NOD mice (n = 8), between 4 and 7 wk old, and new onset diabetic NOD.PD-1$^{-/-}$ mice (n = 8). (**B**) Insulitis scores from nondiabetic or diabetic (DB) NOD and NOD.PD-1$^{-/-}$ mice. Data represent >100 islets from each group. Data are compiled from at least four experiments. *p = 0.01–0.05, **p = 0.001–0.01, ***$p < 0.001$.

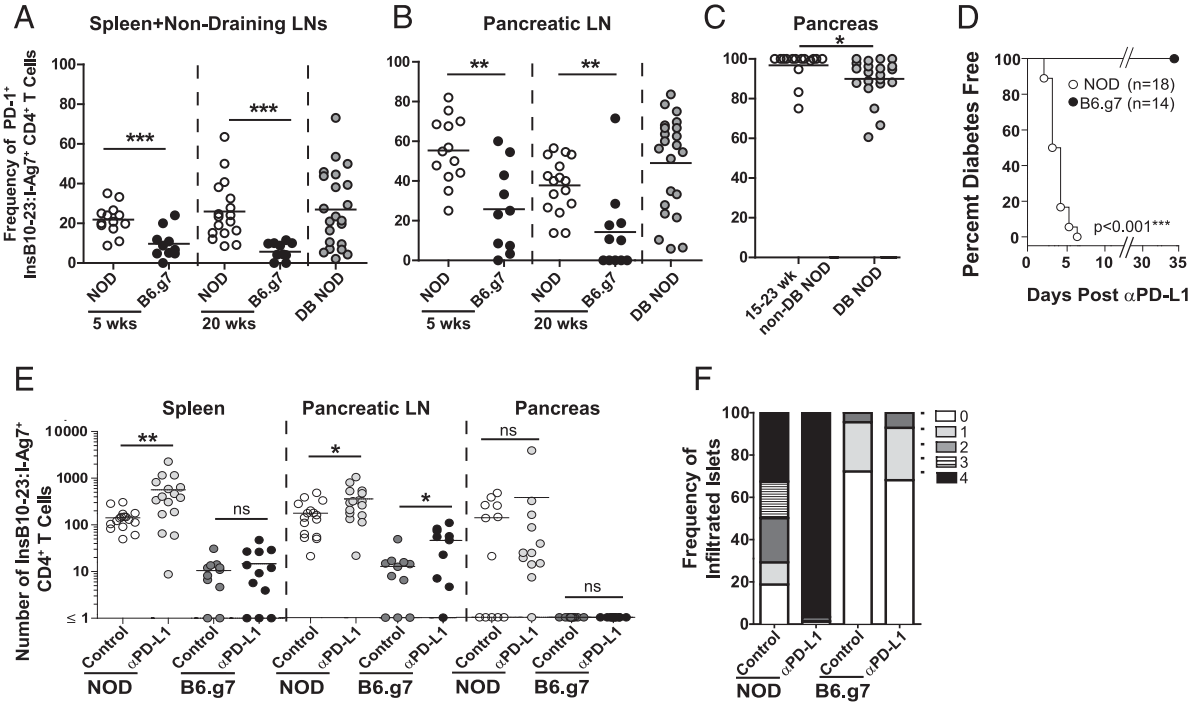

FIGURE 2. PD-L1 blockade differentially impacts insulin-specific CD4 T cells in NOD and B6.g7 mice. Frequency of PD-1$^+$ insulin-specific CD4$^+$ T cells in NOD and B6.g7 mice (**A**) SPL and nondraining LN, (**B**) pancLN, and (**C**) pancreas. (A and B) Nondiabetic NOD mice (n = 13 at 5 wk, n = 17 at 20 wk), diabetic NOD mice (n = 22 at new onset), and nondiabetic B6.g7 mice (n = 10 at 5 wk, n = 11 at 20 wk). Data for (A)–(C) are compiled from at least 10 experiments. (**D**) Diabetes incidence of 15-wk-old NOD (n = 18) or B6.g7 (n = 14) mice after anti–PD-L1. Data are compiled from four experiments. (**E**) Enumeration of CD44^{high} insulin-specific CD4 T cells in SPL, pancLN, and pancreas of 11- to 16-wk-old NOD (n = 16) or B6.g7 (n = 11) mice treated with isotype control or anti–PD-L1. Data were compiled from three or more experiments. (**F**) Insulitis scores from NOD (n = 11) and B6.g7 (n = 12) mice with or without anti–PD-L1. Data represent >100 islets from each group. Data are representative of three or more experiments. *p = 0.01–0.05, **p = 0.001–0.01, ***$p < 0.001$.

in a significant increase in the pancLN. However, insulin-specific CD4 T cells did not infiltrate the pancreas and did not cause T1D (Fig. 2E, 2D). Islet histology was examined and anti–PD-L1 caused severe insulitis in NOD, but not in B6.g7 mice (Fig. 2F).

Differential susceptibility of islet-reactive CD4 T cell subsets to PD-1 blockade

Our results demonstrated that anti-PD-L1 selectively increased Ag-experienced cells (Fig. 2). However, there is notable heterogeneity within the Ag-experienced (CD44^{high}Foxp3$^-$) compartment in pancLN of NOD mice with both effector and anergic cells (13). These populations have been characterized based on expression of two surface proteins, folate receptor 4 (FR4) and CD73 (13, 18). FR4$^+$CD73$^+$ anergic cells produce less effector cytokines than do FR4$^-$CD73$^-$ effector T cells (13, 18). Using these markers we tested the impact of PD-1 signaling in light of our previous work demonstrating that anti–PD-L1 promoted the breakdown of tolerance by inducing the T cell stop signal (17) and reversing tolerance causing diabetes (4).

To investigate the role of PD-1 regulation on anergic and effector T cell subsets, we used our recently characterized adoptive transfer model of diabetes. In this model, a low number of naive BDC2.5 TCR transgenic T cells specific for an islet Ag were transferred into prediabetic NOD mice to mimic the endogenous response (10). Using this model, 70% of the BDC2.5 CD4 T cells develop an anergic phenotype and 30% become effector T cells in the pancLN (Supplemental Fig. 1E). Not surprisingly, these subsets differ in their proliferative capacity, with anergic subsets dividing less than effector T cells (Fig. 3A). Interestingly, PD-1 levels are very similar on anergic and effector T cells (Supplemental Fig. 1H, 1I). We tested whether anti–PD-L1 altered the frequency of anergic or effector T cell subsets. Anti–PD-L1 decreased the frequency of anergic cells (Supplemental Fig. 1E, 1F). However, the decrease was not due to cell loss, but rather to a 3-fold expansion of effector T cells (Supplemental Fig. 1G). We next measured the effect of PD-L1 blockade on anergic cells and predicted these cells would be reinvigorated, given our previous findings with Ag-coupled cell tolerance (4). Contrary to prediction, anergic cells were blunted in their ability to produce IFN-γ. We measured a significant increase in IFN-γ production by effector T cells with anti–PD-L1 (Fig. 3B–D). The mean frequency of IFN-γ$^+$ anergic cells rose from 16.9 to 25.6% after anti–PD-L1 but remained lower than effector cells with and without PD-L1 blockade (43.7 and 58.4%, respectively) (Fig. 3C). If anti–PD-L1 had released the anergic CD4 T cells, we would have observed equal levels of IFN-γ, but this was not the case, indicating there is a subset of anergic CD4 T cells that are not reinvigorated by PD-L1 blockade. Even though the percentage of IFN-γ$^+$ cells changed in the anergic population, the amount of produced IFN-γ did not increase above baseline following anti–PD-L1 (Fig. 3D). Anergic cells made 1.5-fold less IFN-γ than did effector cells in control animals and 2-fold less than did effectors after anti–PD-L1 on a per cell basis (Fig. 3D). Lastly, we measured CXCR3 expression on anergic and effector cells and determined CXCR3 was higher on CD4 effector cells compared with anergic cells, which could mechanistically explain a differential ability of these cells to traffic to sites of inflammation during autoimmunity (Supplemental Fig. 1J).

We have previously reported that CXCR3 expression increased following PD-L1 blockade and did not detect any CXCR3$^+$ anergic cells in the pancreas (13). This is consistent with the idea that anergic cells remain in the periphery and the effector cells traffic to the pancreas to cause T1D (13). Taken together, our results suggest that PD-L1 blockade has the greatest impact on the effector T cell subset allowing enhanced proliferation and IFN-γ production over anergic cells (Fig. 3D). This is consistent with reports of PD-L1–mediated restoration of exhausted CD8 subsets (19).

The differences between anergic and effector cells may be explained by two potential mechanisms. Previous reports implicate that other inhibitory receptors such as CTLA-4, LAG-3, 2B4, and TIM-3 may contribute to the establishment of T cell exhaustion or anergy via nonredundant signaling pathways (20). Alternatively, incomplete TCR stimulation (TCR stimulation without costimulation) leading to a calcium influx–mediated altered gene expression program that includes upregulation of several E3 ubiquitin ligases and T cell anergy may explain the differences between anergic and effector cells (21). What was not known from these studies was whether PD-L1 blockade can reverse this state of anergy and allow for T cell reinvigoration. We conclude from our data that PD-L1 blockade results in differential responsiveness for anergic and effector CD4 T cells.

PD-L1 blockade enhances effector functions of self-antigen–specific CD8 T cells during the induction, but not the maintenance, of tolerance

We next asked whether there was differential PD-L1 dependence of self-antigen–specific CD8 T cells. We transferred naive OVA-specific OT-I CD8 T cells to iFABP-OVA mice expressing OVA as an intestinal self-antigen (11, 12). This system eliminates recent thymic emigrants and synchronizes Ag encounter. Importantly, we evaluated both the induction and maintenance of CD8 T cell tolerance, which has previously been difficult, as deletion of transferred T cells is common. This model overcomes this barrier and results in long-term maintenance of self-specific CD8 T cells, indicating that T cell deletion is not necessarily the outcome of tolerogenic interactions (22).

Previous work using this model suggested a role for PD-1/PD-L1 in the initiation of mucosal CD8 T cell tolerance (23). In the present study, when PD-L1 was blocked early, tolerance induction was prevented. Anti–PD-L1 caused an increase in the frequency and number of OT-I T cells in the SPL and small intestine (IEL) of iFABP-OVA mice and enhanced IFN-γ production from OT-I IEL and granzyme B (Fig. 4A, 4B, Supplemental Fig. 2A, 2B). As a result of PD-L1 blockade, mice died of severe intestinal inflammation by day 10 (data not shown). OT-I cells examined 30 d after transfer had characteristics of anergic T cells: they were Ag experienced, but did not exhibit effector functions and could be found in Ag-rich locations, including the IEL (Fig. 4, Supplemental Fig. 2A, 2B) (11). Similar to BDC2.5 T cells, CXCR3 expression was increased on effector OT-I CD8 T cells compared with anergic cells (Supplemental Fig. 2C). Surprisingly, once anergy was established, PD-L1 blockade did not increase OT-I cell numbers or restore effector functions (Fig. 4). This resistance to treatment was not due to downregulation of the receptor PD-1, because these cells retain expression (Supplemental Fig. 2D). Importantly, anti–

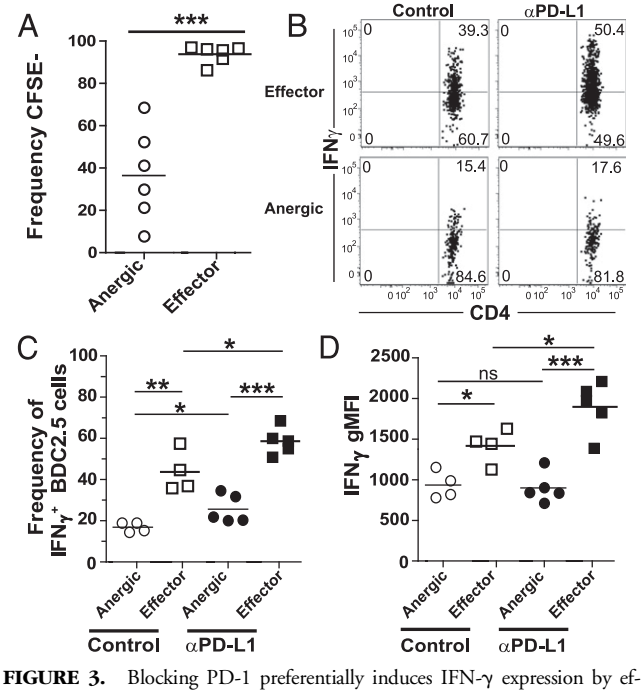

FIGURE 3. Blocking PD-1 preferentially induces IFN-γ expression by effector but not anergic BDC2.5 T cells. (**A**) Frequency of CFSE$^-$ BDC2.5 CD4 T cells in pancLN 3 wk after transfer. (**B**) Concatenated FACS plots showing the frequency of IFN-γ$^+$ anergic and effector BDC2.5 cells in isotype control (n = 4) and anti-PD-L1–treated mice (n = 5). (**C**) Frequency and (**D**) IFN-γ geometric mean fluorescence intensity (gMFI) of IFN-γ$^+$ anergic and effector BDC2.5 CD4 T cells in the pancLN 49 d after transfer. Data are representative of three experiments. *p = 0.01–0.05, **p = 0.001–0.01, ***$p < 0.001$.

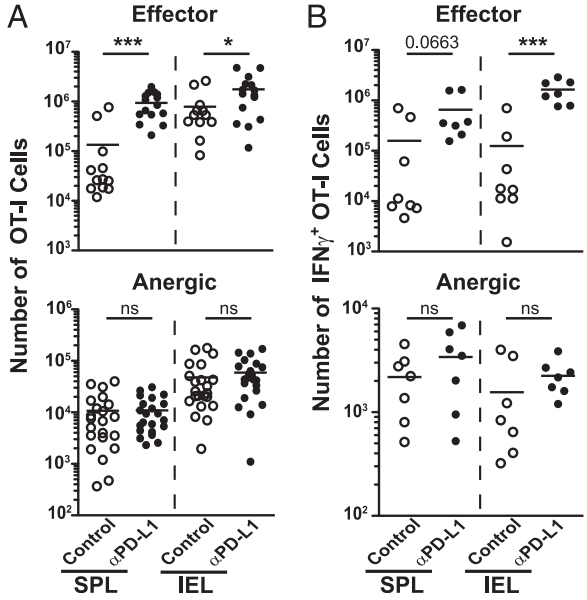

FIGURE 4. PD-L1 signaling is critical for the induction, but not the maintenance, of CD8 T cell tolerance to intestinal self-antigen. Naive OT-I cells were transferred to iFABP-OVA mice and anti–PD-L1 injections began either the day of cell transfer (day 0) or at least 30 d later. Cells were analyzed at day 5 (effector) or at day 45 (anergic) following control or anti–PD-L1. (**A**) Enumeration of OT-I cells from SPL and IEL (effector cells [day 5] isotype, n = 12; anti–PD-L1, n = 14; anergic cells [day 45] isotype, n = 22; anti–PD-L1, n = 20). (**B**) Enumeration of IFN-γ–producing OT-I cells isolated from SPL and IEL (effector [day 5] isotype, n = 8; anti–PD-L1, n = 7; anergic [day 45] isotype, n = 7; anti–PD-L1, n = 7). Data are compiled from three or more experiments. *p = 0.01–0.05, ***$p < 0.001$.

PD-L1 during tolerance maintenance did not promote intestinal pathology (data not shown). These data demonstrate critical differences in the temporal requirement for PD-L1 in CD8 T cell tolerance.

The inhibitory receptor PD-1 has long been considered a central mediator of peripheral tolerance, facilitating T cell inhibition and preventing effector function. In this study, however, we report the unexpected finding that the inhibitory effects of PD-1/PD-L1 signaling and subsequent blockade act on specific subsets of CD4 and CD8 T cells. We found that PD-1/PD-L1 blockade preferentially acts to restore or potentiate the function of effector cells, rather than broadly reversing tolerance in all T cell subsets. These results suggest that effector T cells are restrained via PD-1 inhibitory signals, whereas anergic T cells are not released through PD-L1 blockade alone. These data have important implications for patients receiving PD-1 pathway inhibitors for treatment of cancer or chronic infections, as it suggests that the functional state of T cells will impact patient outcomes. This highlights the importance of autoreactive T cell activation status as an indicator for study exclusion criteria. Additionally, our results indicated that PD-L1 blockade alone did not reverse tolerance of self-reactive anergic T cells to cause autoimmunity. Better clinical efficacy of PD-1 pathway inhibitors may be possible by combining blockade with effective therapeutic vaccination to reverse the tolerant or exhausted state of tumor- or microbe-specific T cells and produce synergistic effects (24). Understanding how PD-1 blockade impacts autoreactive T cells in hosts with varying autoimmune susceptibility is critically important as PD-1 inhibitor use moves forward in the clinic (25, 26).

Disclosures

The authors have no financial conflicts of interest.

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