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CTLA-4 suppresses the pathogenicity of self antigen–specific T cells by cell-intrinsic and cell-extrinsic mechanisms

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The inhibitory immunoregulatory receptor CTLA-4 is critical in maintaining self-tolerance, but the mechanisms of its actions have remained controversial. Here we examined the antigen specificity of tissue-infiltrating CD4⁺ T cells in *Ctla4*$^{–/–}$ mice. After adoptive transfer, T cells isolated from tissues of *Ctla4*$^{–/–}$ mice showed T cell antigen receptor (TCR)-dependent accumulation in the tissues from which they were derived, which suggested reactivity to tissue-specific antigens. We identified the pancreas-specific enzyme PDIA2 as an autoantigen in *Ctla4*$^{–/–}$ mice. CTLA-4 expressed either on PDIA2-specific effector cells or on regulatory T cells was sufficient to control tissue destruction mediated by PDIA2-specific T cells. Our results demonstrate that both cell-intrinsic and non–cell-autonomous actions of CTLA-4 operate to maintain T cell tolerance to a self antigen.

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Cytotoxic T lymphocyte antigen 4 (CTLA-4; A000706) is a structural homolog of the costimulatory molecule CD28 and is a negative regulator required for T cell homeostasis and tolerance[1]. *Ctla4*$^{–/–}$ mice develop a fatal lymphoproliferative disorder characterized by the infiltration of $CD4^+$ T cells into many nonlymphoid tissues[2–4]. Although the importance of CTLA-4 is clear, several aspects of the mechanisms by which it maintains self-tolerance are poorly understood[5]. Specifically, it is not clear if specific self antigens are involved in the expansion of *Ctla4*$^{–/–}$ $CD4^+$ T cell populations or in which lymphocyte populations CTLA-4 must be expressed to prevent the fatal lymphoproliferative disorder.

CTLA-4 might regulate T cell activation by several mechanisms. CTLA-4 could exert cell-intrinsic inhibitory actions by competing with CD28 for their shared ligands, B7-1 and B7-2 (refs. 6,7), by delivering inhibitory signals that induce cell cycle arrest and prevent the production of interleukin 2 (IL-2)[8–10], or by limiting the dwell time of T cells with antigen-presenting cells (APCs)[11]. In addition, experiments with mixed–bone marrow chimeras have shown that *Ctla4*$^{–/–}$ T cells can be controlled by wild-type bone marrow–derived cells[12], which suggests a cell-extrinsic, dominant action of CTLA-4 in promoting tolerance similar to that exerted by regulatory T cells (T_{reg} cells). Indeed, a study has shown that CTLA-4-expressing T_{reg} cells can regulate *Ctla4*$^{–/–}$ T cells *in vivo*[13]. Although CTLA-4 is not required for all normal T_{reg} cell activities *in vitro*[14,15], it seems to be essential for T_{reg} cell function *in vivo*, as T_{reg} cell–specific deletion of CTLA-4 causes spontaneous development of systemic lymphoproliferation and fatal disease in mice[16]. Some evidence suggests that CTLA-4 expression on T_{reg} cells acts to decrease the expression of CD80 and CD86 on dendritic cells[16,17] but it is unclear whether the *in vivo* stimulatory activity of antigen-presenting cells is affected by CTLA-4. Thus, the cellular site of action of CTLA-4 in controlling tolerance requires further examination.

In addition, the antigen specificity of expanding $CD4^+$ T cell populations in *Ctla4*$^{–/–}$ mice has not been examined. Specifically, it is unclear whether these proliferating T cells are reactive generally to self major histocompatibility complex, to ubiquitous antigens and/or to many tissue-specific antigens. Some relationship between the pathology of *Ctla4*$^{–/–}$ mice and T cell antigen receptor (TCR) specificity is suggested by the observation that the introduction of rearranged αβTCR transgenes onto the *Ctla4*$^{–/–}$ genetic background eliminates the fatal lymphoproliferation[18,19]. However, no direct evidence has supported the interpretation that *Ctla4*$^{–/–}$ mice develop an antigen-specific autoimmune disease. For example, spectratype analysis of the TCRβ complementarity-determining region 3 regions from T cell populations expanding in *Ctla4*$^{–/–}$ mice has shown a diverse and unbiased repertoire and has been interpreted as being reflective of antigen-independent T cell activation[20]. Furthermore, no reports so far have directly analyzed the antigen specificity of $CD4^+$ T cell clones obtained from *Ctla4*$^{–/–}$ mice. However, resolving the nature of the TCR repertoire of T cell populations expanding in *Ctla4*$^{–/–}$ mice is important for two reasons. First, mutations in *CTLA4* are associated with several autoimmune diseases, including hypothyroidism and type 1 diabetes[21]. Second, treatment with antibody to CTLA-4 (anti-CTLA-4) is a potential immunotherapeutic approach in the treatment of cancer[22], so it is important to determine the potential for activating self antigen–specific T cells.

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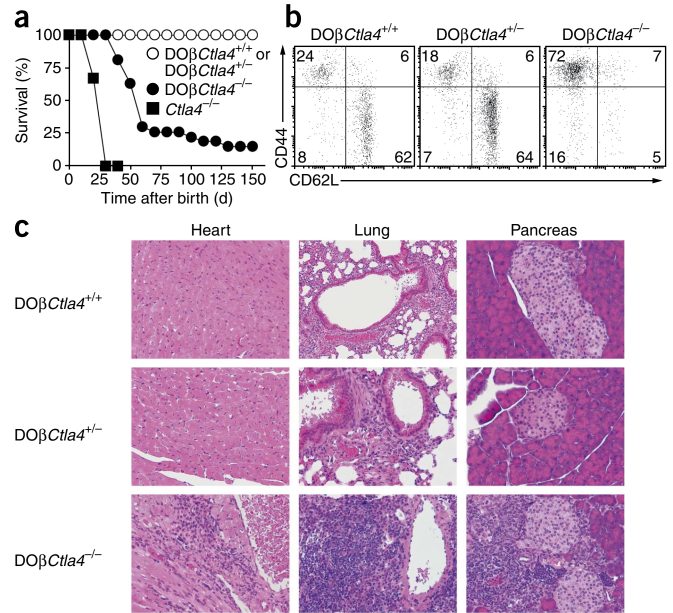

Figure 1 DOβ*Ctla4*$^{-/-}$ mice show spontaneous T cell activation and multiorgan infiltration. (**a**) Survival of DOβ*Ctla4*$^{+/+}$ mice ($n > 30$), DOβ*Ctla4*$^{+/-}$ mice ($n = 30$), DOβ*Ctla4*$^{-/-}$ mice ($n = 27$) and *Ctla4*$^{-/-}$ mice ($n = 9$). Data are representative of two experiments. (**b**) Expression of CD62L and CD44 on splenic CD4$^+$ T cells from 6-week-old DOβ*Ctla4*$^{+/+}$, DOβ*Ctla4*$^{+/-}$ and DOβ*Ctla4*$^{-/-}$ mice. Numbers in quadrants indicate percent cells in each. Data are representative of two experiments with five mice per group. (**c**) Hematoxylin and eosin–stained sections of heart, lungs and pancreas from 12-week-old DOβ*Ctla4*$^{+/+}$, DOβ*Ctla4*$^{+/-}$ and DOβ*Ctla4*$^{-/-}$ mice. Original magnification, ×200. Data are representative of two experiments with three mice per group.

To address those issues, we used an approach similar to that used in analyzing the TCR repertoire of T_{reg} cells[23,24]. By crossing the DO11.10 TCRβ transgene[25] onto the *Ctla4*$^{-/-}$ genetic background to restrict TCRβ specificity, we found that the lethal multiorgan lymphoproliferative disorder and expansion of CD4$^+$ T cell populations in *Ctla4*$^{-/-}$ mice was maintained, but the mice showed a slightly lower rate of death. By analyzing the antigen specificity of the proliferating CD4$^+$ T cells in *Ctla4*$^{-/-}$ mice, we found that *Ctla4*$^{-/-}$ T cells infiltrating peripheral nonlymphoid tissues were composed of separate populations of different tissue-specific T cells. We identified one autoantigen recognized by these tissue-infiltrating *Ctla4*$^{-/-}$ T cells, isolated a specific TCRαβ reactive to this antigen and examined the *in vivo* activity of T cells with this TCR specificity. Our results show that CTLA-4 expressed on the antigen-specific effector T cells greatly diminished their pathogenicity *in vivo* but that CTLA-4 expression by T_{reg} cells was sufficient to control the accumulation of self-reactive, tissue-specific effector T cells in target tissues. Together our findings show both cell-intrinsic and non–cell-autonomous actions of CTLA-4.

RESULTS

Disease in *Ctla4*$^{-/-}$ mice with a fixed TCRβ repertoire

We compared *Ctla4*$^{-/-}$ mice with *Ctla4*$^{-/-}$ mice expressing the DO11.10 TCRβ chain (DOβ*Ctla4*$^{-/-}$ mice; **Fig. 1a**). *Ctla4*$^{-/-}$ mice uniformly died by 4 weeks of age, whereas DOβ*Ctla4*$^{-/-}$ mice had a slower rate of death, with 75% of mice dying by 7 weeks of age, and 10% of mice still surviving by 10 weeks (**Fig. 1a**). Thus, fixing the TCRβ chain prolonged but did not eliminate the disease in *Ctla4*$^{-/-}$ mice. This slower disease course allowed us to better examine the characteristics and tissue specificity of CD4$^+$ T cells. CD4$^+$ T cells in DOβ*Ctla4*$^{-/-}$ mice had an activated surface phenotype (**Fig. 1b**), in agreement with published reports of activation of CD4$^+$ T cells in *Ctla4*$^{-/-}$ mice[2–4]. Specifically, although DOβ*Ctla4*$^{+/-}$ and DOβ*Ctla4*$^{+/+}$ mice had a normal distribution of naive and memory T cells, with 60% of CD4$^+$ splenic T cells having a naive surface phenotype, DOβ*Ctla4*$^{-/-}$ mice had a much lower percentage of naive T cells, with 70% of T cells having an activated-memory phenotype (**Fig. 1b**). Finally, fixation of the TCRβ chain in *Ctla4*$^{-/-}$ mice did not alter the multiorgan nature of disease in *Ctla4*$^{-/-}$ mice, as DOβ*Ctla4*$^{-/-}$ mice had lymphocytic infiltration in the heart, lungs and pancreas, whereas DOβ*Ctla4*$^{+/-}$ mice had normal tissue histology (**Fig. 1c**).

Tissue-specific accumulation of *Ctla4*$^{-/-}$ T cells

So far, tissue-infiltrating T cells from *Ctla4*$^{-/-}$ mice have not been cloned, nor has their antigen specificity been characterized[26,27]. We used DOβ*Ctla4*$^{-/-}$ mice as donors of T cells isolated from various tissues and first set out to examine their pattern of migration and population expansion in recipient mice deficient in recombination-activating gene 2 (*Rag2*$^{-/-}$ mice). Splenic CD4$^+$ T cell populations isolated from DOβ*Ctla4*$^{-/-}$ mice, but not those from DOβ*Ctla4*$^{+/+}$ or DOβ*Ctla4*$^{+/-}$ mice, showed robust expansion

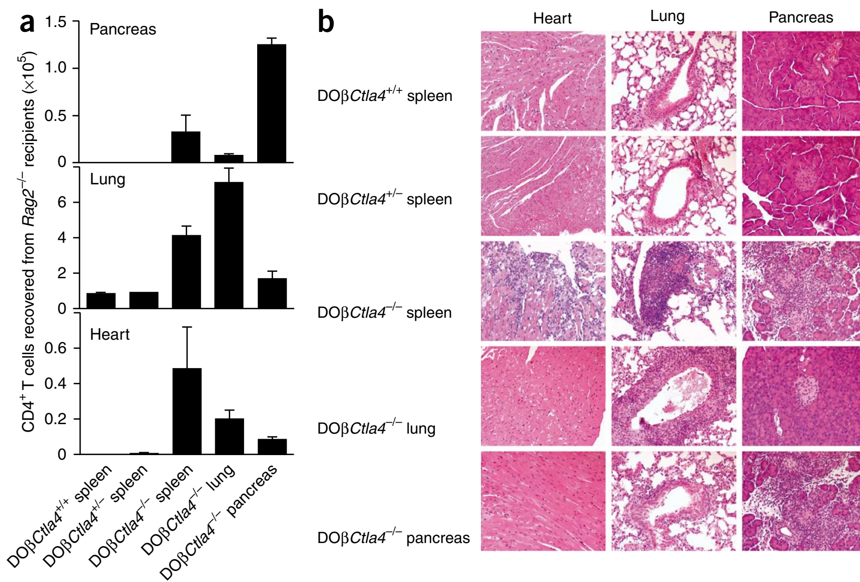

Figure 2 Tissue-infiltrating T cells from DOβ*Ctla4*$^{-/-}$ mice cause tissue-specific inflammation. Analysis of pancreas, lungs and heart removed from *Rag2*$^{-/-}$ mice 3 weeks after transfer of CD4$^+$ T cells (5×10^5 cells) purified from the spleens of DOβ*Ctla4*$^{+/+}$ or DOβ*Ctla4*$^{+/-}$ mice or the spleen, lungs or pancreas of DOβ*Ctla4*$^{-/-}$ mice. (**a**) Recovery of CD4$^+$ T cells from pancreas, lungs and heart of *Rag2*$^{-/-}$ recipient mice. (**b**) Hematoxylin and eosin–stained sections of the pancreas, lungs and heart of *Rag2*$^{-/-}$ recipients. Original magnification, ×100. Data are representative of two independent experiments (mean + s.d. of three mice in **a**).

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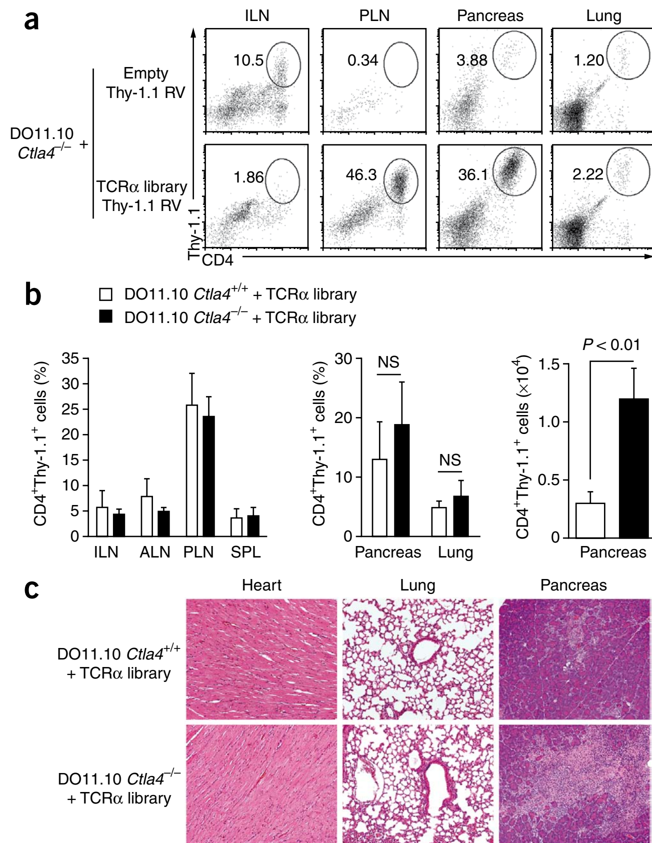

Figure 3 TCRs derived from pancreas-infiltrating T cells confer selective pancreatic accumulation. Analysis of lymphoid and nonlymphoid tissues obtained from *Rag2*$^{-/-}$ mice 3 weeks after transfer of infected CD4$^+$ T cells (1 × 10^6 cells) from DO11.10 *Rag2*$^{-/-}$*Ctla4*$^{+/+}$ or DO11.10 *Rag2*$^{-/-}$*Ctla4*$^{-/-}$ mice infected with empty Thy-1.1$^+$ retrovirus (RV) or the TCRα library Thy-1.1$^+$ retrovirus generated from TCRα cDNA derived from CD4$^+$ T cells infiltrating the pancreas of DOβ *Ctla4*$^{-/-}$ mice. (**a**) Frequency of CD4$^+$Thy-1.1$^+$ cells (numbers adjacent to outlined areas) in inguinal lymph nodes (ILN), pancreatic lymph nodes (PLN), pancreas and lungs of *Rag2*$^{-/-}$ recipients. (**b**) Frequency and number of CD4$^+$Thy-1.1$^+$ cells in inguinal lymph nodes, axillary lymph nodes (ALN), pancreatic lymph nodes, spleen (SPL), pancreas and lungs of *Rag2*$^{-/-}$ recipients. *P* values, Student's *t*-test; NS, not significant. (**c**) Hematoxylin and eosin–stained sections of heart, lungs and pancreas from *Rag2*$^{-/-}$ recipients. Original magnification, ×100. Data represent two independent experiments with three mice per group (mean and s.d. in **b**).

in vivo and migrated into many organs, including the pancreas, lungs and heart (**Fig. 2a**). In contrast, T cells isolated from peripheral organs of DOβ*Ctla4*$^{-/-}$ mice accumulated selectively in their organ of origin (**Fig. 2a**). For example, CD4$^+$ T cells originally isolated from the lungs of DOβ*Ctla4*$^{-/-}$ mice showed a greater accumulation in the lungs than in the pancreas or heart of recipient *Rag2*$^{-/-}$ mice. Notably, CD4$^+$ T cells isolated from the pancreas of DOβ*Ctla4*$^{-/-}$ mice accumulated selectively in the pancreas of recipient *Rag2*$^{-/-}$ mice and not the lungs or heart of recipient *Rag2*$^{-/-}$ mice.

Furthermore, the selective migration of CD4$^+$ T cells isolated from DOβ*Ctla4*$^{-/-}$ mice was associated histologically with the induction of tissue pathology (**Fig. 2b**). Specifically, CD4$^+$ T cells isolated from the spleen of DOβ*Ctla4*$^{-/-}$ mice caused an intense tissue-destructive infiltration in the pancreas, heart and lungs of *Rag2*$^{-/-}$ recipients. CD4$^+$ T cells isolated from the lungs of DOβ*Ctla4*$^{-/-}$ mice elicited a strong peribronchial infiltration with perivascular infiltration and associated epithelial changes in the lungs of *Rag2*$^{-/-}$ recipients (**Fig. 2b**) but caused no changes in the pancreas or heart. Finally, CD4$^+$ T cells isolated from the pancreas of DOβ*Ctla4*$^{-/-}$ mice caused tissue-destructive lesions of the exocrine pancreas in *Rag2*$^{-/-}$ recipients but selectively spared the pancreatic islets, lungs and heart. Tissue lesions caused by the transfer of DOβ*Ctla4*$^{-/-}$ T cells were histologically similar to those caused by *Ctla4*$^{-/-}$ T cells (**Supplementary Fig. 1**), which suggested that the tissue-specific accumulation of DOβ*Ctla4*$^{-/-}$ T cells was not an artifact introduced by transgenic DO11.10 TCRβ chain.

TCR specificity determines tissue-specific accumulation

The tissue-specific accumulation of DOβ*Ctla4*$^{-/-}$ T cells could have been due to either reactivity to tissue-specific antigens or to selective homing properties 'imprinted' after tissue entry, for example, by selective expression of chemokine receptors. To distinguish between those possibilities, we made a TCRα retroviral library from tissue-infiltrating CD4$^+$ T cells of DOβ*Ctla4*$^{-/-}$ mice and tested the migration of T cells expressing TCRs isolated from tissues. We cloned TCRα cDNA made from pancreas-infiltrating T cells of DOβ*Ctla4*$^{-/-}$ mice into a retroviral vector expressing Thy-1.1 as a marker to produce a TCRα library (**Supplementary Fig. 2**). We infected naive *Rag2*$^{-/-}$ DO11.10 T cells expressing or lacking CTLA-4 with the complete TCRα library or with empty retrovirus (**Supplementary Fig. 3**) and adoptively transferred the cells into *Rag2*$^{-/-}$ mice. Thy-1.1$^+$ T cells expressing the TCRα library, but not those expressing the empty retrovirus, showed selective accumulation in pancreatic lymph nodes and pancreas but not in inguinal lymph nodes or lungs of recipient mice (**Fig. 3a**). As pancreas-selective infiltration could reflect either selective entry or selective proliferation *in situ*, we analyzed the kinetics and proliferation of tissue-infiltrating T cells (**Supplementary Fig. 4**). T cells expressing the pancreas-derived TCRα library showed similar early entry into the lungs and pancreas at day 7 after adoptive transfer, although T cells were proliferating more in the pancreas even at this time (**Supplementary Fig. 4a–c**). At later times, this greater accumulation and proliferation in the pancreas than in the lungs persisted and was statistically significant. Analysis by adoptive transfer of cells isolated directly from nonlymphoid tissues of *Ctla4*$^{-/-}$ mice was precluded by the fact that such a large percentage of these cells proliferated immediately after isolation and before adoptive transfer (**Supplementary Fig. 4d,e**). The percentage of TCRα library–expressing T cells that accumulated in pancreatic lymph nodes and pancreas was unaffected by the presence or absence of CTLA-4 (**Fig. 3b**, left and middle). However, we detected significantly more library-derived *Ctla4*$^{-/-}$ cells than library-derived *Ctla4*$^{+/+}$ T cells in the pancreatic tissue (**Fig. 3b**, right). Also, library-derived *Ctla4*$^{+/+}$ T cells caused minimal pancreatic disease, whereas library-derived *Ctla4*$^{-/-}$ T cells caused exocrine-specific tissue destruction (**Fig. 3c**). In summary, TCRα chains derived from pancreas-infiltrating T cells of *Ctla4*$^{-/-}$ mice are sufficient to confer selective accumulation of *Ctla4*$^{-/-}$ T cells in the pancreas as a result of tissue-specific *Ctla4*$^{-/-}$ T cell proliferation. Furthermore, these antigen-specific T cells cause tissue injury in the absence of CTLA-4.

PDIA2 is an autoantigen recognized by *Ctla4*$^{-/-}$ T cells

Ctla4$^{-/-}$ mice and DOβ*Ctla4*$^{-/-}$ mice showed intense lymphocytic infiltration of the exocrine pancreas that largely spared the pancreatic islets (**Fig. 1c** and data not shown). When monitored for development of autoimmune diabetes, DOβ*Ctla4*$^{-/-}$ mice had significantly lower blood glucose concentrations than did DOβ*Ctla4*$^{+/-}$ mice ($P < 0.05$; **Supplementary Fig. 5a**). In addition, when we transferred

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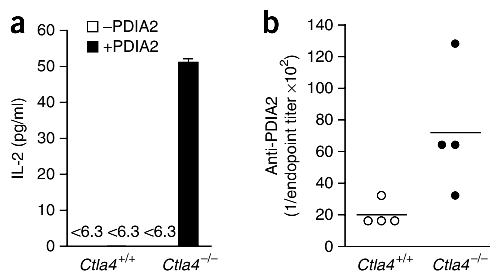

Figure 4 PDIA2 is an autoantigen in *Ctla4*$^{-/-}$ mice. (**a**) Enzyme-linked immunosorbent assay of IL-2 concentrations in supernatants of CD4$^+$ T cells (1×10^5 cells) purified from pancreatic lymph nodes of 20-day-old *Ctla4*$^{+/+}$ or *Ctla4*$^{-/-}$ mice, then cultured for 24 h with (+) or without (–) 10 μM PDIA2 in the presence of irradiated splenocytes (5×10^5 cells). Numbers above left and middle bars indicate IL-2 concentration. Data represent three independent experiments (error bar, s.d.). (**b**) Enzyme-linked immunosorbent assay of anti-PDIA2 titers in serum from 20-day-old *Ctla4*$^{+/+}$ mice ($n = 4$) or *Ctla4*$^{-/-}$ mice ($n = 4$). Each symbol represents an individual mouse; small horizontal lines indicate the mean. Data are representative of two experiments.

CD4$^+$ T cells isolated from pancreas of DOβ*Ctla4*$^{-/-}$ mice into *Rag2*$^{-/-}$ mice, we also observed a significant decrease in blood glucose concentrations over time relative to that of mice that received splenic CD4$^+$ T cells isolated from DOβ*Ctla4*$^{+/-}$ mice (**Supplementary Fig. 5b**). These results suggested that *Ctla4*$^{-/-}$ T cells may react to a pancreas-specific self antigen selectively expressed in acinar tissue.

Published studies have suggested a candidate for such a self antigen[28]. Nonobese diabetic mice develop immune-mediated destruction of beta cells of pancreatic islets, but nonobese diabetic mice deficient in the autoimmune regulator Aire show immune cell reactivity to pancreatic acinar cells and make autoantibodies specific for PDIA2 (protein disulfide isomerase–associated 2), an acinar-specific enzyme[28]. As DOβ*Ctla4*$^{-/-}$ mice showed acinar tissue–restricted autoimmunity, we sought to determine if PDIA2 is an autoantigen in *Ctla4*$^{-/-}$ mice. We isolated T cells from the pancreatic lymph nodes of *Ctla4*$^{+/+}$ or *Ctla4*$^{-/-}$ mice and activated the cells *in vitro* in the presence or absence of PDIA2 protein. T cells from *Ctla4*$^{-/-}$ mice, but not those from *Ctla4*$^{+/+}$ mice, produced a substantial amount of IL-2 in response to PDIA2 (**Fig. 4a**). Furthermore, *Ctla4*$^{-/-}$ mice had antibodies reactive to PDIA2 in the serum, but *Ctla4*$^{+/+}$ mice did not (**Fig. 4b**). Therefore, PDIA2 seems to be an authentic autoantigen in *Ctla4*$^{-/-}$ mice. We tested the reactivity of *Ctla4*$^{-/-}$ T cells to other proteins, such as carbonic anhydrase II, α-amylase, lactoferrin and carboxypeptidase B, which have been suggested to be associated with autoimmune pancreatitis[29,30]. However, none of these proteins elicited detectable secretion of IL-2 from *Ctla4*$^{-/-}$ T cells (**Supplementary Fig. 6**).

PDIA2-specific TCRs from pancreas-infiltrating *Ctla4*$^{-/-}$ T cells

To examine how CTLA-4 regulates autoreactive T cells *in vivo*, we sought to isolate an authentic autoantigen-specific TCR from *Ctla4*$^{-/-}$ T cells. We modified a T cell hybridoma (58α$^-$β$^-$) to express the DO11.10 TCRβ chain along with a green fluorescent protein (GFP) reporter inducible by the transcription factor NFAT. We isolated pancreas-infiltrating CD4$^+$ T cells from DOβ*Ctla4*$^{-/-}$ mice and generated full-length TCRα cDNA, cloned it into a modified retroviral vector and expressed it in this hybridoma (**Supplementary Fig. 7**). We then examined CD3$^+$ hybridoma cells for TCR reactivity to various antigens by assaying for the induction of GFP. As a control, when the DO11.10 TCRα (DOα) was expressed, hybridoma cells reacted robustly to both to anti-CD3 stimulation and to ovalbumin (OVA) peptide; hybridomas expressing the TCRα library responded to anti-CD3 but not OVA (**Fig. 5a**).

First we assessed whether hybridomas expressing the TCRα library had TCRs that reacted to defined antigens from the endocrine pancreas. We found no responses to pancreatic beta cells, insulin, insulin peptide 9–23 or purified glucagon (**Fig. 5b**), consistent with the lack of diabetes in DOβ*Ctla4*$^{-/-}$ mice. Hyridomas also did not respond to the exocrine proteins carbonic anhydrase II, α-amylase, lactoferrin or carboxypeptidase B (**Supplementary Fig. 6**). However, approximately 2% of the hybridomas expressing the TCRα library reacted to PDIA2 (**Fig. 5b**). We isolated these hybridoma populations by sorting GFP$^+$ cells after stimulation with PDIA2 and noted enriched PDIA2 reactivity in sorted populations (**Fig. 5c**). In contrast, we observed no enrichment of insulin reactivity in sorted GFP$^+$ cells stimulated with insulin. The use of PCR in generating the TCRα library and the

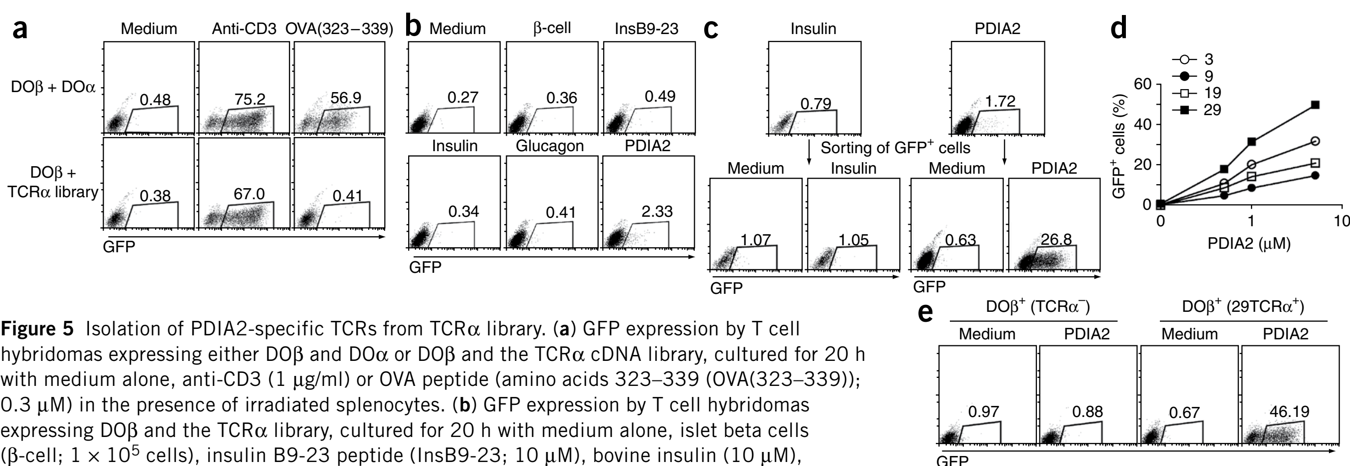

Figure 5 Isolation of PDIA2-specific TCRs from TCRα library. (**a**) GFP expression by T cell hybridomas expressing either DOβ and DOα or DOβ and the TCRα cDNA library, cultured for 20 h with medium alone, anti-CD3 (1 μg/ml) or OVA peptide (amino acids 323–339 (OVA(323–339)); 0.3 μM) in the presence of irradiated splenocytes. (**b**) GFP expression by T cell hybridomas expressing DOβ and the TCRα library, cultured for 20 h with medium alone, islet beta cells (β-cell; 1×10^5 cells), insulin B9-23 peptide (InsB9-23; 10 μM), bovine insulin (10 μM), porcine glucagon (10 μM) or recombinant PDIA2 (10 μM) in the presence of irradiated splenocytes. (**c**) GFP expression by sorted GFP$^+$ cells from T cell hybridomas expressing DOβ and the TCRα library, cultured with PDIA2 or insulin (10 μM) in the presence of irradiated splenocytes; GFP$^+$ cells were sorted after 20 h and, 6 d later, sorted cells were restimulated for 20 h with PDIA2 or insulin. (**d**) PDIA2-specific GFP expression by PDIA2-specific hybridoma clones generated by limiting dilution ($n = 4$ clones; key indicates clone number). (**e**) GFP expression by DOβ$^+$ cells or DOβ$^+$29TCRα$^+$ cells (CD3$^+$ cells sorted from NFAT-GFP–expressing DOβ$^+$ cells infected with a 29TCRα-encoding retrovirus) cultured for 20 h with medium alone or 5 μM PDIA2. Numbers above outlined areas (**a**–**c**,**e**) indicate percent GFP$^+$ cells. Data are representative of three (**a**,**b**) or two (**c**–**e**), experiments.

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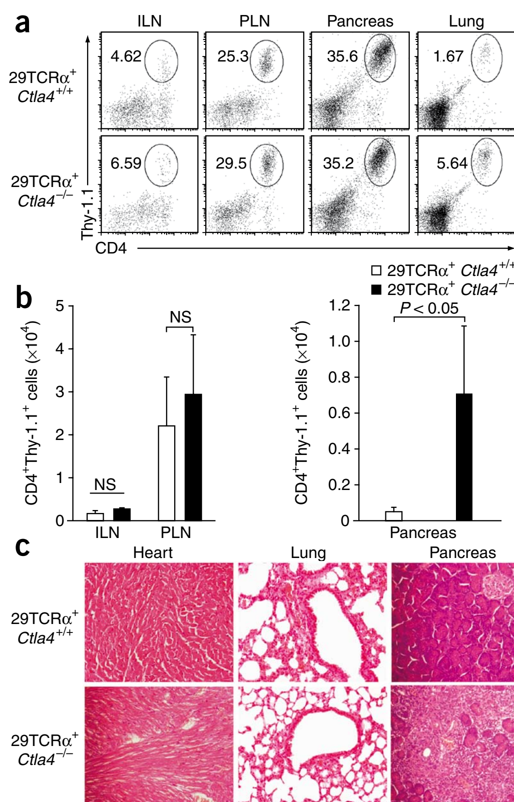

Figure 6 PDIA2-specific $Ctla4^{-/-}$ T cells infiltrate the pancreas. Analysis of inguinal lymph nodes, pancreatic lymph nodes, pancreas and lungs of $Rag2^{-/-}$ recipient mice 3 weeks after transfer of 1×10^6 $CD4^+$ T cells from DO11.10 $Rag2^{-/-}Ctla4^{+/+}$ or DO11.10 $Rag2^{-/-}Ctla4^{-/-}$ mice infected with a 29TCRα Thy-1.1^+ retrovirus (29TCRα$^+$ $Ctla4^{+/+}$ or 29TCRα$^+$ $Ctla4^{-/-}$). (**a**) Frequency of $CD4^+$Thy-1.1^+ cells (numbers adjacent to outlined areas) in inguinal lymph nodes, pancreatic lymph nodes, pancreas and lungs. (**b**) $CD4^+$Thy-1.1^+ cells in inguinal lymph nodes, pancreatic lymph nodes and pancreas (mean + s.d. of three mice). *P* values, Student's *t*-test. (**c**) Hematoxylin and eosin–stained sections of heart, lung and pancreas. Original magnification, ×100. Results are representative of two independent experiments with three mice per group.

limited dynamic range of the hybridoma assay could have introduced a bias in the representation of antigen-reactive T cells identified in this screen. Nevertheless, PDIA-2 was the only autoantigen identified so far by this approach. When we cloned these enriched PDIA2-reactive hybridomas cells by limiting dilution, they continued to show PDIA2 reactivity *in vitro* (**Fig. 5d**).

We used one such hybridoma, clone 29, as a source of a PDIA2-reactive TCRα chain (29TCRα; **Supplementary Fig. 8**). Expression of the 29TCRα chain in the DOβ$^+$ hybridomas regenerated PDIA2-specific reactivity (**Fig. 5e**), which confirmed that this combination of TCRβ and TCRα chains generates a TCR specific for an autoantigen in $Ctla4^{-/-}$ mice. Expression of the 29TCRα chain in naive T cells also conferred PDIA2-specific reactivity (**Supplementary Fig. 9**). We transduced empty Thy-1.1^+ retrovirus or 29TCRα-expressing Thy-1.1^+ retrovirus into $Ctla4^{+/+}$ or $Ctla4^{-/-}$ $Rag2^{-/-}$ DO11.10 transgenic T cells. Expression of the 29TCRα chain endowed T cells with the ability to undergo proliferation in response to PDIA2, but expression of the empty vector did not. In contrast, empty vector and 29TCRα retrovirus left the original TCR reactivity to OVA peptide intact.

PDIA2-specific *Ctla4*$^{-/-}$ T cells infiltrate the pancreas

Expression of the 29TCRα chain in naive DO11.10 $Rag2^{-/-}$ T cells conferred pancreatic accumulation. Specifically, when the 29TCRα chain was expressed in DO11.10 $Rag2^{-/-}$ T cells adoptively transferred into $Rag2^{-/-}$ recipients, we detected substantially greater accumulation of Thy-1.1^+ cells in the pancreatic lymph nodes and pancreas but not in the inguinal lymph nodes, lungs, spleen or axillary lymph nodes (**Fig. 6a** and data not shown). Notably, $Ctla4^{+/+}$ and $Ctla4^{-/-}$ 29TCRα T cells accumulated in equal numbers in pancreatic lymph nodes (**Fig. 6b**), but infiltration of the pancreas itself was greatly affected by the presence of CTLA-4. Approximately tenfold more T cells infiltrated the pancreas when the 29TCRα chain was expressed in $Ctla4^{-/-}$ T cells than when it was expressed in $Ctla4^{+/+}$ T cells (**Fig. 6b**). Again, the pancreatic infiltration was exocrine specific and was not present in the heart or lungs (**Fig. 6c**). Notably, $Ctla4^{+/+}$ T cells expressing 29TCRα did not express the transcription factor Foxp3 in $Rag2^{-/-}$ recipients (data not shown). In addition, 29TCRα$^+$ T cells did not develop into $CD4^+CD25^+$ cells in bone marrow chimeras regardless of CTLA-4 expression (**Supplementary Fig. 10a**). In these bone marrow chimeras, $Ctla4^{+/+}$ 29TCRα$^+$ T cells caused minimal pancreatitis, whereas $Ctla4^{-/-}$ 29TCRα$^+$ T cells induced severe pancreatic tissue destruction (**Supplementary Fig. 10b**). Together these results suggest that CTLA-4 on autoantigen-specific effector T cells diminishes pathogenicity by inhibiting their infiltration into target tissues.

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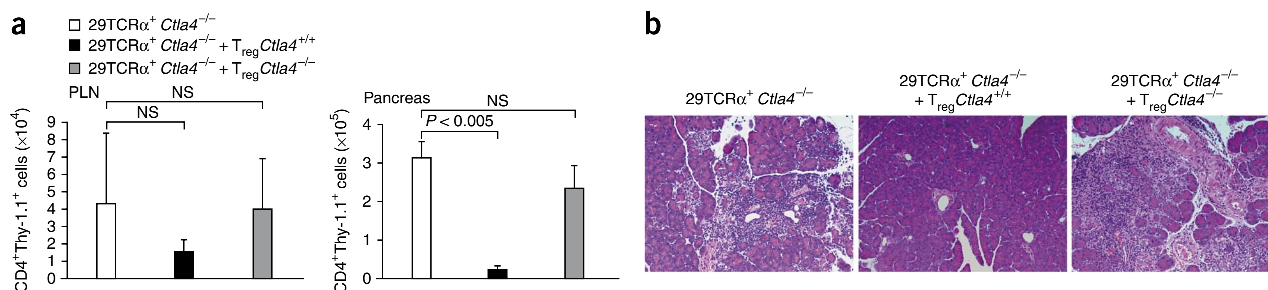

Figure 7 CTLA-4-sufficient T_{reg} cells inhibit pancreatitis induced by $Ctla4^{-/-}$ PDIA2-specific T cells. Analysis of pancreatic lymph nodes and pancreas from $Rag2^{-/-}$recipient mice 3 weeks after transfer of $CD4^+$ T cells (0.15×10^6) from DO11.10 $Rag2^{-/-}Ctla4^{-/-}$ mice infected with 29TCRα Thy-1.1^+ retrovirus; infected cells (29TCRα $^+$ $Ctla4^{-/-}$) were transferred with or without 0.15×10^6 $CD4^+CD62L^{hi}CD25^+$ cells from $Ctla4^{+/+}$ or $Ctla4^{-/-}$ mice (T_{reg} $Ctla4^{+/+}$ or T_{reg} $Ctla4^{-/-}$, respectively). (**a**) $CD4^+$Thy-1.1^+ cells in pancreatic lymph nodes and pancreas (mean + s.d. of three mice). *P* values, Student's *t*-test. (**b**) Hematoxylin and eosin–stained sections of pancreas from $Rag2^{-/-}$ recipient mice. Original magnification, ×100. Results represent two independent experiments with three mice per group.

T_{reg} cells control *Ctla4*$^{-/-}$ PDIA2-specific T cells

Two studies have suggested that CTLA-4 expression by T_{reg} cells is required for their suppressive activity[16,31]. To test if this is true for self antigen–specific T cells, we transferred *Ctla4*$^{-/-}$ 29TCRα$^+$ DO11.10 cells into *Rag2*$^{-/-}$ mice with or without cotransfer of $CD4^+CD62L^{hi}CD25^+$ T_{reg} cells isolated from *Ctla4*$^{+/+}$ or *Ctla4*$^{-/-}$ mice and measured the accumulation of PDIA2-specific T cells in the pancreatic lymph nodes and pancreatic tissue. Cotransfer of *Ctla4*$^{+/+}$ T_{reg} cells did not alter the number of PDIA2-specific T cells that accumulated in pancreatic lymph nodes (**Fig. 7a**). However, cotransfer of *Ctla4*$^{+/+}$ T_{reg} cells resulted in the infiltration of significantly fewer PDIA2-specific T cells into the pancreas (**Fig. 7a**) and prevented the destruction of pancreatic tissue by *Ctla4*$^{-/-}$ PDIA2-specific T cells (**Fig. 7b**). In contrast, cotransferred *Ctla4*$^{-/-}$ T_{reg} cells failed to inhibit the infiltration of PDIA2-specific T cells into the pancreas and destruction of pancreatic tissue (**Fig. 7b**). We confirmed that result in mixed–bone marrow chimeras in which CTLA-4-expressing cells derived from wild-type BALB/c mice controlled pancreatic tissue destruction by *Ctla4*$^{-/-}$ PDIA2-specific T cells (**Supplementary Fig. 10**). These results demonstrate that autoimmune responses by tissue-specific *Ctla4*$^{-/-}$ T cells can be regulated by CTLA-4-expressing T_{reg} cells.

DISCUSSION

At least two questions regarding CTLA-4 have remained unanswered. What is the specificity of $CD4^+$ T cells that proliferate and infiltrate the nonlymphoid organs in *Ctla4*$^{-/-}$ mice? And on what cells does CTLA-4 exert its inhibitory effects? Specifically, it has been unclear whether $CD4^+$ T cells in *Ctla4*$^{-/-}$ mice are reactive to self major histocompatibility complex proteins, to ubiquitous antigens presented by major histocompatibility complex or to particular tissue-specific antigens. Although some evidence has suggested a cell-autonomous action for the CTLA-4 cytoplasmic domain[32,33], other work has supported the idea of a non–cell-autonomous effect of CTLA-4 on cells expressing CTLA-4 ligands[34,35]. Resolving these distinctions is important, as CTLA-4 polymorphisms have been associated with autoimmune diseases[36] and CTLA-4 blockade is an emerging cancer immunotherapy[22].

Some evidence has suggested that proliferating *Ctla4*$^{-/-}$ T cells are not antigen specific[20]. TCR spectratyping of peripheral T cells from *Ctla4*$^{-/-}$ mice and mice deficient in the kinase Jak3 has indicated that Jak3-deficient T cells have a restricted and highly biased TCR repertoire but *Ctla4*$^{-/-}$ T cells have a diverse and unbiased TCR repertoire[20]. This was interpreted to suggest that *Ctla4*$^{-/-}$ T cells are activated by TCR–major histocompatibility complex interactions involved in the survival and/or homeostasis of peripheral T cells[37,38] but not necessarily by tissue-specific antigens. However, another study has identified restricted TCR spectratypes in splenic *Ctla4*$^{-/-}$ T cells[39], which suggests that particular combinations of variable and joining segments analyzed previously were not entirely representative of the *Ctla4*$^{-/-}$ TCR repertoire. Neither study examined *Ctla4*$^{-/-}$ T cells from tissues other than the spleen[20,39]. Therefore, both studies may have missed effects on TCR repertoire bias seen in tissue-infiltrating T cells.

Introducing the DOβ chain onto the *Ctla4*$^{-/-}$ background did not eliminate the lethal multiorgan infiltration of $CD4^+$ T cells, but it did slow disease enough to allow us to examine the specificity of tissue-infiltrating T cells. Unexpectedly, we found that T cells from particular tissues, such as the pancreas, showed selective accumulation in their tissue of origin after adoptive transfer into *Rag2*$^{-/-}$ hosts. Selective accumulation alone does not indicate reactivity to tissue-specific antigens, as these T cells could have acquired expression of chemokine receptors (or other receptors) that mediate such activity. However, a TCRα retroviral library made from pancreas-infiltrating *Ctla4*$^{-/-}$ T cells, when expressed in naive DOβ T cells, also conferred pancreas-specific accumulation, which suggested that the accumulation of *Ctla4*$^{-/-}$ T cells was TCR dependent and antigen specific. These observations are the first to our knowledge to show that tissue-infiltrating $CD4^+$ T cells in *Ctla4*$^{-/-}$ mice are reactive to tissue-specific antigens.

We identified PDIA2 as an autoantigen of *Ctla4*$^{-/-}$ mice. PDIA2 was originally discovered as an autoantigen recognized by serum from *Aire*$^{-/-}$ nonobese diabetic mice[28], which develop exocrine pancreas-specific inflammation that spares islets and does not induce diabetes, similar to our findings in DOβ*Ctla4*$^{-/-}$ mice. How Aire might regulate the emergence of PDIA2-reactive clones is unclear, as PDIA2 expression in thymic epithelial cells is not lower in *Aire*$^{-/-}$ nonobese diabetic mice[28]. Aire might act to eliminate PDIA2-reactive clones in the thymus by influencing the processing and/or presentation of PDIA2, rather than by influencing *Pdia2* transcription[40]. As thymic selection is normal in *Ctla4*$^{-/-}$ mice[4], CTLA-4 might be needed to regulate PDIA2-specific T cell clones that escaped thymic negative selection.

Experiments with OVA-reactive T cells and OVA-transgenic mice[41] have suggested the possibility of T_{reg} cell–dependent and T_{reg} cell–independent actions of CTLA-4. In contrast, our studies have examined the *in vivo* activity of self-reactive T cells that recognize an endogenous self antigen in the setting of CTLA-4 deficiency. By cloning endogenous PDIA2-specific TCRs from pancreas-infiltrating T cells in *Ctla4*$^{-/-}$ mice, we found that CTLA-4 expression on PDIA2-specific T cells limited their *in vivo* pathogenicity by two mechanisms. In the absence of T_{reg} cells, PDIA2-reactive T effector cells specifically accumulated in pancreatic lymph nodes regardless of CTLA-4 expression but infiltrated into the pancreas only in the absence of CTLA-4. This finding demonstrates a cell-autonomous action of CTLA-4, as CTLA-4 expression by the effector T cell was able to limit tissue injury in the absence of T_{reg} cells. However, in the presence of T_{reg} cells, *Ctla4*$^{-/-}$ PDIA2-specific T cells were also prevented from infiltrating the pancreas. This result demonstrates cell-extrinsic regulation of *Ctla4*$^{-/-}$ T cells by T_{reg} cells, as suggested by published studies[12,39].

Although *Ctla4*$^{-/-}$ mice have more $Foxp3^+$ cells[31], these T_{reg} cells are reportedly nonfunctional *in vivo*[31]. Consistent with that finding, conditional deletion of CTLA-4 in T_{reg} cells causes fatal systemic lymphproliferation[16]. However, those findings do not rule out the possibility of a cell-intrinsic action of CTLA-4 on effector cells. Indeed, CTLA-4 regulates effector T cell activation independently of T_{reg} cells, as CTLA-4 expression in effector cells in mice with T_{reg} cell–specific CTLA-4 deficiency can substantially delay the death of *Ctla4*$^{-/-}$ mice[16]. A study of a tumor model has also demonstrated that CTLA-4 blockade not only on T_{reg} cells but also effector T cells is required for maximum antitumor activity[42]. Our results add to that collection of findings, as CTLA-4 expression on PDIA2-specific T cells was sufficient to prevent tissue infiltration in the absence of T_{reg} cells. In summary, we have shown that *Ctla4*$^{-/-}$ T cells infiltrating peripheral nonlymphoid tissues are reactive to tissue-specific self antigens. By examining the activity of an authentic self-reactive T cell in the presence and absence of CTLA-4, and in the presence and absence of T_{reg} cells, we found that CTLA-4 controls the pathogenicity of autoantigen-specific T cells by both cell-autonomous actions in effector T cells and non–cell-autonomous mechanisms in T_{reg} cells.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

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AUTHOR CONTRIBUTIONS

W.I. designed experiments, did research, analyzed and interpreted results and wrote the manuscript; M.K. did TCRα cDNA cloning; K.M.N. and H.M.L. helped generate the TCRα library; A.S. and E.R.U. provided beta cells and contributed to the hybridoma assay; T.L.M. helped sort cells and contributed to the generation of the TCRα library; and K.M.M. directed the study, analyzed and interpreted results and wrote the manuscript.

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ONLINE METHODS

Mice. DO11.10 TCR–transgenic mice[43], DOβ-transgenic mice[25] and *Ctla4*$^{-/-}$ mice[4] have been described. *Ctla4*$^{-/-}$ mice were backcrossed to the BALB/c strain for ten generations. *Rag2*$^{-/-}$ (H-2^d) mice and BALB/c mice were from Taconic. DO11.10 mice were bred onto a *Rag2*$^{-/-}$ background. All animal studies were approved by the Washington University animal study committee.

Antigens. Islet beta cells were purified from BALB/c pancreas as described[44]. Bovine insulin, porcine glucagon, human lactoferrin, human carbonic anhydrase II, porcine α-amylase and porcine carboxypeptidase B were from Sigma. *Pdia2* cDNA was cloned by PCR from BALB/c splenic cDNA, subcloned into the pET28a expression vector (Stratagene) and expressed as a six-histidine fusion protein in *Escherichia coli* strain BL21 (DE3)-RIP (Stratagene). The following primers were used for cloning: 5′ Pdia2-BamHI, 5′-GCCCAGGGATCCATGGACAAGCAG-3′; and 3′ Pdia2-XhoI, 5′-ATTCTC GAGTCCCAATGGCTACAGCTCCTCCT-3′.

T cell preparation and flow cytometry. Heart, lungs and pancreas were minced and then were incubated for 30 min at 37 °C with collagenase B (0.25 mg/ml; Roche) and DNase I (30 U/ml; Sigma). Digested pancreas was further incubated for 10 min at 37 °C with trypsin and EDTA. Lymphocytes were collected by Percoll gradient purification and CD4$^+$ T cell numbers were determined by flow cytometry staining with fluorescein isothiocyanate–conjugated anti–mouse CD4 (RM4-5; Caltag), allophycocyanin-conjugated anti–mouse CD45.2 (104; eBioscience) and phycoerythrin-conjugated anti–mouse Thy-1.1 (HIS51; eBioscience). For Ki67 staining, cells were stained for surface markers, followed by fixation and permeabilization (eBioscience) and the incubation with phycoerythrin-conjugated anti–human Ki67 (B56; BD Bioscience). CD4$^+$ T cells for adoptive transfer were purified with CD4 MACS MicroBeads (Miltenyi Biotec). For T_{reg} cell preparation, CD4$^+$CD62L^{hi}CD25$^+$ T cells were sorted from spleens of BALB/c mice or *Ctla4*$^{-/-}$ mice with a MoFlo (DakoCytomation) by staining with phycoerythrin-conjugated anti–mouse CD25 (7D4; BD Biosciences), fluorescein isothiocyanate–conjugated anti–mouse CD62L (MEL-14; Caltag) and phycoerythrin-indotricarbocyanine–conjugated anti–mouse CD4 (RM4-5; BD Biosciences). Flow cytometry data were collected on a FACSCalibur or FACSCanto II (BD Biosciences) and were analyzed with FlowJo software (TreeStar).

Detection of IL-2. CD4$^+$ T cells (1×10^5 cells) purified from the pancreatic lymph nodes of *Ctla4*$^{+/+}$ or *Ctla4*$^{-/-}$ mice were cultured with or without 10 μM PDIA2 in the presence of irradiated BALB/c splenocytes (5×10^5 cells). Culture supernatants were collected 24 h later and IL-2 concentrations were measured with the Mouse IL-2 OptEIA ELISA set (BD Bioscience).

Determination of PDIA2-specific immunoglobulin titers. Serum was collected from *Ctla4*$^{+/+}$ or *Ctla4*$^{-/-}$ mice and PDIA2-specific antibody responses were measured by enzyme-linked immunosorbent assay against plate-coated PDIA2 with serial dilution of serum and horseradish peroxidase–conjugated goat anti–mouse immunoglobulin secondary antibodies (Southern Biotech). PDIA2-specific antibody titers are presented as the greatest serum dilution that provided an average absorbance exceeding 1.5-fold the average background absorbance at 405 nm.

Histology. Mouse organs were fixed with 10% (vol/vol) buffered formalin and were embedded in paraffin. Sections were stained with hematoxylin and eosin and were examined with a microscope.

TCRα library and retroviral constructs. RNA from pancreas-infiltrating CD4$^+$ T cells in DOβ*Ctla4*$^{-/-}$ mice was isolated with an RNeasy kit (Qiagen), then cDNA was synthesized with the SMART cDNA Library Construction kit (Clontech) and a Cα primer specific for the constant region of the TCRα chain (5′-ATCCAGGTGGGATTGTGAATCAGGGCCAAC-3′). Full-length TCRα cDNA was amplified with a 5′ PCR primer from the SMART cDNA Library Construction kit (Clonetech) and the Sfi-Cα PCR primer (5′-TAG GCCGAGGCGGCCAACCAGACCCCAGACAGC-3′). PCR products were digested with SfiI, were cloned into a murine stem cell virus (MSCV) retrovirus vector with or without a cassette for an internal ribosomal entry site and Thy-1.1 (IRES–Thy-1.1) and were transduced into XL-10 Gold (Stratagene) for a library transcript complexity of 2×10^5. MSCV retrovirus lacking IRES–Thy-1.1 was generated by removal of the IRES–Thy-1.1 cassette from the MSCV IRES–Thy-1.1 retrovirus (a gift from W. Sha) by digestion with EcoRI, followed by blunting with Vent polymerase and religation. The retroviral reporter hCD4-pA-GFP-NFAT-RV (encoding human CD4 (hCD4)) was constructed by replacement of the GFP–IL-2p cassette of retrovirus hCD4-pA-GFP-IL-2p[45] with the GFP-NFAT cassette from pKS-NFAT-GFP[46] (a gift from M. Iwashima). The plasmid DOβ-IRES-mCD4-RV was constructed as follows: first, mouse CD4 (mCD4) was cloned by PCR from splenic cDNA. GFP cDNA in IRES-GFP-RV[47] was replaced with mCD4 to create IRES-mCD4-RV; then, DOβ was excised from DOβ-IRES-GFP-RV[48] and subcloned into IRES-mCD4-RV. DOα-RV was created by PCR cloning of DOα cDNA with DOα-IRES-hCD4-RV[48] as the template and ligation of DOα into MSCV retrovirus lacking IRES–Thy-1.1.

Retrovirus infection. Retroviral vectors were transfected into Phoenix E cells as described[49]. T cell hybridomas were infected with the viral supernatants in the presence of polybrene (2 μg/ml) by spin infection for 30 min at 1,400*g*. CD4$^+$ T cells purified from DO11.10 mice were activated by plates coated with anti-CD3 (145-2C11) and anti-CD28 (37.51; both from Bio X Cell). On days 1 and 2, fresh viral supernatant was added, followed by centrifugation for 90 min at 1,400*g*. in the presence of polybrene (6 μg/ml). Infected CD4$^+$ T cells were collected on day 5 for adoptive transfer.

Bone marrow chimeras. Bone marrow cells were collected from DO11.10 or DO11.10 *Ctla4*$^{-/-}$ mice 2 d after injection of 5-fluorouracil (150 μg per g body weight; Sigma) and were cultured for 2 d in the presence of mouse IL-3 (6 ng/ml), mouse IL-6 (10 ng/ml) and mouse stem cell factor (100 ng/ml; Pepro Tech). Bone marrow cells were then spin-infected for 90 min at 1,400*g*. with viral supernatants in the presence of polybrene (6 μg/ml). Recipient *Rag2*$^{-/-}$ mice received a total of 480 rads of whole-body irradiation and then were injected intravenously with 0.5×10^6 infected bone marrow cells with or without 5×10^6 bone marrow cells freshly isolated from BALB/c mice.

Generation of T cell hybridomas and assay. The 58α$^-$β$^-$ hybridoma cell line[50] was infected with hCD4-pA-GFP-NFAT-RV and then cells positive for human CD4 were sorted by MoFlo 2 d after infection. Cells were next infected with DOβ-IRES-mCD4 retrovirus and cells positive for mouse CD4 were sorted. Finally, cells were infected with a retroviral TCRα library. CD3$^+$ cells were sorted to obtain TCRαβ$^+$ cells. TCR-reconstituted hybridomas (2.5×10^4 cells) were cultured for 20 h withantigens and irradiated splenocytes (5×10^5 cells) in 96-well round-bottomed plates. GFP expression on the hybridomas was analyzed by flow cytometry. Retrovirus-specific inserts were recovered from cDNA of hybridomas by PCR as described[49].

Statistical analysis. The significance of differences in frequency or numbers of T cells between groups was calculated by the Student's *t*-test with the statistical analysis function of Prism 4.03 (GraphPad Software).

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