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Non-overlapping roles of PD-1 and FoxP3 in maintaining immune tolerance in a novel autoimmune pancreatitis mouse model

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Abstract

PD-1, an immune-inhibitory receptor required for immune self-tolerance whose deficiency causes autoimmunity with variable severity and tissue specificity depending on other genetic factors. PD-1 is expressed on activated T cells, including the FoxP3+ Treg cells known to play critical roles in maintaining immune tolerance. Yet, whether PD-1 expression by the Treg cells is required for their immune regulatory function, especially in autoimmune settings is still unclear. We found that mice with partial FoxP3 insufficiency developed early-onset lympho-proliferation and lethal autoimmune pancreatitis only when PD-1 is absent. The autoimmune phenotype was rescued by the transfer of FoxP3-sufficient T cells, regardless of whether they were derived from WT or PD-1-deficient mice, indicating that Treg cells dominantly protect from development of spontaneous autoimmunity without intrinsic expression of PD-1. The absence of PD-1 combined with partial FoxP3 insufficiency however, led to generation of ex-FoxP3 T cells with pro-inflammatory properties and expansion of effector/memory T cells that contributed to the autoimmune destruction of target tissues. Altogether, the results suggest that PD-1 and FoxP3 work collaboratively in maintaining immune tolerance mostly through non-overlapping pathways. Thus, PD-1 is modulating the activation threshold and maintaining the balance between regulatory and effector T cells, while FoxP3 is sufficient for dominant regulation through maintaining the integrity of the Treg function. We suggest that genetic or environmental factors that even moderately affect the expression of both PD-1 and FoxP3 can cause life-threatening autoimmune diseases by disrupting the T cell homeostasis.

Significance statement

We investigated the effect of PD-1 a negative immunoreceptor on the function of FoxP3+ Treg cells. When a moderately down-modulated FoxP3 knockin mouse, which did not show any autoimmunity, were made PD-1 deficient, the mice developed severe pancreatitis and died soon after birth. PD-1 deficiency also induced instability of Treg cell status and increased so-called ex-Treg cells which showed the helper effector activity with self antigen recognition. PD-1 deficient T reg cells, however, did not compromise their function but rather increased their suppressive activity. Thus, PD-1 and Treg cells mutually influence and both are required for protection from autoimmunity.
Introduction

Peripheral immune tolerance is maintained by multiple mechanisms. Activation of T cells for example, is regulated by activation-induced cell death, clonal anergy, negative costimulation and regulatory T cells (Treg)-mediated suppression, among others.

Many regulatory pathways are partially redundant, as in the case of the costimulatory receptor CTLA-4 and the nuclear factor FoxP3, both inhibiting other T cells in a cell-extrinsic fashion. FoxP3 is a hallmark for Treg, driving either transcriptional repression or activation of more than 100 targets (1, 2). FoxP3 directly promotes the expression of CTLA-4, a receptor member of the CD28 family (3). CTLA-4 suppresses the activation of T cells through competitive binding to CD80 and CD86 ligands, thus preventing their interaction with the co-activator receptor CD28 (3, 4). CTLA-4 also plays a critical role in Treg function. Indeed, deficiency of CTLA-4 or FoxP3 in humans causes lethal autoimmunity (5, 6). In mice, genetic ablation of CTLA-4 causes a lympho-proliferative disease that phenocopies FoxP3 deficiency. Moreover, deletion of CTLA-4 specifically on FoxP3+ T cells results in autoimmunity and early death of mice (3). These results support the notion that CTLA-4 and FoxP3 cooperate for the proper function of Tregs and that both molecules need to be co-expressed in T cells to prevent lymphoproliferation (7).

PD-1 is another cell surface receptor belonging to the CD28/CTLA-4 family that inhibits activation of T cells. PD-1 has a typical immune receptor tyrosine based switch motif (ITSM) (8) in its cytoplasmic tail that is phosphorylated upon engagement of PD-1 by its ligands (PD-L1 and PD-L2) (9). The phosphorylated ITSM recruits the tyrosine phosphatase SHP-2, leading to dephosphorylation of the
antigen receptor complex (10). In this way, PD-1 causes the rheostatic inhibition of signaling through TCR, which results in reduced proliferation, cytokine secretion and cytotoxic activities of T cells (11, 12). PD-1 contributes to maintaining immune tolerance, as autoantigen-specific T cells are maintained in an anergic state in vivo for long term in a PD-1 dependent manner (13). Accordingly, the deficiency of PD-1 causes autoimmunity in mice, and PD-1 is a genetic risk factor in humans and mice, albeit with less dramatic outcomes than CTLA-4 or FoxP3 deficiency (14).

FoxP3+ Treg cells constitutively express PD-1 under diverse conditions, in both mice and humans. In fact, Treg cells isolated from mouse germinal centers, as well as from chronically HCV-infected patients or cancer patients express high levels of PD-1 on the surface (15-17). However, the precise role of PD-1 in homeostasis and function of Treg cells is still unclear. Its high expression levels suggest that PD-1 acts directly on FoxP3+ T cells in a way similar to CTLA-4. Supporting this view, PD-1 stimulation inhibits IL-2 production by activated T cells and induces anergy, which may account for the naturally anergic phenotype of Tregs (18). PD-1 may also regulate other aspects of Treg cell biology. Since PD-1 controls the strength of the TCR signal, the absence of PD-1 on FoxP3+ T cells may alter the nature of these cells, their behavior and function. It is accepted that helper T cells possess remarkable plasticity in vivo (19). T helper cells derived from FoxP3+ T cells, generally referred to as ex-FoxP3 cells, can be identified using a complex genetic strategy that allows for the fate mapping of FoxP3 expressing cells in vivo (20). Unlike Treg cells, the ex-FoxP3 cells are rather pathogenic cells with an effector/memory phenotype. Indeed, ex-FoxP3 cells were shown to produce inflammatory cytokines and to contribute to autoimmunity (20).

We aimed to elucidate the role of PD-1 and FoxP3 to immune tolerance. For this purpose, we bred mice deficient for PD-1 (PD-1 KO) with mice carrying the
FoxP3-GFPcre knock-in (KI) locus and Rosa26-tdRFP transgene that can report the history of FoxP3 expression (21). Serendipitously, we observed that the FoxP3-GFPcre reporter strain showed reduced transcription of FoxP3, which led to early death by autoimmune pancreatitis of PD-1 KO mice but not PD-1 sufficient mice. The lethal autoimmunity was prevented by the transfer of FoxP3 sufficient Treg cells regardless of whether cells were sufficient or deficient for PD-1, indicating that PD-1 expression is not required for the suppressive functions of Treg cells. However, deficiency of PD-1 affected the Treg compartment by increasing considerably the generation of ex-FoxP3 cells and contributing to the expansion of effector/memory T cells capable of inducing autoimmunity. Our results indicate that PD-1 regulates the relative abundance of Treg and effector T cells to maintain immune tolerance.
Results

Lympho-proliferation and lethal autoimmune pancreatitis in FoxP3-GFPcre\textsuperscript{Kl} PD-1 deficient mice. We bred PD-1 KO mice with mice carrying the IRES-GFPcre reporter knock-in at the 3’ untranslated region of the FoxP3 gene (hereafter FoxP3-GFPcre\textsuperscript{Kl}) (21) (Fig. S1A). During the course of breeding, we found that some of the litters died soon after weaning. The cumulative data revealed that all male mice with the genotype of FoxP3-GFPcre\textsuperscript{Kl} PD-1 KO died within 3 months of age (Fig. 1A). Histological analyses revealed the presence of mononuclear infiltrates in multiple organs such as the pancreas, liver, lung or heart of FoxP3-GFPcre\textsuperscript{Kl} PD-1 KO but not FoxP3-GFPcre\textsuperscript{Kl} PD-1 WT mice (Fig. 1B, Table S1). Remarkably, the pancreas was atrophic and its exocrine structure was almost completely lost in most of the mice analyzed. Indeed, the level of serum lipase was significantly reduced in FoxP3-GFPcre\textsuperscript{Kl} PD-1 KO mice (Fig. S1C). Interestingly, the pancreatic islets remained intact in FoxP3-GFPcre\textsuperscript{Kl} PD-1 KO mice. The total number of cells and frequencies of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells were considerably higher in pancreas and pancreatic lymph nodes (LN) (Fig. 1C, Fig. S1D). There was a significant increase in CD4\textsuperscript{+}CD44\textsuperscript{+} and CD8\textsuperscript{+}CD44\textsuperscript{+} effector/memory T cells, especially in the pancreatic LN (Fig. 1C, Fig. S1E). The moribund FoxP3-GFPcre\textsuperscript{Kl} PD-1 KO mice did not show any kidney or joint pathology as observed previously in PD-1 KO mice on a C57BL/6 background (22), probably because the early onset and lethal outcome of exocrine pancreatitis. Nephritis and arthritis begin after three months of age (22).

Examination of lymphoid organs of FoxP3-GFPcre\textsuperscript{Kl} PD-1 KO mice revealed enlarged spleen (SPL) and LN with significantly increased cellularity (Fig. 1D, Fig. S1D). There was a significant increase in frequencies of both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, especially those expressing CD44 in the SPL and LN of FoxP3-GFPcre\textsuperscript{Kl} PD-1 KO mice compared with those in PD-1 WT mice, indicating a preferential expansion of


effector/memory T cell populations (Fig. 1E, Fig. S1F, G). Interestingly, the GFP⁺ (FoxP3⁺) T cells were also increased in SPL and LN of FoxP3-GFPcreKl/Y PD-1 KO mice (Fig. 1E, Fig. S1F, G). The ratios of CD4⁺CD44⁺/Treg cells and CD8⁺CD44⁺/Treg cells were significantly higher in FoxP3-GFPcreKl/Y PD-1 KO mice (Fig. 1E, Fig. S1G) indicating that unbalanced T cells expansion with dominance of effector/memory T cells versus Treg cells occurs in the absence of PD-1 control.

Taken together, the analyses revealed that PD-1 deficiency caused lympho-proliferation in lymphoid and non-lymphoid organs in FoxP3-GFPcreKl/Y mice, resulting in their early death from T cell-mediated autoimmune destruction, especially autoimmune pancreatitis.

Transcription of FoxP3 is reduced in the FoxP3-GFPcreKI mice. Two groups reported that FoxP3 knock-in reporter mice harboring GFP fused to the amino-terminus of FoxP3 (GFP-FoxP3KI) display a partial loss of FoxP3 function, promoting autoimmune phenotypes (23, 24). We thought that the current genetic modification of the FoxP3 locus could also affect the endogenous FoxP3 expression and consequently the function of Treg cells. In the case of GFP-FoxP3 KI mice, the transcriptional level of FoxP3 was unaffected, yet the mature GFP-FoxP3 fusion protein had altered binding to various cofactors (23, 24). In contrast, our FoxP3-GFPcreKI line showed reduced expression of FoxP3 at both transcriptional and protein levels, which was more obvious in male FoxP3-GFPKl/Y than in female FoxP3-GFPcreKI/WT mice (Fig. 2A, B). The expression of CD25, a marker that usually defines activated T cells and Treg cells was reduced on FoxP3⁺ T cells in lymphoid organs from male FoxP3-GFPcreKl/Y mice (Fig. 2C). However, the levels of CTLA-4 were comparable between FoxP3⁺CD25⁺ Treg cells from FoxP3-GFPcreKl/Y mice and those from WT mice (Fig. 2C). At the population level, the frequency of CD25⁺ Treg
but not that of CTLA-4+ Treg cells was reduced in male FoxP3-GFPcre<sup>KI/Y</sup> mice (Fig. S2A, B).

Importantly, female FoxP3-GFPcre<sup>KI/WT</sup> PD-1 KO mice were healthy, presumably because a half of Treg cells express normal level of FoxP3 transcripts from the WT allele (due to random X-inactivation), thus providing a dominant protection (Fig. 1A). The allelic difference in FoxP3 expression was represented by higher CD25+ expression in GFP+ T cells compared with GFP+ T cells in female FoxP3-GFPcre<sup>KI/WT</sup> mice (Fig. S2C). In contrast, the female PD-1 KO mice with the genotype of FoxP3-GFPcre KI on both alleles (FoxP3-GFPcre<sup>KI/KI</sup> PD-1 KO) died with kinetics similar to those of male FoxP3-GFPcre<sup>KI/Y</sup> PD-1 KO (Fig. 1A). Thus, the reduced FoxP3 expression in Treg cells is likely contributing to the autoimmune phenotype observed in FoxP3-GFPcre<sup>KI</sup> PD-1 KO mice.

**PD-1-deficient Treg cells efficiently rescue the autoimmune phenotype of FoxP3-GFPcre<sup>KI/Y</sup> PD-1 KO mice.** To determine if the partial insufficiency of FoxP3 was the cause of fatal autoimmunity in male FoxP3-GFPcre<sup>KI/Y</sup> PD-1 KO mice, we tested whether supplying FoxP3-sufficient Treg cells could rescue the FoxP-3-GFPcre<sup>KI/Y</sup> PD-1 KO mice from death. We transferred CD4+CD25+ T cells (>98% of which were Treg cells) sorted from SPL and LN of WT mice into neonatal FoxP3-GFPcre<sup>KI/Y</sup> PD-1 KO mice at day 3 after birth. More than 70% of mice that received FoxP3-sufficient Treg cells survived over 3 months without any signs of disease (i.e. wasting) (Fig. 3A). We next asked whether PD-1 expression on transferred Treg cells was needed for such rescue. To address this issue, we performed transfer experiments with CD4+CD25+ T cells sorted from PD-1 KO mice. Remarkably, the injection of FoxP3-sufficient PD-1-deficient Treg cells prevented the development of the wasting disease in FoxP3-GFPcre<sup>KI/Y</sup> PD-1 KO mice (Fig. 3A). Although we could
not directly assess the expansion of transferred Treg cells in this particular system, the histological features of the pancreas indicate that PD-1 deficient Treg cells had probably enhanced suppressive activity compared to WT Treg cells even 6 months after transfer (Fig. 3B). Indeed, while both transfers associated with a significant decrease of CD4+ and CD8+ T cells, including the CD44+ effector/memory T cells in SPL and LN of the recipient mice, such reduction was much more pronounced in mice that received PD-1-deficient Treg cells (Fig. 3C, Fig. S3A). The suppressive function of PD-1 deficient Treg cells might be augmented by their accelerated expansion and/or due to their increased FoxP3 and CTLA-4 expression (Fig. S3B). Altogether the results suggest that Treg cells exert a dominant protection and that PD-1 expression is not necessary for this protection as long as they maintain FoxP3 expression at normal or higher levels.

**PD-1 deficiency promotes the generation of effector T cells with history of FoxP3 expression.** The above conclusion raises the question as to why PD-1 deficiency causes severe autoimmunity in FoxP3-GFPcreK/Y PD-1 KO mice, in spite of generation of Treg cells with increased function. We hypothesized that some Treg cells may contribute to the enrichment of effector/memory T cell compartment with autoimmune potentials after losing their FoxP3 expression. The breeding of FoxP3-GFPcreK with Rosa26-YFP mice, a CRE-reporter strain allows stable in vivo labeling of the cells that once expressed GFP (20). Thus, the GFP-YFP+ cells reporting the previous expression of FoxP3 are designated as ex-FoxP3 (20). We took advantage of this system and bred FoxP3-GFPcreK PD-1 KO mice with Rosa26 RFP strain, instead of Rosa26-YFP (25) in order to monitor the development of ex-FoxP3 cells in mice with PD-1 deficiency (Fig. S1B). We first compared the effect of PD-1 on Treg cells in healthy female FoxP3-GFPcreK/W PD-1 KO Rosa-RFP mice.
Consistent with a PD-1-deficient phenotype, we observed expansion of Treg cells in FoxP3-GFPcre<sup>KI/WT</sup> Rosa-RFP mice in the absence of PD-1 (Fig. 4A). Since the homeostatic control of Treg cell expansion is normally quite solid, such significant changes in the frequency of Treg cells suggests the existence of powerful regulatory mechanism of Treg cell homeostasis by PD-1.

Interestingly, there was a significant increase of CD4<sup>+</sup>GFP<sup>-</sup>RFP<sup>+</sup> cells (ex-FoxP3) in the peripheral lymphoid organs as well as the thymus of heterozygous female FoxP3-GFPcre<sup>KI/WT</sup> mice bearing the Rosa26-RFP transgene and the deletion of PD-1 compared to the same mice with the WT PD-1 allele (Fig. 4A, B). When activated in vitro, sorted GFP-RFP<sup>-</sup>ex-FoxP3 T cells showed increased production of various cytokines compared with GFP<sup>+</sup>RFP<sup>+</sup>T cells, confirming previous observations of their effector potentials (20) (Fig. 4C). PD-1 deficient ex-FoxP3 secreted higher levels of pro-inflammatory (IFN-γ, IL-17A) as well as anti-inflammatory (IL-10) cytokines than PD-1-sufficient ex-FoxP3 cells (Fig. 4C). These data clearly indicate that in non-inflammatory conditions, PD-1 expression restricts the expansion of Treg cells, contributes to maintenance of FoxP3 expression and prevents the generation of ex-FoxP3 cells with the powerful effector function.

**Ex-FoxP3 cells in PD-1 KO FoxP3-GFPcre<sup>KI/Y</sup> Rosa-RFP mice contain autoreactive cells.** We next evaluated the characteristics of Treg cells including the generation and effector function of ex-FoxP3 T cells in inflammatory autoimmune settings by examining male PD-1 KO FoxP3-GFPcre<sup>KI/Y</sup> Rosa-RFP mice. Remarkably, before the onset of lympho-proliferation, the 2 weeks old pups PD-1 deficient fate-mapped mice showed a significant increase in ex-FoxP3 cells compared to the PD-1 WT counterparts not only in the SPL and LN but also in pancreatic LN and pancreas (Fig. 5A, B). In addition, the frequency of GFP<sup>+</sup> cells was increased, but
these cells may be of a different nature, as many of them did not express CD25 (Fig. 5A).

Interestingly, after the onset of CD4+ T cell expansion and of the autoimmune phenotypes, the frequency of ex-FoxP3 T cells became closer to the controls by unknown reasons (Fig. 5C). However, the frequency of CD44+GFP-RFP+ ex-FoxP3 T cells with effector/memory phenotype was significantly increased in lymphoid tissues of PD-1 deficient fate-mapped mice (Fig. 5C). This result clearly shows an increased flow of cells that once expressed FoxP3 into the effector/memory T cells compartment.

We next tested the pathogenic potential of ex-FoxP3 T cells from pancreas and pancreatic LN of male PD-1 KO FoxP3-GFPcre^K/Y Rosa RFP in vivo, by transferring them with or without CD8+ T cells into Rag-2 KO mice. Because only a small number of ex-FoxP3 T cells can be isolated from these tissues, we first expanded them in vitro. Then, we re-sorted CD4+GFP-RFP+ cells before the transfer, as some of the expanded ex-FoxP3 re-expressed FoxP3, as previously reported (21) (Fig. S4A). Interestingly, 2 weeks after transfer the mice that received ex-FoxP3 T cells, and ex-FoxP3 T cells together with CD8+ T cells but not CD8+ T cells alone, began to lose weight and had severe diarrhea (Fig. 6A). Mice were examined 4 weeks after the transfer, revealing that the large intestine were significantly shorter and swollen in Rag-2 KO mice that received ex-FoxP3 T cells or ex-FoxP3 together with CD8+ T cells but not CD8+ T cells only (Fig. 6B). Histological analysis confirmed the presence of severe colitis, with huge infiltrates of mononuclear cells and disruption of colonic structures only in mice receiving ex-FoxP3 T cells alone or the combination of ex-FoxP3 and CD8+ T cells (Fig. 6C, Table S2). Similar mononuclear infiltrates were observed in the pancreas, especially around pancreatic duct and vessels (Fig. 6C lower panels). Unexpectedly, the more pronounced infiltrates were detected in Rag-2 KO mice.
transferred with ex-FoxP3 T cells only (Fig. 6C lower panels, Table S2). This could be due to better expansion of ex-FoxP3 T cells by more abundant IL-2 in the absence of competition with CD8+ T cells, as suggested by the increased numbers of ex-FoxP3 (CD4+) T cells in LN and pancreatic LN in these mice compared to those receiving the combination of ex-FoxP3 T cells together with CD8+ T cells (Fig. S4B, C). These results clearly indicate that ex-FoxP3 T cells have pathogenic potential and contribute to pathology either directly or indirectly by helping other effector T populations, like cytotoxic CD8+ T cells.
Discussion

We found that the “genetic surgery” of the FoxP3 locus fortuitously resulted in mildly reduced expression of endogenous FoxP3 without obvious functional consequences in FoxP3-GFPcre\textsuperscript{KI} mice. However, when crossed with PD-1-deficient mice, the FoxP3-GFPcre\textsuperscript{KI} male mice, developed acute and lethal autoimmunity. These mice provided a useful model to address how PD-1 modulates the Treg cell biology in autoimmune settings. The early death was prevented by the transfer of FoxP3-sufficient Treg cells, irrespective of whether they were from WT or PD-1-deficient donors. It should be noted that the Treg cells from PD-1 KO mice provided even better rescue compared to Treg cells from WT mice, by causing a statistically significant reduction of effector/memory CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. Sage et al. have demonstrated that PD-1 restricts the number and the function of follicular Tregs (Tfr) (26). Our study expands this finding to conventional Treg cells. FoxP3 expression and Treg function both require TCR recognition of self-antigens (27, 28). We speculate that, as PD-1 attenuates TCR downstream signaling, PD-1 deficiency may enhance TCR signaling in Treg cells, which induces their proliferation and in turn strengthen their regulatory function in vivo.

We demonstrated that PD-1 negatively regulates the appearance of ex-FoxP3 cells. Previous studies reported the appearance of ex-FoxP3 cells, especially at the inflammatory sites such as the pancreatic LN and islets of NOD mice (20), the joints in a collagen induced arthritis mouse model (29) and CNS in a EAE model (30). It was proposed that the ex-FoxP3 cells derive from genetic reprogramming of committed Treg cells (20, 31). On the other hand, Rubtsov et al. showed that FoxP3\textsuperscript{+} Treg cells are stable (32). Beside the two opposing models, Miyao et al. proposed that ex-FoxP3 cells did not come from the “genuine” Treg cells, but rather from conventional CD4\textsuperscript{+} T cells that transiently expressed FoxP3 as a consequence of
strong self-interaction \textit{in vivo} (21). We found the increase of ex-FoxP3 cells in the PD-1 KO mice in C57BL/6 background, before onset of nephritis or arthritis reported in these mice (22). As PD-1 is indisputably an attenuator for antigen receptor signaling, possibly PD-1-deficient Tregs would receive stronger TCR stimulation \textit{in vivo} and could convert into FoxP3 negative cells with strong self-reactivity. It is also possible that “promiscuous expression” of FoxP3 in conventional T cells is augmented in the absence of PD-1, resulting in increase of cells with a history of transient FoxP3 expression. We cannot exclude either possibility at this stage.

In the diseased FoxP3-GFPcre\textsuperscript{KI} PD-1 KO, we initially thought it unlikely that the ex-FoxP3 T cells could directly cause autoimmunity. However, at least in the Rag-2 transfer system, ex-FoxP3 T cells clearly induced autoimmune destruction in the pancreas and also in the colon, which was as severe as non-FoxP3 experienced CD44\textsuperscript{+} effector/memory T cells (Fig. S5, Table S2). The presence of CD8\textsuperscript{+} T cells aggravated the autoimmune phenotype in the colon. The targeting of the colon in the Rag-2 KO mice but not in the FoxP3-GFPcre\textsuperscript{KI} PD-1 KO mice could be due to a repertoire shift of the ex-FoxP3 cells after their activation and skewed expansion in vitro, among other possibilities. Taken together, our results indicate that PD-1 signaling maintains homeostasis of the Treg cells and the fine-tuned balance between the regulatory and effector T cell compartment. Stronger TCR signaling may induce FoxP3 expression but could also cause additional transcriptional modifications leading to down-regulation and eventually loss of FoxP3 expression, and acquisition of effector functions (i.e. cytokine production) by ex-FoxP3 T cells. Such regulation of Treg homeostasis is consistent with the feed back control perspective rather than the lineage perspective (33).

It is interesting that even a modest deficiency of FoxP3 expression by Treg cells in FoxP3-GFPcre\textsuperscript{KI} mice results in a spontaneous and accelerated autoimmune
phenotype with fatal outcome when combined with PD-1 deficiency. PD-1 deficient mice developed autoimmune phenotypes, which were strain-specific (12, 22, 34, 35). In C57BL/6 background, PD-1 deficiency induced arthritis and nephritis (22), while in BALB/c background mostly myocarditis and gastritis (12, 34). Neonatal thymectomy in PD-1 KO mice on the BALB/c background was shown to cause lethal autoimmune hepatitis, which was attributed to combined deficiency of Treg cells and PD-1 (34). However, thymectomy can cause other effects such as severe lymphopenia, which can support autoimmunity. PD-1 deficient MRL strain of mice showed rapid and fatal autoimmune myocarditis for undefined reason (35). A mild functional deficiency of Treg cells reported in this strain (36) might be synergizing with PD-1 deficiency for the development of lethal phenotype. Besides Treg instability, reduced FoxP3 expression on Tregs might have caused excessive activation of conventional T cells. Under such conditions self-reactive T cells, may develop into the effector population. This could be an additive, Treg non-autonomous effect of PD-1. In any case, we now show that indeed, deficiency of PD-1 combined with even a mild insufficiency of FoxP3+ Treg cells results in an unexpectedly strong phenotype. In humans, both polymorphisms of FoxP3 and PD-1 associate to autoimmunity. Although the mutations that affect the exon of FoxP3 results in IPEX by itself, others mutations affecting intronic or non-coding regions that might alter the expression levels are relatively mild (6, 37). The lessons from our mice show that even such mild SNPs, if both molecules are affected, can provoke severe autoimmunity. It also suggests the requirement of careful immunological monitoring of cancer patients who are treated with both anti-CTLA4 and anti-PD-1 antibodies.

Taken together, our data suggest that PD-1 and FoxP3 work together for maintenance of peripheral tolerance, but mostly through different pathways. PD-1 is involved in rheostatic modulation of the threshold of TCR signaling that prevents the
development of auto-reactive effector T cells and the following tissue destruction, while FoxP3 is sufficient for dominant regulation through maintaining the integrity of Treg function. Accumulative defects in these independent genetic factors can cause life-threatening autoimmune diseases in humans.

Materials and Methods

Animal. All the experiments were carried out using the mice in the C57BL/6 background. The PD-1 KO, Rosa-RFP\textsuperscript{K/FWT}, and FoxP3-GFP\textsuperscript{K/Y} mice were as described previously (21, 25, 38). PD-1 KO mice were initially crossed with reporter strains, and resulting F1 generation was extensively intercrossed to obtain PD-1 KO in reporter strains. Littermate or the appropriate age/sex-matched mice were used as control. All mice are housed in specific pathogen-free facility, and all procedures followed protocols approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University. All the other methods and materials are described in legends or supplementary materials.

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References


**Figure legend**
Fig. 1. PD-1 deficient FoxP3-GFPcre mice show lethal autoimmunity. (A) Survival curve of male GFPcre^KI/Y^PD-1~WT~(n=20), male GFPcre^KI/Y^PD-1~KO~(n=8), female GFPcre^KI/WT^PD-1~WT~(n=20), female GFPcre^KI/WT^PD-1~KO~(n=23), female GFPcre^KI/KI^PD-1~WT~(n=11), female GFPcre^KI/KI^PD-1~KO~(n=6) mice. (B) HE staining of tissues (magnification 50× and 200×). (C) FACS analysis of pancreas of 3~10 weeks old FoxP3-GFPcre^KI/Y^PD-1~WT~or~PD-1~KO~mice. (D) Appearance of spleen (SPL) and lymph-nodes (LN). (E) FACS analysis of splenocytes. Data are representative of 3~7 mice per group. Bars represent the means (±SEM). ***P<0.001, **P<0.01, *P<0.05.

Fig. 2. Reduced expression of endogenous FoxP3 in FoxP3-GFPcre mice. (A) CD4^+^ cells from SPL and LN of 2~3 months old indicated mice were subjected for quantitative real-time PCR (qRT-PCR) for the expression levels of endogenous FoxP3. (B) Intracellular staining for FoxP3 gated on LN CD4^+^ T cells. (C) FACS analysis of CD25 and CTLA-4 expression from LN cells of 2 months old male WT or FoxP3-GFPcre^KI/Y^ mice. Statistical analysis of CD25 MFI and CTLA-4 MFI from SPL and LN of each mouse in indicated genotype. Data are representative of 3~4 mice per group. Bars represent the means (±SEM). **P<0.01, *P<0.05, ns, not significant.

Fig. 3. PD-1-deficient Tregs efficiently rescue FoxP3-GFP^KI/Y^PD-1~KO~mice from lethal autoimmunity. (A) Tregs (CD4^+^CD25^+) were sorted from C57BL/6 PD-1 WT or PD-1 KO mice and injected into 3 days old mice as indicated (GFPcre^KI/Y^PD-1~KO~+~WT-Treg: n=9; GFPcre^KI/Y^PD-1~KO~+~PD-1~KO-Treg: n=9). Mice that did not receive Treg cells (GFPcre^KI/Y^PD-1~WT: n=6; GFPcre^KI/Y^PD-1~KO: n=5) served as controls. (B) HE staining of pancreas from the mice in A. (C) The FACS analysis of
SPL from 12–16 weeks old mice in A. (B, C) Note that the data from untreated GFPcre^KI/Y PD-1 KO is collected at 3–10 weeks old due to early death of this group. Data are representative of 4–6 mice per group. Bars represent the means (±SEM). **P<0.01, *P<0.05, ns, not significant.

Fig. 4. The increase of ex-FoxP3 T cells in female FoxP3-GFPcre^KI/WT PD-1 KO mice. (A) GFP and RFP expression from spleen of 4–8 weeks old FoxP3-GFPcre^KI/WT Rosa-RFP PD-1 KO mice. (B) Percent GFP RFP^− (ex-FoxP3) T cells within RFP^+ T cells (Treg plus ex-FoxP3) of CD4^+ T cells, or CD4^−CD8^+ thymocytes (Thy). (C) The same number of naïve, GFP^+RFP^+ and GFP^−RFP^+ of CD4^+ T cells were sorted from mice in A, and stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, the level of cytokine was measured. Data are shown from 3–6 mice per group. Bars represent the means (±SEM). ***P<0.001, **P<0.01, *P<0.05.

Fig. 5. ex-FoxP3 T cells in autoimmune FoxP3-GFPcre^KI/Y PD-1 KO mice. (A) FACS analysis of Tregs, ex-FoxP3 T cells, and CD25^+ T cells from SPL of 2 weeks old male FoxP3-GFPcre^KI/Y PD-1 KO mice. (B) Statistical analysis of the ex-FoxP3 (GFP-RFP^−) cells within RFP^+ T cells in A. (C) FACS analysis of SPL cells from 3–7 weeks old male FoxP3-GFPcre^KI/Y Rosa-RFP PD-1 KO mice. Data are from 3–6 mice per group. Bars represent the means (±SEM). **P<0.01, *P<0.05.

Fig. 6. ex-FoxP3 T cells contain autoreactive cells with pathogenic potential. (A) CD8^+, ex-FoxP3 or the combination of CD8^+ and ex-FoxP3 (1:1) T cells from pancreatic LN and pancreas of FoxP3-GFPcre^KI/Y Rosa-RFP PD-1 KO mice were transferred into Rag-2 KO mice (See supplementary materials and methods). Body weight was monitored for 4 weeks. The data is shown as a ratio of weight before
injection. Statistical analysis was performed at 4 weeks. (B) large intestine of the recipients at 4 weeks. (C) HE staining of large intestine and pancreas of mice in B (magnification 50× and 200×). Representative data of 3~4 mice per each group are shown. Bars represent the means (±S.D.). ***P<0.001, *P<0.05.
Fig. 1

**A**

Survival (%)

Days

Male FoxP3-GFPcre\textsuperscript{KI/Y} PD-1 WT
Female FoxP3-GFPcre\textsuperscript{KI/Y} PD-1 KO

Male FoxP3-GFPcre\textsuperscript{KI/WT} PD-1 WT
Female FoxP3-GFPcre\textsuperscript{KI/WT} PD-1 KO

Female FoxP3-GFPcre\textsuperscript{KI/KI} PD-1 WT
Female FoxP3-GFPcre\textsuperscript{KI/KI} PD-1 KO


**B**

Male FoxP3-GFPcre\textsuperscript{KI/Y}

PD-1 WT
Liver

PD-1 KO

Lung

Pancreas

x50

x200


**C**

Pancreas

Percentage FoxP3+

Ratio CD44+/FoxP3+

PD-1 KO
PD-1 WT

T cells

CD4\textsuperscript{+} CD8\textsuperscript{+} CD4\textsuperscript{+} CD8\textsuperscript{+} T cells in:

SPL

Percentage cells

PD-1 WT
PD-1 KO

CD4\textsuperscript{+} CD8\textsuperscript{+} CD4\textsuperscript{+} CD8\textsuperscript{+} CD44\textsuperscript{+} T cells in:

**D**

Male FoxP3-GFPcre\textsuperscript{KI/Y}

PD-1 WT
PD-1 KO


**E**

SPL

Percentage cells

CD4\textsuperscript{+} CD8\textsuperscript{+} CD4\textsuperscript{+} CD8\textsuperscript{+} CD44\textsuperscript{+} T cells in:

Percentage FoxP3+

Ratio CD44+/FoxP3+

PD-1 KO
PD-1 WT

T cells

CD4\textsuperscript{+} CD8\textsuperscript{+} CD4\textsuperscript{+} CD8\textsuperscript{+}
**Fig. 2**

**A** FoxP3 mRNA level in CD4⁺ T cells

- Male WT
- Male FoxP3-GFPcreKI/Y
- Female WT
- Female FoxP3-GFPcreKI/WT

Relative to Male WT

**B**

LN

WT

FoxP3-GFPcreKI/Y

Male

WT

FoxP3-GFPcreKI/WT

Female

FoxP3

CD4 (gated on CD4⁺ T cells)

**C**

Gated on FoxP3⁺ T cells

Gated on FoxP3⁺ CD25⁺ T cells

Counts

CD25 MFI

CD25

SPL

LN

Counts

CTLA-4 MFI

CTLA-4

SPL

LN

\textbf{WT}

\textbf{FoxP3-GFPcre}^{KI/Y}

\textbf{ns}

\textbf{ns}
**Fig. 3**

A) Survival curve showing the percentage of survival over time for different genotypes. The curves are as follows:
- Black line with square markers: FoxP3-GFPcre$^\text{KI/Y}$ PD-1 WT
- Red line with circle markers: FoxP3-GFPcre$^\text{KI/Y}$ PD-1 KO
- Blue line with triangle markers: FoxP3-GFPcre$^\text{KI/Y}$ PD-1 KO + WT Treg
- Orange line with diamond markers: FoxP3-GFPcre$^\text{KI/Y}$ PD-1 KO + PD-1 KO Treg

B) Images of pancreas sections showing different genotypes:
- FoxP3-GFPcre$^\text{KI/Y}$ PD-1 WT at 6 mo
- FoxP3-GFPcre$^\text{KI/Y}$ PD-1 KO at 2.5 mo
- FoxP3-GFPcre$^\text{KI/Y}$ PD-1 KO + WT Treg at 6 mo
- FoxP3-GFPcre$^\text{KI/Y}$ PD-1 KO + PD-1 KO Treg at 6 mo

C) Bar graphs showing the number of different types of T cells in the pancreas:
- **CD4$^+$ T cells**
- **CD44$^+$CD4$^+$ T cells**
- **CD8$^+$ T cells**
- **CD44$^+$CD8$^+$ T cells**

The graphs compare the following genotypes:
- Black bars: FoxP3-GFPcre$^\text{KI/Y}$ PD-1 WT
- Red bars: FoxP3-GFPcre$^\text{KI/Y}$ PD-1 KO
- Blue bars: FoxP3-GFPcre$^\text{KI/Y}$ PD-1 KO + WT Treg
- Orange bars: FoxP3-GFPcre$^\text{KI/Y}$ PD-1 KO + PD-1 KO Treg

The significance levels are indicated as follows:
- *: p < 0.05
- **: p < 0.01
- ***: p < 0.001
- ns: not significant
**Fig. 4**

**A**
Female FoxP3-GFPcre\textsuperscript{KI/WT}Rosa-RFP

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**B**
Gated on CD4\(^+\) T cells

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**C**

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- Naive GFP+RFP\(^+\), GFP-RFP\(^+\), GFP-RFP\(^-\), RFP+RFP\(^+\), RFP-RFP\(^+\), RFP-RFP\(^-\)
- *p < 0.05, **p < 0.01, ***p < 0.001
A

Male
FoxP3-GFPcre<sup>KI/Y</sup> Rosa-RFP
2 wks old

WT
PD-1KO

Counts

RFP (gated on CD4<sup>+</sup> T cells)

CD25 (gated on GFP<sup>+</sup> T cells)

B

Gated on CD4<sup>+</sup> T cells

PD-1 WT
PD-1 KO

Counts

RFP (gated on GFP<sup>+</sup> T cells)

C

Male
FoxP3-GFPcre<sup>KI/Y</sup> Rosa-RFP
3-7 wks old

WT
PD-1KO

Counts

RFP (gated on GFP<sup>+</sup> T cells)
Fig. 6

A

Large intestine of Rag-2 KO 4 wks after transfer of:

- CD8+ exFoxP3
- exFoxP3 + CD8+

B

Large intestine of Rag-2 KO 4 wks after transfer of:

CD8+  exFoxP3  exFoxP3  + CD8+

C

Rag-2 KO 4 wks after transfer of:

CD8+  exFoxP3  exFoxP3  + CD8+

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Supporting Information

SI Materials and Methods

Antibodies and Reagents. The antibodies conjugated with various fluorophores for flow cytometry included CD4 (clone: GK1.5), CD8 (53-6.7), CD25 (PC61.5), CD44 (1M7). The intracellular staining for CTLA-4 (UC10-4B9) and FoxP3 (FJK-16s) was performed by FoxP3 intracellular staining kit (e-Bioscience). For cytokine detection, cells were stimulated with 50ng/ml PMA and 500ng/ml ionomycin for 3 days, and the supernatant was measured by the mouse Th1/Th2/Th17 Cytokine Kit (BD).

Quantitative RT-PCR. Total RNA was extracted by RNeasy Mini Kit (QIAGEN) and reverse-transcribed using reagents included in the QuantiTect™ SYBR Green RT-PCR Master Mix (QIAGEN). 7500 Fast Real-Time PCR system (Applied Biosystems) was used for real-time PCR. Gene expression was normalized to the Gapdh expression. The following primers were used: FoxP3 forward 5’-GGCCCTTCTCCAGGACAGA-3’; reverse 5’-GCTGATCATGGCTGGGTTGT-3’ (39).

Adoptive transfer. SPL and LN cells were isolated from PD-1 WT or PD-1 KO mice, CD4+ T cells were enriched by CD4 micro-beads and autoMACS (Miltenyibiotec), then 1x10^6 Treg (CD4+CD25+) cells were further sorted on FACSaria™II (BD Sciences) and injected intra-peritoneally into FoxP3-GFPcre^KI/Y PD-1 KO male mice at day 3 after birth. For Rag-2 KO mice transfer experiment, CD8 T cells and ex-FoxP3 (GFP-RFP+) cells were sorted from pancreatic LN and pancreas of male FoxP3-GFPcre^KI/Y Rosa-RFP^KI/WT PD-1 KO mice, and expanded in vitro by
Dynabeads mouse T-Activator CD3/CD28 (VERITAS) in the present of recombinant human IL2 (PEPROTECH), after 10 days’ culture, each group of cells was sorted again, then 1x10^6 CD8+, ex-FoxP3 or the combination of CD8+ and ex-FoxP3 (1:1) T cells were transferred intravenously into Rag-2 KO mice.

**Preparation of mononuclear cells from pancreas.** The tissue was cut into about 3-5 mm thick pieces and incubated 30 min in RPMI 1640 medium (Life Technologies, serum free) with 0.25 mg/ml DNAse I (Roche) and 2 mg/ml collagenase D (Roche) at 37°C on a rotator. The tissue was dissociated with a P1000 by pipetting 20 times (need to cut tip) and incubated 10 min at 37°C on a rotator, then the tissue was further dissociated with a P1000 by pipetting 20 times (uncut tip). Digested tissue was washed twice with RPMI 1640 medium (2% (v/v) FBS) and strained through a 70µm cell strainer (Greiner bio-one). Cells were analyzed in a flow cytometer (FACSCantoII, BD Biosciences).

**Statistical analysis.** The survival curves were compared using a log-rank test and means of groups were analyzed by two-tailed Student’s t-test or One-way ANOVA. P values <0.05 were considered significant.
SI Figure Legend

**Fig. S1.** Schematic representation of reporter mice and lympho-proliferation of FoxP3-GFPcre<sup>KI/Y</sup> PD-1 KO mice. (A) Map of FoxP3-GFPcre locus is shown. Filled boxes, exons; gray box, 3’UTR of Foxp3 gene; I, IRES. (B) Principle of Cre-Lox-mediated tdRFP. (C) 3~11 weeks old male FoxP3-GFPcre<sup>KI/Y</sup> PD-1 WT or PD-1 KO mice were analyzed for serum lipase concentration (*excluding the maximum and minimum values*). (D) Total number of mononuclear cells in pancreas, pancreatic LN, SPL and LN of 3~10 weeks old FoxP3-GFPcre<sup>KI/Y</sup> PD-1 WT or PD-1 KO mice. (E) The number of CD4<sup>+</sup> and CD8<sup>+</sup> T cells from pancreas and pancreatic LN, and the number of CD44<sup>+</sup> in CD4<sup>+</sup> or CD8<sup>+</sup> T cells from pancreatic LN of mice as in D. (F) The number of CD4<sup>+</sup>, CD8<sup>+</sup>, CD44<sup>+</sup>CD4<sup>+</sup>, CD44<sup>+</sup>CD8<sup>+</sup> and FoxP3<sup>+</sup>(GFP<sup>+</sup>) T cells from spleen of mice as in D. (G) LN cells were analyzed for the frequency and number of CD4<sup>+</sup>, CD8<sup>+</sup>, CD44<sup>+</sup>CD4<sup>+</sup>, CD44<sup>+</sup>CD8<sup>+</sup>, FoxP3<sup>+</sup> (GFP<sup>+</sup>) T cells, and for the ratio of CD44<sup>+</sup> (gated on CD4<sup>+</sup> or CD8<sup>+</sup> T cells) and FoxP3<sup>+</sup> (GFP<sup>+</sup>) T cells of mice as in D. Data are representative of 3~7 mice per group and shown as the means (±SEM). ***P<0.001, **P<0.01, *P<0.05.

**Fig. S2.** CD25<sup>+</sup> T cells are decreased in FoxP3-GFPcre mice. (A) FACS analysis of LN cells from 2~3 months old male WT or FoxP3-GFPcre<sup>KI/Y</sup> mice for expression of FoxP3, CD25 and CTLA-4 gated on CD4<sup>+</sup> T cells. Numbers in plots indicate percent cells in each gate. (B) Statistical analysis of FoxP3<sup>+</sup>CD25<sup>+</sup> frequency and cell number in CD4<sup>+</sup> T cells (left panel), and FoxP3<sup>+</sup>CTLA-4<sup>+</sup> frequency and cell number in CD4<sup>+</sup> T cells (right panel) from SPL and LN of mice as in A. (C) Statistical analysis of CD25 MFI within CD25<sup>+</sup>GFP<sup>-</sup> or CD25<sup>+</sup>GFP<sup>+</sup> T cells from SPL and LN of female FoxP3-GFPcre<sup>KI/WT</sup> mice. Data are representative of 3~4 mice per group. Bars represent the means (±SEM). ***P<0.001, **P<0.01, *P<0.05, ns, not significant.
**Fig. S3.** PD-1 deficient Treg cells showed increased CTLA-4 expression and suppressive activity in vivo. (A) The number of CD4⁺, CD8⁺, CD44⁺CD4⁺ and CD44⁺CD8⁺ T cells in LN from indicated mice as in Fig. 3C. (B) FACS analysis of FoxP3 and CTLA-4 expression gated on CD4⁺ T cells from SPL and LN of 2 months old PD-1 WT or PD-1 KO mice on the C57BL/6 background. Numbers in plots indicate percent cells in each gate. Data are representative of 3–6 mice per group. Bars represent the means (±SEM). ***P<0.001, **P<0.01, *P<0.05, ns, not significant.

**Fig. S4.** PD-1 deficient CD8 and ex-FoxP3 transferred Rag-2 KO mice. (A) Before transfer, CD8⁺ and ex-FoxP3 T cells as in Fig. 6A, were re-sorted and analyzed for FACS. (B) FACS analysis of CD8 and ex-FoxP3 expression in SPL, LN and pancreatic LN of Rag-2 KO mice 4 weeks after transfer of each indicated cells. (C) The absolute number of ex-FoxP3 T cells in SPL, LN and pancreatic LN of mice as in B. Numbers in plots indicate percent cells in each gate. Representative data of 3–4 mice per each group are shown. Bars represent the means (±SEM). **P<0.01, *P<0.05.

**Fig. S5.** Proinflamatoy activites of PD-1 deficient CD44⁺RFP⁻ T cells in Rag-2 KO mice. (A) CD8⁺ and CD44⁺RFP⁺GFP⁻CD4⁺ (CD44⁺RFP⁺) T cells were sorted from pancreatic LN and pancreas of male FoxP3-GFPcreKI/Y Rosa-RFP PD-1 KO mice, and expanded in vitro by CD3/CD28-beads in the present of IL2 for 10 days, each group of cells was re-sorted and CD44⁺RFP⁻ or the combination of CD8⁺ and CD44⁺RFP⁻ (1:1) T cells were respectively transferred into Rag-2 KO mice. Body weight was monitored during the 3–4 weeks of each group of mice. The mean weight is shown as
a ratio of weight before injection. Statistical analysis was performed between each
group after 3 weeks injection. (B) Picture of large intestine of Rag-2 KO mice
transferred with indicated cells for 3 or 4 weeks. (C) HE staining of large intestine and
pancreas of mice as in B (magnification 50×, 200× and 400×). Mitosis in crypt is
marked by red circle. Representative data of 3~4 mice per each group are shown. Data
are shown as the means (±S.D.). *P<0.05.
A. FoxP3-GFPcre

B. Rosa26-tdRFP

C. Serum lipase (U/L)

D. Cell number (x10^5)

E. Pancreas

F. SPL

G. LN

Fig. S1
**Fig. S2**

A

**WT** vs. **FoxP3-GFPcre<sup>KI/Y</sup>**

- **LN**
  - **FoxP3**
  - **CD25** (gated on CD4<sup>+</sup> T cells)
  - **CTLA-4** (gated on CD4<sup>+</sup> T cells)

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B

- **Percentage FoxP3<sup>+</sup>CD25<sup>+</sup> in CD4<sup>+</sup> T cells**
- **Cell number of FoxP3<sup>+</sup>CD25<sup>hi</sup> in CD4<sup>+</sup> T cells**

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Cell number of FoxP3<sup>+</sup>CD25<sup>hi</sup> (x10<sup>5</sup>):

- **SPL**
  - WT: 3.0 ± 0.5
  - FoxP3-GFPcre<sup>KI/Y</sup>: 3.0 ± 0.5
- **LN**
  - WT: 3.0 ± 0.5
  - FoxP3-GFPcre<sup>KI/Y</sup>: 3.0 ± 0.5

C

- **Female FoxP3-GFPcre<sup>KI/WT</sup>**
  - **CD25 MFI**

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A  
LN  
CD4+ T cells  
CD44+CD4+ T cells  
CD8+ T cells  
CD44+CD8+ T cells  

Cell number (x10^5)  

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**Fig. S3**
**Fig. S4**

### A

Before transfer

- **CD8^+**
- **exFoxP3**

### B

**Rag-2 KO 4 wks after transfer of:**

- **SPL**
  - **CD8^+**
  - **exFoxP3**
  - **exFoxP3 + CD8^+**

### C

- **Cell number of exFoxP3 T cells (x10^3)**
  - **SPL**
  - **LN**

- **Cell number of exFoxP3 T cells (x10^5)**
  - **Pancreatic LN**

Rag-2 after transfer of:
- **exFoxP3**
- **exFoxP3 + CD8^+**

- **RFP (gated on CD4^+CD8^- T cells)**

---

**Notes:**
- Variations in cell counts and subgroups are indicated with statistical significance symbols (*, **).
Fig. S5

A

Percentage of initial weight

Days

0 10 20 30

CD44+ RFP-

CD44+ RFP+ CD8+

B

4 wks after transfer of CD44+ RFP-

3 wks after transfer of CD44+ RFP+ CD8+

C

4 wks after transfer of CD44+ RFP-

3 wks after transfer of CD44+ RFP+ CD8+

Large intestine

Pancreas

x50

x200

x400
Table S1. Histological examination of mice.

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<tr>
<td>FoxP3-</td>
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<td>4</td>
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<tr>
<td>GFPcre&lt;sup&gt;KI/Y&lt;/sup&gt;</td>
<td>47</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PD-1 KO</td>
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<td>4</td>
<td>1</td>
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</tbody>
</table>

Table summarizes the score of inflammation of organs from male FoxP3-GFPcre<sup>KI/Y</sup> PD-1 WT or male FoxP3-GFPcre<sup>KI/Y</sup> PD-1 KO mice at the indicated age (days). Score 0: absence of lymphocytic infiltrates; Score 1: mild infiltrates; Score 2: moderate infiltrates; Score 3: marked infiltrates; Score 4: almost complete destruction of organs. ND, not done.
Table S2. Histological examination of Rag-2 KO mice with adoptive transfer.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Donor cells</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD8⁺</td>
<td>0~1</td>
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<tr>
<td>Large intestine</td>
<td>ex-FoxP3</td>
<td>2~3</td>
</tr>
<tr>
<td></td>
<td>ex-FoxP3 + CD8⁺</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>CD44⁺RFP⁻</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>CD44⁺RFP⁻ + CD8⁺</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organ</th>
<th>Donor cells</th>
<th>Inflammatory cell infiltration in the exocrine pancreas</th>
<th>Insulitis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD8⁺</td>
<td>0~1</td>
<td>0</td>
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<tr>
<td>Pancreas</td>
<td>ex-FoxP3</td>
<td>2</td>
<td>0~1</td>
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<tr>
<td></td>
<td>ex-FoxP3 + CD8⁺</td>
<td>1</td>
<td>0~1</td>
</tr>
<tr>
<td></td>
<td>CD44⁺RFP⁻</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CD44⁺RFP⁻ + CD8⁺</td>
<td>1~2</td>
<td>0~1</td>
</tr>
</tbody>
</table>

Table summarizes the score of inflammation of Large intestine and pancreas from Rag-2 KO mice 3 weeks after transfer of the combination of CD44⁺RFP⁻ + CD8⁺, or 4 weeks after transfer of other indicated T cells from pancreatic LN and pancreas of male FoxP3-GFPcre<sup>KI/Y</sup> Rosa-RFP PD-1 KO mice. Score 0: normal colon mucosa or absent of lymphocytic cell infiltration; Score 1: mild mucosal changes or mild infiltrates; Score 2: mucosal abrasion or moderate infiltrates; Score 3: mucosal erosion, ulceration or marked infiltrates; Score 4: mucosa atrophy and few mitosis in crypt, or almost complete destruction of organs.