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Shunsuke Chikuma; ... et. al

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PD-1-Mediated Suppression of IL-2 Production Induces CD8$^+$ T Cell Anergy In Vivo[1]

Shunsuke Chikuma,* Seigo Terawaki,* Tamon Hayashi,† Ryusuke Nabeshima,† Takao Yoshida,† Shiro Shibayama,† Taku Okazaki,*‡ and Tasuku Honjo[2]*

Accumulating evidence suggests that PD-1, an immuno-inhibitory receptor expressed on activated T cells, regulates peripheral T cell tolerance. In particular, PD-1 is involved in the induction and/or maintenance of T cells' intrinsic unresponsiveness to previously encountered Ags, although the mechanism is yet to be determined. We used a simple experimental model to dissect the mechanism for anergy establishment, in which 2C TCR transgenic rag2$^{-/-}$ PD-1$^{+/+}$ mice were anergized by a single injection of a cognate peptide. Interestingly, 2C rag2$^{-/-}$ PD-1$^{-/-}$ mice were totally resistant to anergy induction by the same treatment; thus, PD-1 was responsible for anergy induction. Furthermore, PD-1 expression was induced within 24 h of the initial Ag exposure. The establishment of anergy was associated with a marked down-regulation of IL-2 from the CD8$^+$ T cells. In fact, IL-2 blockade resulted in anergy even in 2C rag2$^{-/-}$PD-1$^{-/-}$ T cells. Furthermore, the complementation of the IL-2 signal in 2C rag2$^{-/-}$ PD-1$^{+/+}$ mice reversed the anergy induction. We propose that CD8$^+$ T cell anergy is induced by a reduction of cell-autonomous IL-2 synthesis, which is caused by the quick expression of PD-1 in response to Ag stimulation and the subsequent stimulation of this receptor by its ligands on surrounding cells. *The Journal of Immunology,* **2009, 182: 6682–6689.**

Peripheral T cell tolerance is an essential property of the immune response that is regulated by the cooperation of the intrinsic anergy of T cells with active suppression by other cells. T cell anergy is a state in which T cells fail to respond to previously encountered antigenic stimulation by functional APCs (reviewed in Ref. 1). Initially, T cell anergy was found to be induced in a CD4$^+$ T cell clone in vitro, when the cells were stimulated by TCR (first signal) without a costimulatory CD28 signal (second signal) (2). Because such cells lose the ability to produce IL-2 upon secondary stimulation in vitro, and recover from the anergic state when exposed to exogenous IL-2, T cell anergy is defined as the state in which T cells lose the ability to produce IL-2 autonomously (2). To date, various experimental systems have been designed to mimic T cell anergy induction, including the transfer of TCR transgenic (Tg)[3] cells into an Ag-bearing host, the transfer of Ag into TCR Tg mice, and the stimulation of naive T cells by ionomycin in vitro, among others (1).

Anergic T cells have a defect in their Ras signaling, which leads to impaired AP-1 activation. This defect might be caused by the induction of ubiquitin ligase proteins, such as grail or itch, that degrade some signaling components required for proper T cell activation (3). In the case of CD8-positive T cells, the encounter of naive cells with Ag-bearing APCs leads to several rounds of cell division. However, within 3 or 4 days, the cells lose their ability to proliferate, which is called "split anergy" or activation-induced nonresponsiveness, and this state is maintained without significant deletion of the T cells (reviewed in Ref. 4). The systemic anergic state of CD8$^+$ T cells can be broken by virus-induced inflammatory cytokines, such as IL-12 or IFNα (4), which explains why systemic viral infections often cause autoimmunity (5). However, the fundamental mechanisms by which potentially autoreactive CD8$^+$ T cells initially fall into an anergic state (anergy induction) and how the anergic state is maintained (anergy maintenance) are yet to be characterized.

Programmed cell death-1 (PD-1), a negative coreceptor containing a tyrosine-based inhibitory motif, is an Ig-like molecule that structurally resembles the negative costimulatory receptor CTLA-4, and is known to play a critical role in the suppression of autoimmunity (6–9). Although, CTLA-4 has little or no role in the CD8$^+$ T cell intrinsic immune response in various model systems (10, 11), the inhibitory function of PD-1 seems quite important in CD8$^+$ T cells. For instance, strong expression of PD-1 is observed on virus-specific CD8$^+$ T cells in chronic infections, and Ab-mediated blockade of the PD-1/PD-1-ligand interaction reverses the tolerance of CD8$^+$ T cells and contributes to viral clearance (12). The reversal of "exhausted T cells" occurs even in CD4$^+$ T cell-depleted hosts (12), suggesting a cell-intrinsic regulation of CD8$^+$ T cells by PD-1. In a self-tolerance model, resting dendritic cells (DC) induce peripheral CD8$^+$ T cell anergy against a Tg viral Ag in a PD-1-dependent manner (13). Finally, OVA-specific TCR-Tg CD8$^+$ T cells with PD-1 blockade are resistant to high dose peptide-mediated anergy induction in a transfer model (14), and rapidly cause diabetes when they are adoptively transferred into syngeneic hosts that express the Tg OVA Ag in pancreatic β cells (15, 16), suggesting that PD-1-mediated CD8$^+$ T cell anergy is important for the prevention of aggressive destruction in an autoimmune setting.

*Department of Immunology and Genetic Medicine, Graduate school of Medicine, Kyoto University, Sakyo-ku, Kyoto, Japan; †Exploratory Research Laboratories, Ono Pharmaceutical Company, Tsukuba, Ibaraki, Japan; and ‡Division of Immune Regulation, Institute for Genome Research, University of Tokushima, Tokushima, Japan

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[2] Address correspondence and reprint requests to Tasuku Honjo, Department of Immunology, and Genetic Medicine, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto, Japan. E-mail address: honjo@mfour.med.kyoto-u.ac.jp

[3] Abbreviations used in this paper: Tg, transgenic; DC, dendritic cell; CAG-GFP, chicken β-actin promoter driven enhanced GFP Tg mice; PD-1, programmed cell death-1; LN, lymph node.

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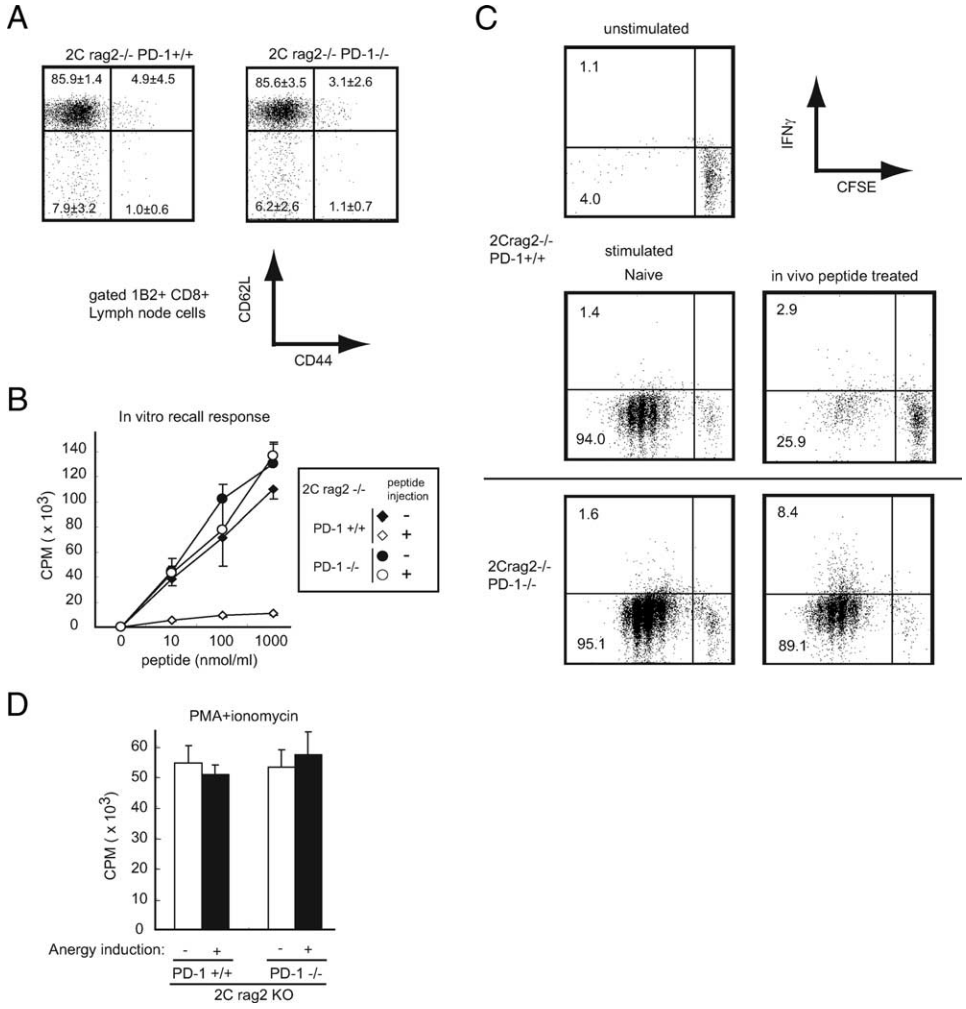

FIGURE 1. 2C TCR Tg rag2$^{-/-}$ PD-1$^{-/-}$ T cells are resistant to peptide-induced anergy in vivo. *A*, Lymph node cells from 2C rag2$^{-/-}$ PD-1$^{+/+}$ or PD-1$^{-/-}$ mice were analyzed for Alexa 488-conjugated 1B2 (2CTCR chronotypic antibody), PerCP-CD8, CD62L-APC, and CD44-PE. CD62L and CD44 staining is shown from 1B2^{+}CD8^{+} population. The numbers indicate average ± SD from three mice per genotype. The experiment is performed once. *B*, 2C rag2$^{-/-}$ PD-1$^{+/+}$ or PD-1$^{-/-}$ mice were given an i.p. injection of PBS or 25 nmol of SIYRYYGL peptide. Lymph node cells were harvested 7 days after the treatment. Isolated 2C^{+} cells were stimulated in vitro, and their proliferation was measured as described in *Materials and Methods*. The symbols and error bars represent the average and SD from triplicate wells. One representative data from >10 experiments is shown. *C*, Isolated 2C^{+} cells from B were CSFE labeled, and the CFSE dilution and IFN-γ secretion simultaneously measured by FACS 4 days after the secondary stimulation. The data were representative of two experiments using isolated cells from two mice combined for each group. *D*, The same T cells used in B were stimulated by PMA plus ionomycin. The data shown are representative of >10 experiments that yielded similar results.

In this study, we addressed the role of PD-1 in intrinsic CD8^{+} T cell tolerance, using MHC class I-restricted TCR Tg PD-1-deficient mice. We found that PD-1-deficient CD8^{+} T cells were resistant to anergy induction in vivo. Furthermore, IL-2 produced during the early activation of CD8^{+} T cells was strictly suppressed by the rapid up-regulation of PD-1. The IL-2 blockade during peptide stimulation resulted in absolute T cell anergy, while IL-2 complementation rescued the cells from the anergic state, irrespective of their PD-1 expression. Taking these findings together, we propose a novel model in which PD-1 controls the anergy induction of CD8^{+} T cells by limiting the autocrine IL-2 production, which primarily determines the anergy vs effector/memory differentiation of CD8 T cells.

Materials and Methods

Experimental animals

All the mice were kept under specific pathogen-free conditions in the animal facility at Kyoto University. The PD-1 knockout mice have been described previously and were bred to the 2C TCR Tg mice as described (7), and further bred to the Rag2 background to obtain 2C rag2$^{-/-}$ PD-1$^{-/-}$ mice. Chicken β-actin promoter driven enhanced GFP Tg mice (CAG-GFP) were purchased from Japan SLC. The animal experiments in this study were approved by the Animal Research Committee of Kyoto University.

Abs and reagents

The mAbs for flow cytometric analysis were PerCP-conjugated anti-CD8 (BD Biosciences), PE-anti-CD44 (eBioscience), biotinylated-anti-PD-1 (eBioscience), biotinylated-anti-CTLA-4 (eBioscience), and allophycocyanin anti-FoxP3 (eBioscience). An anti-2C TCR Ab (clone 1B2) was conjugated with fluorescent dyes Alexa 488, and Alexa 647, using commercially available kits from Molecular Probes. The binding of biotinylated Abs was detected by labeling with PE-conjugated streptavidin (eBioscience). For intracellular CTLA-4 and FoxP3 detection, cells were pretreated with CytoFix/CytoPerm reagents (BD Biosciences) and FoxP3 staining solutions (eBiosciences), respectively, before immunostaining. RPMI 1640 medium (Sigma-Aldrich) supplemented with 2 mM L-glutamine, 55 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS was used for in vitro cell culture. The cognate peptide for 2C TCR (aa. SIYRYYGL) was synthesized by Sigma-Aldrich Japan. The anti-IL-2 Ab (clone JES6–5) was from BD Biosciences. The purified anti-mouse IL-2 receptor α-chain Ab (clone PC61) was provided by Dr. Jeffrey Bluestone (University of California, San Francisco, CA). The purified anti-PDL1 (clone 1–111) and anti PDL2 (clone 54–1) Abs for in vivo blocking experiments were obtained from eBioscience and Medical and Biological Laboratories, respectively.

In vivo anergy induction model

Systemic anergy induction was performed as described in Frauwirth et al. (11), with slight modification. In brief, 2C rag2$^{-/-}$ PD-1$^{-/-}$ mice and 2C rag2$^{-/-}$ PD-1$^{+/+}$ mice were given an i.p. injection of PBS or 25–30 nmol of SIYRYYGL peptide and sacrificed 7 or 8 days later, unless noted otherwise. The lymph node T cells were labeled with CD8^{+} T cell isolation reagents (Miltenyi Biotec) followed by negative selection by AUTO MACS (Miltenyi Biotec). The sorting usually yielded chronotype 1B2-positive T cells with >97% purity. The cells were subsequently stimulated with mitomycin-C-treated C57BL/6 splenocytes and with the SIYRYYGL peptide, in a 96-well flat-bottom plate at a density of 2×10^4 T cells and 1×10^5 splenocytes per well. [^{3}H]labeled thymidine incorporation was measured during the last 6 h of a 72-h culture.

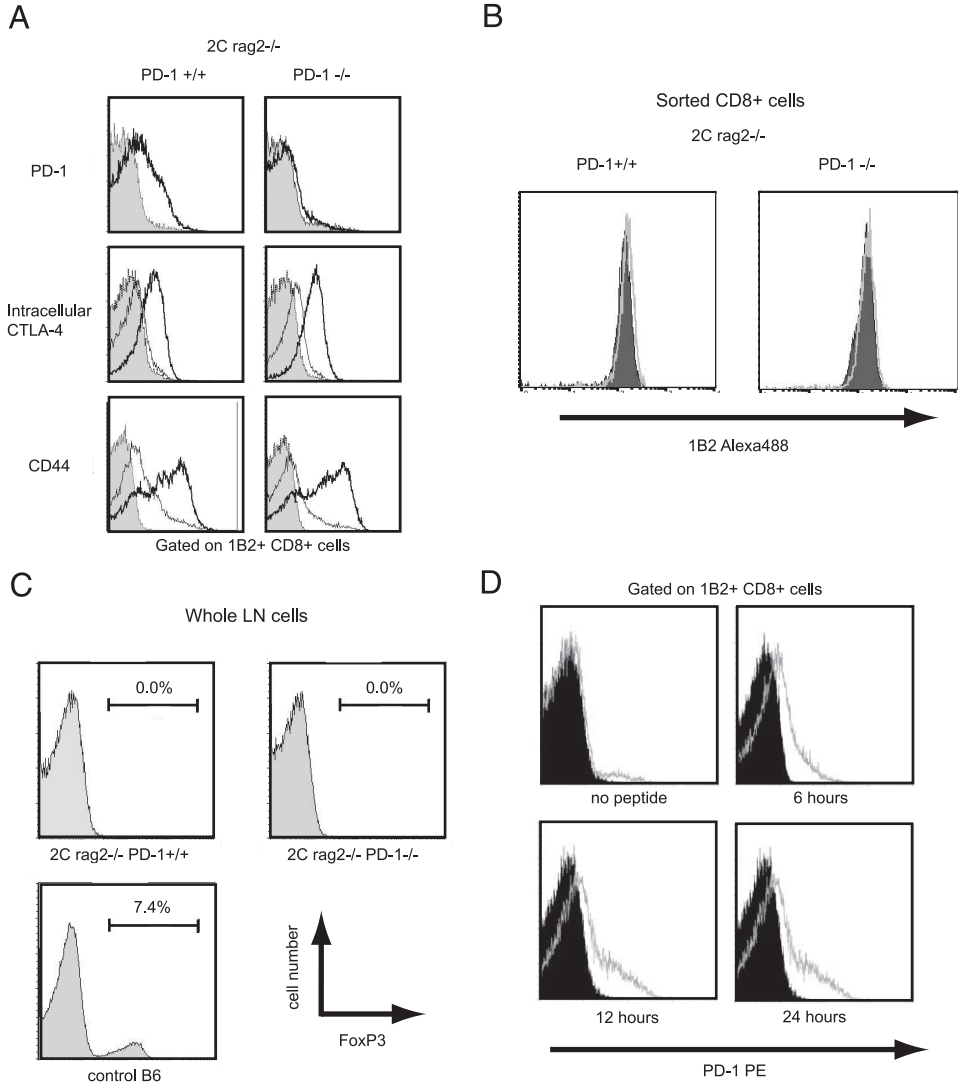

FIGURE 2. Selective involvement in and rapid up-regulation of PD-1 during anergy induction in 2C rag2$^{-/-}$ mice. *A*, Lymph node cells from peptide-treated (thick line) and PBS-treated (thin line) 2C rag2$^{-/-}$ PD-1$^{+/+}$ and PD-1$^{-/-}$ mice were isolated on day 8, stained with Alexa 488-conjugated 1B2, PerCP-CD8, and PE-conjugated anti-PD1, CTLA-4, or CD44, and analyzed by FACS. Histograms of the gated 1B2^{+} CD8^{+} cells are shown. The data were representative of three experiments. *B*, Sorted CD8^{+} cells from Fig. 1*B* was examined for Alexa 488–1B2 staining. Shaded and empty histograms are from PBS and peptide treated mice, respectively. The experiment was performed >10 times. *C*, Lymph-node cells from peptide-treated 2C rag2$^{-/-}$ PD-1$^{+/+}$ and PD-1$^{-/-}$ mice were stained with an anti-FoxP3 Ab and analyzed by FACS. The experiment was done twice. *D*, LN cells from peptide-treated 2C rag2$^{-/-}$ PD-1$^{+/+}$ mice were isolated at the indicated time points and analyzed as described in *A*. Shown data were representative of from three experiments.

In vivo cytokine measurement

Serum cytokine-Ab complexes during anergy induction were measured by BioPlex assay (Bio-Rad) and with an in vivo cytokine capture assay kit (BD Biosciences), according to the manufacturers' protocols. For the in vivo cytokine capture assay, experimental mice were given an i.p. injection of 10 μg/head of both biotinylated-anti-IL-2 and biotinylated-anti-IFN-γ mAbs 1 day before the peptide injection. Serum samples obtained 24 and 48 h after the peptide injection were analyzed by specific ELISAs. The serum from Ab-injected, PBS-treated mice was used as a 0-h control.

rIL-2 treatment in vivo

Recombinant murine IL-2 (Wako) was mixed with NaN_3-free anti-murine IL-2 mAb in PBS for 15 min in a polystyrene tube. The resulting IL-2/anti-IL-2mAb complex, which was composed of 1.5 μg IL-2 and a 50-μg mAb equivalent, was injected i.p. into one mouse per treatment.

Adoptive transfer experiments

Donor CD8^{+} cells were isolated by AUTO-MACS using CD8^{+} T cell isolation kit (Miltenyi Biotec) and i.p. injected into recipient mice. The mice immediately received i.p. injection of SIYRYYGL peptide with or without anti-IL-2 receptor Ab. Seven days later, lymph node (LN) and spleen cells were stained with anti Alexa 647 conjugated anti-2CTCR (1B2) and sorted by FACS ARIA (BD Bioscience) based on GFP and Alexa 647 fluorescence. For the experiments using rag2$^{+/+}$ C57BL/6 mice as recipients, combined LN and spleen cells were pre-enriched for CD8 T cells using AUTOMACS using anti-CD8 microbeads (Miltenyi Biotec), and subjected for FACS-based isolation.

Results

PD-1 regulates anergy induction of CD8^{+} T cells in vivo

To dissect PD-1's involvement in T cell anergy experimentally, we bred the PD-1$^{-/-}$ genotype into 2C rag2$^{-/-}$ mice, taking advantage of an established peptide-inducible anergy model (11). 2C TCR Tg T cells recognize a mimotope peptide (SIYRYYGL) in the context of syngeneic MHC H-2K^{b}. In 2C rag2$^{-/-}$ mice, there are no endogenously rearranged Ag receptor-positive CD8^{+} T cells, CD4^{+} T cells, or B cells, allowing us to test anergy induction in a genuine CD8^{+} T cell with single-Ag specificity in vivo (11). Chlonotype Ab (1B2)$^{+}$, CD8^{+} T cells from 2C rag2$^{-/-}$ PD-1$^{-/-}$ mice and 2C rag2$^{-/-}$ PD-1$^{+/+}$ mice consistently showed CD44^{low} CD62L^{high} phenotype (Fig. 1*A*), suggesting that the T cells in each case remained naive in vivo until the peptide injection.

We attempted to induce CD8^{+} intrinsic anergy in vivo, by injecting highly purified (>95% purity) antigenic SIYRYYGL peptide i.p. into 2C rag2$^{-/-}$ PD-1$^{-/-}$ and 2C rag2$^{-/-}$ PD-1$^{+/+}$ mice. One week after the peptide injection, the purified CD8^{+} T cells from the control 2C rag2$^{-/-}$ PD-1$^{+/+}$ mice showed a reduced proliferative response to stimulation with the same peptide in vitro, compared with the cells from naive animals with the same genetic background, suggestive of a typical T cell anergy induction (Fig. 1*B*). In sharp contrast, the T cells from peptide-injected 2C

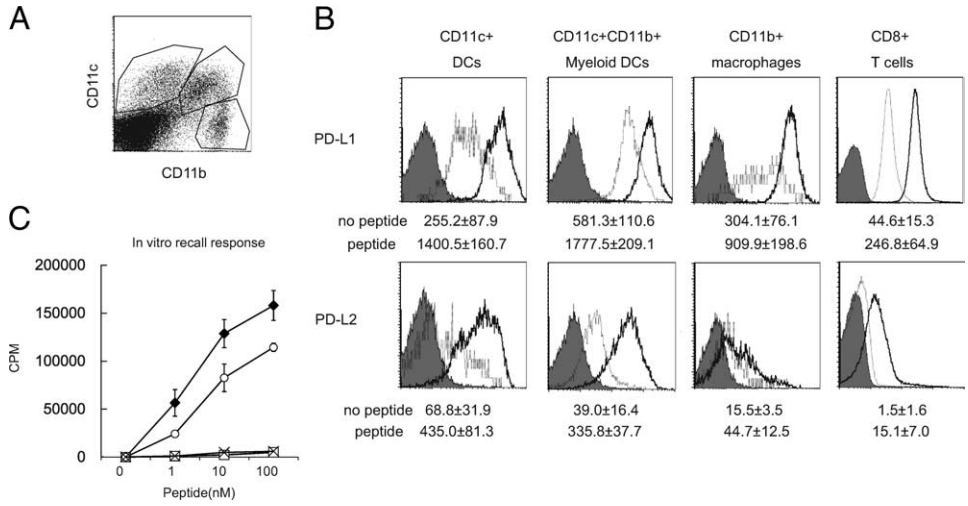

FIGURE 3. Blocking PD-1/PD-L1 interaction reverses $CD8^+$ T cell anergy. *A*, Mesenteric LN cells were immune-stained with CD11c FITC, CD11b APC, CD8 PerCP, and PE-conjugated anti-PD-L1 or PD-L2 Abs. Representative dot plot for CD11c and CD11b is shown. *B*, Mesenteric LN cells from untreated ($n = 5$; thin line) or peptide treated ($n = 5$; thick line) mice were gated as in *A*. Representative histograms for PD-ligands staining in each gating are depicted. Numbers represent average mean fluorescent intensity (MFI) after subtracting the background MFI (shaded histograms) ± SD. *C*, 2C $rag2^{-/-}PD\text{-}1^{+/+}$ were either untreated (◆) or injected with peptide (□, ○, and X). Some mice were further treated with anti 200 μg PD-L1 mAb (○) or 200 μg anti PD-L2 mAb (X) on the day of and 1 day after peptide injection. The secondary response was measured as in Fig. 1*B*. The data was representative of two independent experiments.

$rag2^{-/-}$ $PD\text{-}1^{-/-}$ mice proliferated almost as well as those from nontreated mice (Fig. 1*B*). Because CFSE-Labeled, Ag-experienced 2C $rag2^{-/-}$ $PD\text{-}1^{-/-}$ cells ex vivo produced more IFN-γ than naive 2C $rag2^{-/-}$ $PD\text{-}1^{-/-}$ cells during the secondary stimulation (Fig. 1*C*), these cells were not simply kept naive upon peptide recognition, but somehow "experienced" the Ag in vivo. Regardless of the Ag stimulation in vivo, the T cells from both the 2C $rag2^{-/-}$ $PD\text{-}1^{+/+}$ and 2C $rag2^{-/-}$ $PD\text{-}1^{-/-}$ mice proliferated similarly in response to PMA and ionomycin in vitro, suggesting that the reduced proliferation of 2C $rag2^{-/-}$ $PD\text{-}1^{+/+}$ T cells in response to the peptide was not due to reduced cell viability (Fig. 1*D*).

Lack of CTLA-4 or FoxP3 involvement in PD-1 regulated anergy of $CD8^+$ T cells

A previous report that compared 2C $rag2^{-/-}$ $CTLA\text{-}4^{+/+}$ and 2C $rag2^{-/-}$ $CTLA\text{-}4^{-/-}$ mice showed that a CTLA-4 deficiency allowed the induction of anergy by the i.p. injection of the SIYRYYGL peptide (11). We too observed the up-regulation of intracellular CTLA-4, together with the activation marker CD44, in both anergy-susceptible 2C $rag2^{-/-}$ $PD\text{-}1^{+/+}$ cells and anergy-resistant 2C $rag2^{-/-}$ $PD\text{-}1^{-/-}$ T cells (Fig. 2*A*). Equal level of TCR expression was observed between naive and peptide treated mice, excluding the reduced proliferation in secondary stimulation was due to peptide-induced TCR internalization (Fig. 2*B*). According to the previous report, the neonatal acquisition of the anergic phenotype may involve the de novo synthesis of FoxP3 positive regulatory $CD8^+$ T cells (17). However, even 72 h after anergy induction, no FoxP3-positive cells could be detected in the whole lymph node, which excludes the involvement of newly generated FoxP3-positive $CD8^+$ cells in this system (Fig. 2*C*). Although we cannot totally exclude the presence of yet-to-be defined $CD8^+$ FoxP3 negative Treg cells in our system, it is likely that PD-1 regulates the induction of CD8 T cell anergy, independent of CTLA-4 or regulatory $CD8^+FoxP3^+$ cell development.

Rapid up-regulation of PD-1 during anergy induction

We next examined the time course of PD-1 expression during anergy induction by FACS. PD-1 is expressed on activated T cells in vitro, 24–72 h after TCR stimulation. We found obvious PD-1 up-regulation 6 h after the i.p. peptide injection. The PD-1 expression peaked at 24 h, and was sustained for 8 days after the peptide injection (Fig. 2, *A* and *D*). Although we previously reported that PD-1 is up-regulated on thymocytes from C57BL/6 mice 24 h after the mice received an injection of anti-CD3 Ab (18), we found even earlier PD-1 expression in the peripheral lymph nodes upon Ag encounter in the present system.

Blockade of PD-1/PD-L1 interaction reverses T cell anergy

We next examined whether known PD-1 ligands (namely PD-L1 and PD-L2) were involved in the anergy induction in the current system. Immunostained lymph node cells were gated into four populations as follows; $CD11c^+$ (DC), $CD11b^+$ (macrophages), CD11c CD11b double positive cells (myeloid DC), and CD11c-CD11b-$CD8^+$ cells (CD8 T cells) (Fig. 3*A*). Upon peptide challenge, up-regulation of both PD-L1 and PD-L2 in all populations was observed (Fig. 3*B*). Notably, constitutive high expression of PD-L1, but not PD-L2 was seen in cells from untreated animals. Furthermore, administration of a blocking anti-PD-L1, but not anti PD-L2 Ab during first 2 days of anergy induction significantly restored the secondary response, suggesting PD-L1, but not PD-L2 triggers PD-1 for anergy induction (Fig. 3*C*).

Down-regulation of IL-2 expression by PD-1 during anergy induction

PD-1 is reported to be induced on T cells before their first division following Ag encounter in vivo, and the administration of blocking Abs for PD-1 or PD-L1 at the time of Ag encounter markedly enhances the production of effector cytokines from the T cells 4 days later (19). Together with our observations, this finding suggests that the rapid induction of PD-1 and constitutive expression of its ligand PD-L1, observed in Ag-injected 2C $rag2^{-/-}$ $PD\text{-}1^{+/+}$ mice, is important in anergy induction. Therefore, to examine PD-1's function in cytokine production after peptide injection in vivo, we used the BioPlex cytokine array, which allowed us to obtain simultaneous measurements of various cytokines from the sera of peptide-injected mice. Notably, we found that 2C $rag2^{-/-}$ $PD\text{-}1^{-/-}$ but not 2C $rag2^{-/-}$ $PD\text{-}1^{+/+}$ mice produced massive

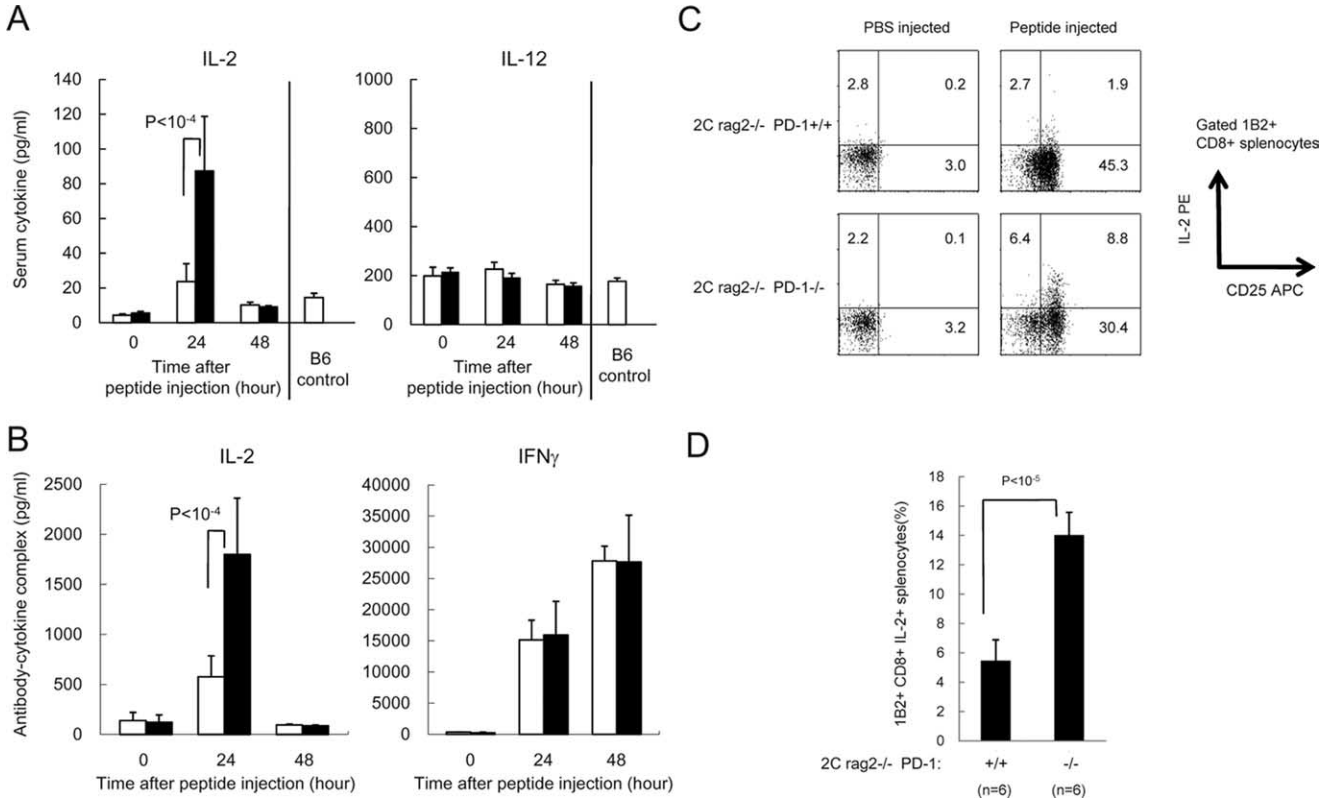

FIGURE 4. Selective down-regulation of IL-2 by PD-1 during anergy induction. *A*, Serum was collected from peptide-treated 2C rag2$^{-/-}$PD-1$^{+/+}$ (open bars) and $^{-/-}$ (closed bars) mice at the indicated time points and analyzed by a BioPlex cytokine array. Results shown are the average and S.D. from 4 mice from each group and from age-matched C56BL/6 mice (B6 control). This screening experiment was performed once. *B*, In vitro cytokine capture assays were conducted on serum samples from peptide-treated 2C rag$^{-/-}$ PD-1$^{+/+}$ (□) and PD-1$^{-/-}$ mice as described in *Materials and Methods*. Results shown are the average and SD from five mice. The data were representative of four experiments. *C*, Splenocytes from PBS or peptide injected mice were collected 6 h after the treatment. The cells were immediately fixed, permeabilized, and examined for 1B2, PerCP-CD8, APC-CD25, and PE-IL-2. Gated 1B2$^+$CD8$^+$ cells were shown for CD25 vs IL-2 staining. *D*, Percent 1B2$^+$ CD8$^+$ IL-2$^+$ cells from *C* was depicted. A student *t* test was performed for statistics. The shown data in *C* and *D* are representatives of three experiments.

amounts of IL-2 within the first 24 h following the injection (Fig. 4*A*, *left*). In contrast, a typical inflammatory cytokine, IL-12, was not induced in the same mice, suggesting that no overt inflammation occurred during anergy induction (Fig. 4*A*, *right*).

To confirm the production of IL-2 during the primary Ag encounter of PD-1-sufficient and PD-1-deficient 2C rag2$^{-/-}$ cells, we used a newly developed in vivo cytokine capture assay (20). In this method, biotin-labeled anti-cytokine Abs are injected into mice before the peptide injection; long-lived soluble cytokine-Ab complexes accumulate in the serum, enabling a much more efficient detection of cytokines by ELISA than by other conventional assays. The IL-2 production profile assessed by ELISA was parallel to that measured by the BioPlex assay, and peaked 24 h after the peptide injection (Fig. 4*B*, *left*), when the 2C rag2$^{-/-}$PD-1$^{-/-}$ mice produced ~3 times more IL-2 than the 2C rag2$^{-/-}$ PD-1$^{+/+}$ mice. Interestingly, by 48 h, there was little or no detectable IL-2 production from either the 2C rag2$^{-/-}$ PD-1$^{+/+}$ or the 2C rag2$^{-/-}$ PD-1$^{-/-}$ mice (Fig. 4*B*, *left*). We detected no difference in IFN-γ production between the PD-1-sufficient and PD-1-deficient 2C rag2$^{-/-}$ mice in simultaneous measurements (Fig. 4*B*, *right*). Our in vivo capture assay constantly resulted in higher values than BioPlex assay, that might be because it detected cytokine-Ab complex that have higher m.w. than native cytokines, or the in vivo capture Ab stabilized more cytokines as original findings suggested (20). Furthermore, to compare actual cytokine secretion by T cells from 2C rag2$^{-/-}$ PD-1$^{+/+}$ and the 2C rag2$^{-/-}$ PD-1$^{-/-}$ mice ex vivo, we immediately isolated and fixed splenocytes from the peptide-challenged mice 6 h after peptide injection, and intracellularly stained IL-2. Surprisingly, not only we detected more IL-2 from peptide-experienced 2C rag2$^{-/-}$ PD-1$^{-/-}$ than 2C rag2$^{-/-}$ PD-1$^{+/+}$ cells, but also we found that T cells from both 2C rag2$^{-/-}$ PD-1$^{-/-}$ cells and 2C rag2$^{-/-}$ PD-1$^{+/+}$ up-regulated IL-2 receptor α-chain (CD25) within 6 h of i.p. Ag injection (Fig. 4, *C* and *D*). The up-regulation of IL-2 secretion in 2C rag2$^{-/-}$ PD-1$^{-/-}$ cells, but not 2C rag2$^{-/-}$ PD-1$^{+/+}$ supported IL-2 might play important roles in determining secondary responses between two T cell populations.

Functional involvement of IL-2 in PD-1 regulated anergy of CD8$^+$ T cell

Although PD-1 may regulate IFN-γ or TNF-α in the effector phase of an immune response, our data indicated that PD-1 suppresses autonomous IL-2 production in CD8$^+$ T cells during anergy induction. A previous report suggested that foreign Ags displayed on APCs can prime CD8$^+$ T cells to become fully functional as early as 6–12 h following Ag exposure, in vivo (21). The inverse correlation we observed between PD-1 expression and IL-2 production suggested that the early inhibition of IL-2 production by CD8$^+$ cells might be a consequence of PD-1 stimulation. We therefore hypothesized that the inhibition of IL-2 production from 2C CD8$^+$ cells within 24 h was critical for CD8$^+$ T cells to acquire the anergic phenotype.

To examine whether the high IL-2 production by 2C rag2$^{-/-}$ PD-1$^{-/-}$ T cells is responsible for their anergy-resistant phenotype

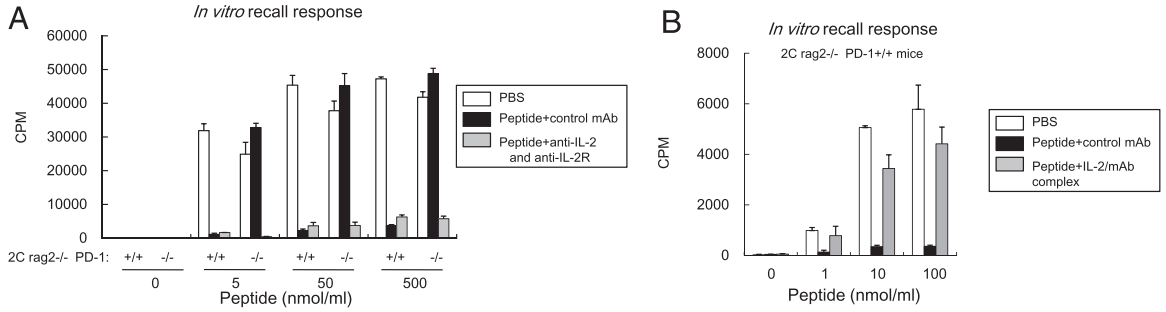

FIGURE 5. The importance of PD-1 regulation of IL-2 in T cell anergy induction in vivo. *A*, 2C rag2$^{-/-}$PD-1$^{+/+}$ and 2C rag2$^{-/-}$PD-1$^{-/-}$ mice were given an i.p. injection of control rat Ig or a combination of anti-IL-2 and IL-2 receptor mAbs (250 μg each) on days -1, 0, 1, 3, and 5, with day 0 defined as the day of the peptide injection. The in vitro proliferation of isolated 1B2^{+} cells was measured on day 8 as described in *Materials and Methods*. The data were representative of three experiments. *B*, 2C rag2$^{-/-}$ PD-1$^{+/+}$ mice received an injection of rIL-2/IL-2mAb complex on the day of peptide-injection and were analyzed as described in *A*. The data were representative of three experiments.

in vivo, an effective IL-2/IL-2R blockade was performed using specific mAbs. As shown in Fig. 5*A*, injections of a combination of neutralizing anti-IL-2 and IL-2 receptor Abs before and during anergy induction prevented the anergy resistance in the 2C rag2$^{-/-}$ PD-1$^{-/-}$ cells, which showed a dramatically reduced proliferative response 1 wk after the peptide injection.

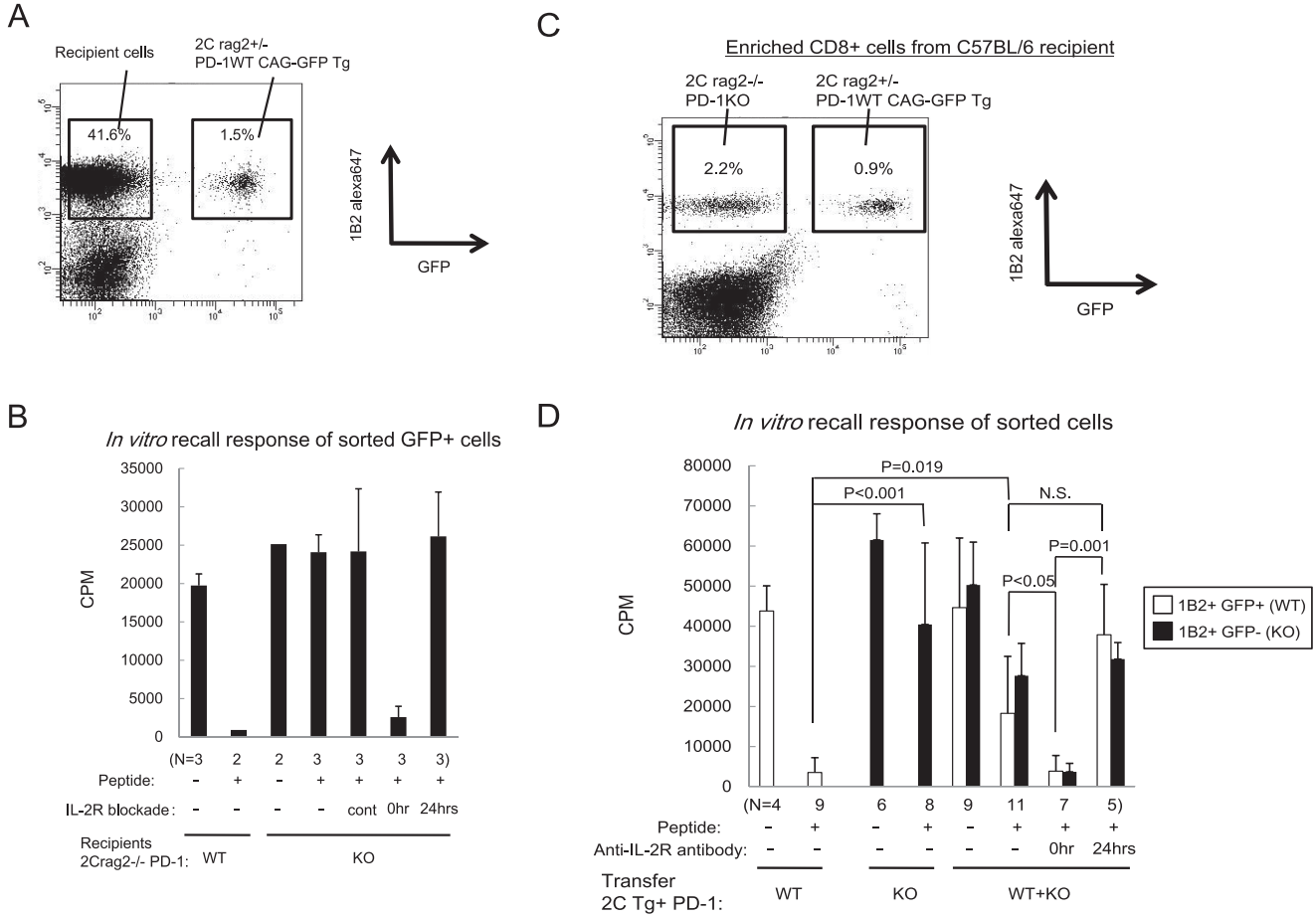

FIGURE 6. IL-2 dependent, in trans rescue of 2C PD-1 wild-type cells by 2C PD-1KO cells in vivo. *A*, One $\times$ 10^7 CD8$^+$ cells from 2C rag$^{+/-}$ PD-1$^{+/+}$ GFP Tg mice were adoptively transferred into 2C rag2$^{-/-}$ PD-1$^{+/+}$ or 2C rag2$^{-/-}$ PD-1$^{-/-}$ mice. Some recipients received nonspecific rat Ig or anti-IL-2 receptor Ab at the time of or 24 h after peptide challenge. One week later, transferred GFP$^+$ 2C$^+$ cells were sorted from combined LN and spleen cells. *B*, Two $\times$ 10^4 sorted cells from *A* were stimulated by syngeneic splenocytes and 50 nM peptide. [^{3}H]Thymidine uptake from groups containing singlet well per mouse was measured and depicted. Result was shown as average $\pm$ SD. The data were representative of two experiments. *C*, Total 2 $\times$ 10^7 CD8$^+$ cells from 2C rag$^{+/-}$ PD-1$^{+/+}$ GFP Tg, 2C rag$^{-/-}$ PD-1$^{-/-}$ mice (1 $\times$ 10^7 each) were cotransferred into C57BL/6 mice. Some recipients received anti-IL-2 receptor Ab (0.5 mg/body) at the time of or 24 h after peptide challenge. One week later, transferred cells were separately sorted based upon Alexa 647 1B2$^+$ and GFP fluorescence from combined LN and spleen cells. *D*, Two $\times$ 10^4 sorted cells from *C* were stimulated by syngeneic splenocytes and 50 nM peptide. [^{3}H]Thymidine uptake from groups containing singlet well per mouse was measured and depicted. Data from four independent experiments that included one to three mice per treatment group were combined. Total numbers of mice accumulated are indicated. Results are shown as average $\pm$ SD after excluding the highest and the lowest value from each group. Student *t* tests were performed for statistics.

If the IL-2 down-regulation caused by PD-1 were the mechanistic basis of anergy induction in the 2C rag2$^{-/-}$ PD-1$^{+/+}$ T cells, complementation of the IL-2 signal during peptide injection should reverse the anergic state. To test this hypothesis, 2C rag2$^{-/-}$ PD-1$^{+/+}$ mice received an injection of rIL-2 complexed with anti-IL-2 mAb (clone JES6–5), which was recently reported by Sprent and colleagues (22) to enhance the potency of IL-2 in vivo. We found that the administration of the IL-2/anti-IL-2 mAb complex to mice at the time of peptide-treatment significantly rescued the proliferative response of the T cells in vitro (Fig. 5*B*), suggesting that the treatment overcame the PD-1-mediated anergy induction.

PD-1 deficient CD8$^+$ T cells rescue PD-1+ CD8$^+$ T cell from anergy in an IL-2 dependent manner

Although we saw clear effect of Ab mediated blockade of IL-2-IL-2 receptor pathway or IL-2/anti-IL-2 mAb treatment controlled 2C T cell anergy, we wondered whether 3-fold difference of IL-2, detected from serum of 2C rag2$^{-/-}$ PD-1$^{+/+}$ vs 2C rag2$^{-/-}$ PD-1$^{-/-}$ mice, brought critical qualitative change during Ag experience. To directly clarify whether over-produced IL-2 by 2C rag2$^{-/-}$ PD-1$^{-/-}$ could rescue the anergy induction of PD-1 sufficient 2C cells in vivo, we genetically labeled 2C rag2$^{-/-}$ PD-1$^{+/+}$ cells by crossing them onto Chicken β-actin (CAG) promoter driven GFP Tg mice to allow the adoptive transfer experiments. The i.p. transferred 2C PD-1$^{+/+}$ CAG-GFP cells were challenged immediately by i.p. injection of SIYRYYGL peptide. One week later, transferred 2C PD-1$^{+/+}$ CAG-GFP cells, sorted from 2C rag2$^{-/-}$ PD-1$^{+/+}$ recipients lost in vitro response, while ones transferred into 2C rag2$^{-/-}$ PD-1$^{-/-}$ recipients totally retained response (Fig. 6, *A* and *B*, first to fourth bars). In this setting, an administration of the IL-2 receptor Ab at the time of peptide challenge completely blocked "rescue of anergy" of 2C rag2$^{-/-}$ PD-1$^{+/+}$ cells in the 2C rag2$^{-/-}$ PD-1$^{-/-}$ recipient, while Ab administration at 24 h did not have any effects (Fig. 6*B*; fifth to seventh bars). This result has three implications: 1) 2C rag2$^{-/-}$ PD-1$^{-/-}$ cells prevented anergy induction of 2C rag2$^{-/-}$ PD-1$^{+/+}$ in trans, 2) the effect was IL-2 dependent, and 3) IL-2 blockade at the peak of IL-2 production by 2C rag2$^{-/-}$ PD-1$^{-/-}$ cells only dampened 2C rag2$^{-/-}$ PD-1$^{-/-}$ cells dependent 2C rag2$^{-/-}$ PD-1$^{+/+}$ anergy-resistance.

PD-1-regulated IL-2 dependent anergy occurs in nonlymphopenic mice

Finally, we tested whether PD-1-regulated IL-2 dependent anergy occurred in nonlymphopenic, rag2-sufficient, mice. Cotransfer of 2C rag2$^{+/-}$ PD-1$^{+/+}$ (CAG-GFP positive) and 2C rag2$^{-/-}$ PD-1$^{-/-}$ (GFP negative) cells into a syngeneic C57BL/6 recipients, and the extensive sorting of cells allowed us to follow the fate of transferred cells (Fig. 6*C*). Singly transferred 2C rag2$^{-/-}$ PD-1$^{+/+}$ cells, but not 2C rag2$^{-/-}$ PD-1$^{-/-}$ cells lost their responsiveness after the transfer and challenge with SIYRYYGL peptide (Fig. 6*D*; second and fourth groups, $p < 0.001$). However, when two cell populations were cotransferred, 2C rag2$^{-/-}$ PD-1$^{+/+}$ cells retained a significant response at the secondary stimulation (Fig. 6*D*; second vs sixth groups, $p = 0.019$). The retention of the response was reversed by anti IL-2 receptor Ab administration at the time of peptide challenge (Fig 6*D*, sixth vs seventh groups, $p < 0.05$), but not by the Ab treatment 24 h later (Fig. 6*D*, fifth vs seventh groups, $p = 0.019$). Thus, IL-2 down-regulation by PD-1 induced CD8$^+$ T cell anergy in nonlymphopenic hosts.

Discussion

It had not been previously established whether anergy is induced and/or maintained by negative regulatory molecules. In this study, we clearly demonstrated that PD-1, and not CTLA-4, was the critical inhibitory receptor for the induction of anergy in CD8 T cells. In our current system, the PD-1-mediated tolerance did not involve thymic negative selection, CD4 help, or the de novo synthesis of FoxP3$^+$ regulatory population(s). Instead, we found that PD-1 down-regulated the autonomous IL-2 production by CD8$^+$ T cells, and this occurred within the first 24 h. Irrespective of the PD-1 expression on T cells, IL-2 blockade during anergy induction resulted in T cell anergy, and IL-2 complementation resulted in anergy resistance. We therefore propose that PD-1 plays a central role in anergy induction by negatively regulating the autonomous production of IL-2 by CD8$^+$ T cells. Because our current system relies on a unique MHC-class I restricted TCR Tg mice system in which the most T cells recognize a single specific peptide, it would be interesting to generalize the findings using other Tg model (14) as well as endogenous CD8 T cells using MHC-tetramer as detection reagents.

We found that most cell populations within lymph node constitutively expressed high PD-L1, and PD-L2 to the lesser extent. Although both PD-L1 and PD-L2 were up-regulated upon peptide challenge, in vivo blocking experiment clearly demonstrated PD-L1, but not PD-L2 is a functional ligand of PD-1 in the anergy induction. The data are consistent with previous reports, in which blocking anti-PD-L1 mAb, but not anti-PD-L2 mAb reversed anergy induction/maintenance in a CD8 (14) or CD4$^+$ T cell system (23). As other example, NOD mice deficient for PD-L1, but not PD-L2 become accelerated spontaneous autoimmune diabetes, suggesting a role of PD-L1/PD-1 pathway, but not PD-L2 in peripheral tolerance (24). Currently, it is not clear why two known PD-1 ligands have functional differences in vivo. Considering PD-1 mediated anergy occurs within 24 h after Ag exposure, it is tempting to speculate that highly expressed PD-L1, but not PD-L2 during first 24 h of Ag encounter preferentially engages PD-1 for the rapid establishment of anergy. We previously showed that resting DC induce peripheral CD8$^+$ T cell anergy against a Tg viral Ag in a PD-1-dependent manner (13) Although PD-L1 is expressed on most cells within the lymph node, it will be interesting to determine which APCs (or CD8$^+$ T cells themselves) provide PD-L1 for anergy induction.

When a pathogen infects an animal, inflammatory cytokines such as IL-12 or IFNα secreted by activated DC break CD8$^+$ T cell tolerance, causing the cells to differentiate into effector T cells. In contrast, the common γ-chain related cytokines, such as IL-2, IL-7, and IL-15, are the primary determinants of CD8$^+$ T cell homeostasis and memory-cell formation. For instance, although IL-2 was initially reported not to play an important role in the primary CD8$^+$ T cell response in vivo, a recent report showed that IL-2 receptor-deficient CD8$^+$ T cells have a largely impaired secondary, but not primary, response against virus infection compared with IL-2 receptor-sufficient CD8$^+$ T cells (25). In a gain-of-function experiment, the injection of a stimulatory IL-2-anti-IL-2 mAb complex during a primary viral challenge augments the programming of CD8 T cells for a massive secondary response (25). In the present system, neither IL-12 nor IFNα was detected in the sera of peptide-injected animals (Fig. 3*A* and data not shown). Our current results expand on the previous findings by supporting the idea that IL-2 not only regulates memory formation during pathogen infection (25), but also regulates anergy induction when no overt inflammation occurs.

Recently, PD-1 was suggested to play a critical role in the maintenance of the anergic T cell phenotype in individuals with chronic viral infections or tumors. Such cells have defective IFN-γ and TNF α production, a phenomenon known as "functional T cell exhaustion" (12). Interestingly, these exhausted T cells are functionally rescued by a blockade of the PD-1/PD-ligand pathway in vivo. In this study, we have provided a simple explanation for T cell anergy mediated by PD-1. It will be interesting to determine whether such functional exhaustion against persistent Ags is also maintained by the continuous blockade of IL-2 production by PD-1 and/or its associated signaling pathways.

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Disclosures

The authors have no financial conflict of interest.

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