Programmed death-1 (PD-1) modulates regulatory T cell homeostasis during low-dose IL-2 therapy

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- PD-1 regulates IL-2-induced Treg proliferation and prolongs Treg survival in murine models and in patients receiving low-dose IL-2 therapy

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Abstract:

CD4+Foxp3+ regulatory T cells (Tregs) play a central role in the maintainance of immune tolerance after HSCT. We previously reported that low-dose IL-2 therapy increased circulating Tregs and improved clinical symptoms of chronic GVHD, however, the mechanisms which regulate Treg homeostasis during IL-2 therapy have not been well studied. To elucidate these regulatory mechanisms, we examined the role of inhibitory coreceptors on Tregs during IL-2 therapy in a murine model and in patients with chronic GVHD. Murine studies demonstrated that low-dose IL-2 selectively increased Treg and simultaneously enhanced the expression of Programmed death-1 (PD-1), especially on $CD44^{+}CD62L^{+}$ central-memory Treg, while expression of other inhibitory molecules including CTLA-4, LAG-3 and TIM-3 remained stable. PD-1 deficient Tregs showed rapid Stat5 phosphorylation and proliferation soon after IL-2 initiation but thereafter Tregs became pro-apoptotic with higher Fas and lower Bcl-2 expression. As a result, the positive impact of IL-2 on Treg was completely abolished and Treg levels returned to baseline despite continued IL-2 administration. We also examined circulating Tregs from patients with chronic GVHD receiving low-dose IL-2 and found that IL-2-induced Treg proliferation was promptly followed by increased PD-1 expression on memory Treg. Notably, clinical improvement of GVHD was associated with elevated levels of PD-1 on Tregs, suggesting that the PD-1 pathway supports Treg-mediated tolerance. These studies indicate that PD-1 is a critical homeostatic regulator for Treg by modulating proliferation and apoptosis during IL-2 therapy. Our findings will facilitate the development of therapeutic strategies that modulate Treg homeostasis to promote immune tolerance.

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) provides curative therapy for patients with various hematologic malignancies, bone marrow failure syndromes and congenital immune deficiencies. Improvements in immune suppressive therapy and supportive care have improved patient outcomes but chronic graft-versus-host disease (GVHD) remains a major problem affecting long-term survivors.[1-3] The clinical and laboratory manifestations of chronic GVHD closely resemble those of autoimmune diseases and both T and B cell responses play a role in disease pathogenesis, suggesting that chronic GVHD reflects a general loss of immune tolerance including abnormalities in the function of $CD4^{+}Foxp3^{+}$ regulatory T cells (Tregs).[4-9]

Tregs are a functionally distinct subset of mature T cells, which have a critical role in the development and maintenance of immune tolerance.[10-12] Tregs are physiologically primed and constitutively proliferate in the presence of interleukin-2 (IL-2) and promptly mediate active suppression to prevent excessive inflammation.[13-15] Patients with chronic GVHD and other autoimmune diseases have a relative deficiency of Tregs[16-21] and enhancement of Treg function can prevent allograft rejection and suppress autoimmune activity[22-24], indicating Tregs play an essential role in the establishment of life-long tolerance between recipient tissues and donor-derived immunity after allogeneic HSCT.[25]

In contrast to the genetic Treg deficiency in patients with IPEX syndrome[26], Tregs after HSCT are derived from genetically-normal healthy donors and Treg deficiency in this setting appears to be a consequence of homeostatic abnormalities in the post-transplant lymphopenic environment wherein increased proliferation of Tregs is not sufficient to compensate for reduced thymic output and increased susceptibility to apoptosis.[18,21,27] Importantly, abnormal

Treg homeostasis in patients with chronic GVHD can be restored by the supplemental administration of low-dose IL-2.[28-30] In previous clinical trials, daily therapy with low-dose IL-2 for 8-12 weeks in patients with chronic GVHD led to a rapid increase in circulating Tregs, without a significant increase in $CD4^+$ or $CD8^+$ effector T cells. This was associated with improvement of clinical GVHD symptoms in more than 50% of patients.[28,29] Similar results of low dose IL-2 therapy have been reported in healthy donors as well as patients with various autoimmune diseases.[31-34].

Prolonged therapeutic intervention is often needed in patients with chronic GVHD as well as other autoimmune-based disorders. In our previous studies, patients with clinical improvement were eligible to continue low-dose IL-2 therapy for prolonged periods and some patients have continued IL-2 treatment over 1 year. In these patients, increased levels of circulating Tregs are maintained for the entire duration of IL-2 therapy and contribute to clinical improvement.[29] However, the mechanisms which regulate Treg homeostasis under the pressure of exogenerous IL-2 are not well understood. Since IL-2 can induce apoptosis of T cells with an activated/memory phenotype[14], cell-intrinsic inhibitory pathways appear to be needed to prevent apoptosis of activated Tregs and maintain homeostasis during prolonged IL-2 therapy. Although inhibitory co-receptors are known to play important roles in the regulation of effector T cells[35], their functions in activated Tregs have not been well characterized.

In the present study, we examined the role of inhibitory receptors on Treg homeostasis in vivo. Among these inhibitory receptors, we found that exogenous IL-2 induced Treg expression of PD-1 without increased expression of CTLA-4, LAG-3 or Tim-3. Expression of PD-1 was most evident in central-memory Tregs and increased PD-1 expression was

maintained during IL-2 therapy. PD-1 blockade negated these effects of IL-2, promoted Treg apoptosis and reduced Treg numbers in vivo. In contrast, PD-1 expression did not reduce the suppressive activity of Tregs. These results demonstrate that PD-1 signaling has a critical role in maintaining Treg homeostasis and contributes to the maintenance of immune tolerance.

Methods

Mice

C57BL/6 (B6) mice were purchased from CLEA Japan (Tokyo, Japan). PD-1 deficient mice (PD-$1^{-/-}$) with a B6 background were purchased from RIKEN BRC.[36-38] All mice were maintained under specific-pathogen-free conditions and used at the age of 8-12 weeks. All animal experiments were performed according to the regulations of the Animal Care and Use Committee, Okayama University Advanced Science Research Center. For in vivo experiments, recombinant IL-2 (teceleukin) and anti-mouse PD-1 antibody (RMP1-14) were used. IL-2 was administered by subcutaneous injection in either wild type or PD-$1^{-/-}$ mice without transplant conditioning or transplantation of hematopoietic stem cells.

Flow cytometry for murine experiments

Single cell suspensions were first incubated with the following directly conjugated monoclonal antibodies (obtained from eBioscience unless otherwise stated) for 20 min at 4°C: eFlour450 conjugated anti-CD4 (RM4-5) and anti-CD3 (17A2); FITC, PE or PE-Cy7 conjugated anti-CD25 (FITC, 7D4; BD Biosciences, PE and PE-Cy7, PC61.5); FITC conjugated anti-CD62L (MEL14) anti-PD-L2 (122), and anti-LAG-3 (eBioC9B7W); PE conjugated anti-PD-1 (RMP1-30), anti-PD-L1 (MIH5), anti-Tim-3 (RMT3-23) anti-Fas (15A7) and Annexin-V; PerCP-Cy5.5 conjugated anti-CD8 (53-6.7) and anti-CD11c (N418); PE-Cy7 conjugated anti-MHC Ⅱ-1A/1E (M5/114.15.2); APC conjugated CD19 (MB19-1); APC-eFlour780 conjugated anti-CD8 (53-6.7) and anti-CD44 (IM7). For intracellular staining, we processed cells using Foxp3 staining buffer set (eBioscience) and incubated with FITC conjugated anti-Bcl2 (10C4) and anti-CTLA-4 (UC10-4B9) and APC conjugated anti-Foxp3 (FJK-16S) for 30 min at 4°C. Bcl-2 and Fas expression were calculated as follows: Bcl-2 (ratio) = Bcl-2 (MFI) of IL-2 treated mice / Bcl-2 (MFI) of control mice and

Fas (ratio) = Fas (MFI) of IL-2 treated mice / Fas (MFI) of control mice. Samples were analyzed on MAQSQuant flow cytometer (Miltenyi Biotec) and data were analyzed using Flowjo software (Tree Star).

In vitro suppression assay

$CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ Tcons were isolated from murine spleen cells by $CD4^+CD25^+$ regulatory T cell isolation kit and autoMACS Pro Separator (Miltenyi Biotec). Sorted cells were confirmed to be more than 95% pure. Responder Tcons purified from naïve B6 mice were labeled with CellTrace™ Violet according to the manufacturer's protocols, and were cultured with suppressor Tregs in the presence of anti-CD3/28 dynabeads (Dynal) in 96-well round-bottom plates. After 3 days, cells were harvested and incubated with PerCP-Cy5.5 conjugated anti–CD4 (RM4-5; eBioscience). Proliferations of Tcon were analyzed on MAQSQuant flow cytometer.

Patient characteristics

Laboratory studies were undertaken in 14 patients with chronic GVHD who were enrolled in a Phase1 clinical trial of low-dose IL-2 at the Dana-Farber Cancer Institute, Boston, MA (Table1) [28]. The clinical protocol was approved by the Human Subjects Protection Committee of the Dana-Farber/Harvard Cancer Center. Written informed consent was obtained from each patient before sample collection, in accordance with the Declaration of Helsinki. Recombinant IL-2 (aldesleukin) was administrated subcutaneously once daily for 8 weeks. Seven patients received 3.0×10^5 IU IL-2/m2 per day, and seven received 1.0×10^6 to 1.5×10^6 IU IL-2/m2 per day. Blood samples were obtained before and 1, 2, 4, 6, 8, and 12 weeks after starting IL-2.

Flow cytometry for human subjects

PBMCs were incubated with the following directly conjugated monoclonal antibodies for 20 min at 4°C: Pacific Blue conjugated anti-CD4 (RPA-T4; BD Biosciences), PE-Cy7 conjugated anti–CD25 (M-A251; BD Biosciences), FITC conjugated anti-CD45RA (ALB11; Beckman Coulter), PE conjugated anti-PD1 (eBioJ105; eBioscience), and APC-Flour750 conjugated anti-CD127 (eBioRDR5; eBioscience). To detect intracellular Foxp3, we incubated them with PE conjugated anti-Foxp3 (PCH101) for 30 min at 4°C by using Foxp3 staining buffer set (eBioscience). Samples were analyzed on FACSCanto II (BD Biosciences) and data were analyzed using Flowjo software (Tree Star).

In vitro proliferation assay

For in vitro proliferation assay of human lymphocytes, $CD45RA^+$ Treg and Tcon cells were isolated from a patient receiving low-dose IL-2 by cell sorting with FACSAria. Sorted cells were confirmed to be more than 95% pure. Cells were labeled with CFSE (Invitrogen) according to the manufacture's protocol and were cultured separately in the presence of anti-CD3 antibody (0.1 ug/ml) (OKT3; eBioscience), anti-CD28 antibody (1 ug/ml) (L293; BD Biosciences) and functional anti-PD-L1 antibody (MIH1; eBioscience) in 96-well round-bottom plates at a concentration of 1×10^4 T cells per well. After 4 days, cells were harvested and incubated with Pacific Blue-conjugated anti-CD4 (RPA-T4; BD Biosciences), PE-conjugated anti-PD-1 (eBioJ105; eBioscience) and PECy7-conjugated anti-CD45RA (HI100; BD Biosciences). Cell death was assessed by APC-conjugated Annexin-V staining and forward to side scatter profiles.

Statistical analysis

In murine experiments, results are presented as means +/- SEM. The Student's t test was used

to assess statistical significance between two groups and 1-way ANOVA was used to compare >2 groups. *P* values < 0.05 were taken to indicate statistically significant. In human subject analyses, the Wilcoxon signed rank test was performed for paired group comparisons. All tests were two-sided at the significance level of 0.05.

Results

Low dose IL-2 administration selectively activates Tregs in a murine model.

We first determined the dose of IL-2 needed to activate Tregs in a murine model. Each T cell subset was defined as shown in Fig. 1A. To compare the response of T cell subsets to IL-2 in vitro, spleen cells were stimulated with IL-2 for 30 minutes and phosphorylation of Stat5 was evaluated by flow cytometry. High concentrations of IL-2 (100 to 10000 IU/ml) induced Stat5 phosphorylation all T cell subsets. However, lower concentrations of IL-2 (1 to 10 IU/ml), only induced Stat5 phosphorylation in Tregs (Fig. 1B, left). To compare the response of T cell subsets to IL-2 in vivo, a single dose of IL-2 was administered to naïve mice and pStat5 was measured in splenic T cells. Consistent with in-vitro experiments, Stat5 was phosphorylated in all T cell subsets after treatment with relatively high doses of IL-2 (>20,000 IU/mouse). In contrast, Stat5 phosphorylation was observed only in Tregs after injection of low dose IL-2 (<5,000 IU/mouse) (Fig. 1B, right). To evaluate effects of prolonged treatment on T cells, mice received control vehicle, 5,000 IU or 20,000 IU IL-2 once daily for 14 days. Administration of 5,000 IU IL-2/day selectively increased Treg proliferation, leading to the expansion of this subset. In contrast, administration of 20,000 IU IL-2/day induced proliferation of other T cell subsets including CD8 T cells and CD4 Tcons (Fig. 1, C and D). Administration of 5,000 IU IL-2/day did not affect either CD8 T cells or Tcons (Fig. 1E-G). To determine whether IL-2-expanded Tregs maintain suppressive activity, we purified Tregs from IL-2-treated mice and control mice and compared their suppressive function in-vitro. As shown in Fig. 1H, IL-2-expanded Tregs suppressed the proliferation of responder Tcons and the suppressive activity was comparable to control Tregs (Fig. 1I). These results indicate that administration of 5,000 IU IL-2/day is sufficient to provide Tregs

with homeostatic signals to initiate proliferation without affecting other T cell subsets.

In vivo IL-2-expanded Tregs exhibit central memory phenotype with enhanced PD-1 expression.

To examine the effect of low dose IL-2 on Treg differentiation, we examined the phenotype of IL-2-expanded Tregs. Mice received control vehicle or 5,000 IU IL-2 once daily for 14 days and spleen cells were analyzed on day15. At this dose, IL-2 treatment did not affect the phenotype of CD8 T cells or CD4 Tcons. However, compared to control mice, IL-2-treated mice had decreased frequency of Treg with a naïve phenotype ($CD44^{low}CD62L^{high}$) and increased frequency of Tregs with a central-memory phenotype ($CD44^{high}CD62L^{high}$) (Fig. 2, A and B). IL-2 induced proliferation of both $CD44^{high}CD62L^{high}$ central-memory and $CD44^{high}CD62L^{low}$ effector memory Tregs but expression of Ki67 was significantly higher in the central memory Treg subset (Fig. S1, A and B). We did not observe any change in expression of CCR7 and CCR4 chemokine receptors on central-memory Treg suggesting that low-dose IL-2 did not affect cell migration (Fig. S1, C). Interestingly, central-memory Tregs in IL-2-treated mice expressed higher levels of PD-1 compared to vehicle-treated mice (Fig. 2, C and D). In contrast, Treg expression of other inhibitory molecules including CTLA-4, LAG-3 was not increased and TIM-3 expression was only slightly increased after IL-2 administration (Fig. 2E). We also examined the effect of IL-2 therapy on PD-Ligand expression on each T cell subset and antigen presenting cells. These studies demonstrated that low dose IL-2 resulted in a small increase of PD-L1 expression on Tregs (Fig. S2, A and B). PD-L1 and PD-L2 expression on $CD11c^{+}$ MHC class II^{+} dendritic cells did not change during IL-2 administration (Fig. S2, C and D).

PD-1 blockade results in failure of durable Treg expansion after IL-2 stimulation.

To examine the role of increased PD-1 expression on Tregs during low dose IL-2 therapy, we inhibited the PD-1 pathway using anti-PD-1 antibody. C57BL/6 mice received anti-PD-1 antibody twice weekly for a total of 4 doses and/or IL-2 once daily for 14 days. Peripheral blood cells were collected at day 0, 4, 8, 11, and 15, to evaluate the proliferation of each subset. Proliferation of Tregs was significantly greater on day 7 in mice that received both IL-2 and PD-1 antibody compared to mice that received IL-2 alone. However, vigrous Treg proliferation was not maintained thereafter (Fig. 3A). Similarly, combined treatment with IL-2 and PD-1 antibody induced a rapid increase in the frequency of Treg, but this was not maintained. In contrast, IL-2 treatment without PD-1 blockade resulted in a durable increase in the frequency of Tregs (Fig. 3B). These data indicate that PD-1 plays an important role in maintaining increased levels of Treg during IL-2 administration.

PD-1 signaling prevents central-memory Tregs from differentiating into apoptosis-prone effector-memory Tregs.

We next explored the mechanisms whereby PD-1 contributes to Treg homeostasis using PD-1$^{-/-}$ mice. PD-1 deficient mice and control wild-type (WT) mice received IL-2 once daily for 4 weeks and splenic T cell subsets were analyzed weekly. In WT mice, the percentage of Treg continued to increase during the first 14 days and remained stable thereafter (Fig. 4A). Consistent with PD-1 blocking experiments, the initial peak of Treg expansion was higher in PD-1 deficient mice. Indeed, after 1 week of IL-2 therapy, pStat5 expression in Tregs increased more in PD-1 deficient mice than in control wild-type mice, resulting in the rapid

increase of proliferation, percentage and absolute number of Tregs (Fig. 4B-G). However, the number of Tregs in PD-1 deficient mice returned to baseline levels by day 14. In contrast, Tregs continued to increase in PD-1^{WT} mice (Fig. 4A). On day 14, Tregs in PD-1 deficient mice treated with IL-2 were predominantly $CD44^{high}CD62L^{low}$ effector-memory type. In contrast, Tregs in PD-1^{WT} mice treated with IL-2 were predominantly $CD44^{high}CD62L^{high}$ central-memory type (Fig. 4, H and I). Notably, accumulating $CD44^{high}CD62L^{low}$ effector-memory Tregs in PD-1 deficient mice were Annexin-V positive (Figure 4J). IL-2 treated PD-$1^{-/-}$ Tregs also showed decreased expression of anti-apoptotic Bcl-2 (Fig. 4K) and increased expression of pro-apoptotic Fas (CD95) (Fig. 4L). As shown in Fig. S3A, IL-2 treated PD-$1^{-/-}$ Tregs isolated at week 2 suppressed proliferation of responder wild type Tcons (Fig. S3B) that was identical to Tregs isolated from PD-1^{WT} mice, indicating that IL-2 treated PD-$1^{-/-}$ Tregs maintain suppressive activity. These results suggest that PD-1 signaling stabilizes Treg expansion during IL-2 intervention by maintaining Tregs in a central-memory phenotype and inhibits their differentiation to an apoptosis-prone effector-memory phenotype.

Enhanced expression of PD-1 on human Tregs during low dose IL-2 therapy modulates long term stable homeostasis.

To examine the role of PD-1 expression on human Tregs, we studied peripheral blood samples from 14 patients enrolled in a Phase 1 clinical trial of low-dose IL-2 for chronic GVHD.[28] Within the CD4 T cell gate, Tregs were identified as $CD25^{med\text{-}high}CD127^{low}$ and Tcons were identified as $CD25^{neg\text{-}low}CD127^{med\text{-}high}$. Tregs and Tcons were further divided into subsets with $CD45RA^{+}$ naïve and $CD45RA^{-}$ activated/memory phenotype, as shown in Fig.

5A. Before IL-2 therapy, both CD45RA$^+$ naïve Tregs and Tcons showed little expression of PD-1. In contrast, both CD45RA$^-$ activated/memory Tregs and Tcons showed significantly higher expression of PD-1 than their naïve counterparts and there was no significant difference in PD-1 expression between CD45RA$^-$ Tregs and Tcons. After starting IL-2, expression of PD-1 rapidly increased in CD45RA$^-$ Tregs, while PD-1 expression did not change in other CD4 T cell subsets, including CD45RA$^+$ Tregs, CD45RA$^+$ Tcons and CD45RA$^-$ Tcons (Fig. 5B). PD-1 expression on CD45RA$^-$ Tregs reached maximal levels 4 weeks after starting IL-2 and remained elevated during the entire 8 week treatment period. PD-1 expression in CD45RA$^-$ Tregs returned to baseline levels 4 weeks after stopping IL-2 therapy.

Though PD-1 expression increased in CD45RA$^-$ activated/memory Tregs, PD-1 expression did not increase in CD45RA$^+$ naïve Tregs during IL-2 therapy. To examine the proliferative potential of CD45RA$^+$PD-1^{neg} naïve cells during IL-2 therapy, we purified CD45RA$^+$ Tregs and Tcons by cell sorting from a patient receiving IL-2 and cultured each fraction separately in the presence of CD3/CD28 stimulation with or without PD-1 blockade. CD3/CD28 stimulation resulted in limited proliferation of naïve Tregs from patients during IL-2. However, vigorous naïve Treg proliferation was observed when the PD-1 pathway was blocked (Fig. 6A). Rapidly proliferating Tregs expressed high levels of Annexin-V (Fig. 6, B and C). In contrast, the effect of PD-1 blockade on CD45RA$^+$ naïve Tcons was relatively small (Fig. 6A).

To evaluate the impact of early upregulation of PD-1 on clinical outcome, we compared

expression of PD-1 in 6 patients with clinical improvement of chronic GVHD (clinical responders) with 5 patients without clinical improvement (clinical non-responders) after IL-2 therapy. Two weeks after IL-2 therapy began, PD-1 was more highly expressed on Tregs compared to Tcons in clinical responders (Fig. 7A). In contrast, the level of PD-1 expression was similar on Tregs and Tcons in clinical non-responders (Fig. 7A). When PD-1 expression at 2 weeks was compared to PD-1 expression prior to starting IL-2, there was no change in Treg-PD-1/Tcon-PD-1 ratio in non-responders. In contrast, Treg-PD-1/Tcon-PD-1 ratio increased significantly ($p=0.03$) in clinical responders 2 weeks after beginning low-dose IL-2 therapy (Fig. 7B). These results suggest that the rapid increase of PD-1 expression on Tregs after starting IL-2 therapy contributes to the maintenance of Treg expansion which faciliates the clinical response to IL-2.

Discussion

In inflammatory microenvironments, activated effector T cells produce IL-2 which supports the further expansion of activated effector T cells in a positive feedback loop. However Tregs also respond to secreted IL-2 to inhibit effector T cells and suppress inflammation.[39] The constitutive expression of high-affinity IL-2 receptors enables Tregs to promptly respond to low concentrations of IL-2 without antigen-specific activation of T cell receptors.[40] However, the homeostatic mechanisms that regulate the Treg response to IL-2 are not well understood.

PD-1 is a co-inhibitory receptor of the B7:CD28 family that negatively regulates T cell activation after interaction with specific ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC).[41-44] Expression of PD-1 is up-regulated on effector T cells during chronic antigen stimulation in the context of persistent viral infections. Effector T cells also often express PD-1 in the tumor microenvironment and PD-1 mediated "immune exhaustion" of effector T cells is associated with immune dysfunction and disease progression.[45,46] In patients with cancer, recent clinical trials have demonstrated that blockade of the PD-1 pathway by in vivo administration of anti-PD-1 or anti-PD-L1 antibodies can reverse PD-1 mediated T cell dysfunction resulting in durable tumor regression.[47-52] While many studies have examined the role of PD-1 in the suppression of effector T cells and NK cells[53], the role of PD-1 in the regulation of Tregs has not been established.

To examine the role of PD-1 in Treg homeostasis in vivo, we developed a murine model of low dose IL-2 therapy and determined that daily administration of 5,000 IU IL-2 was sufficient to selectively activate and expand Tregs in vivo without inducing expansion of

other T cell subsets. Importantly, daily IL-2 administration at this low dose resulted in the expansion of central memory Tregs that was similar to the effects of daily IL-2 therapy previously observed in patients with chronic GVHD. Further characterization of expanded Tregs demonstrated that PD-1 expression was increased on these cells while other immune inhibitory molecules including CTLA-4, LAG-3 and Tim-3 remained stable. When administration of IL-2 was combined with anti-PD-1 antibody, the initial response to IL-2 was enhanced. Notably, Tregs examined after 7 days of treatment exhibited higher levels of proliferation and the % Treg in peripheral blood was increased compared to mice that received IL-2 alone. However, this high level of Treg proliferation was not sustained, and by day 15, the frequency of Tregs in PBMC was lower in mice that received IL-2 plus anti-PD-1 compared with mice that received IL-2 alone. Similar results were obtained when daily IL-2 was administered to PD-1 deficient mice. In this setting, PD-$1^{-/-}$ Tregs that expanded after IL-2 therapy were predominately effector memory Tregs with a significantly higher fraction of apoptotic cells. When compared to PD-1^{WT} Tregs, PD-$1^{-/-}$ Tregs expressed lower levels of anti-apoptotic protein Bcl-2 and increased levels of pro-apoptotic FAS (CD95). With continued daily IL-2 for 4 weeks, there was no expansion of PD-$1^{-/-}$ Tregs. Taken together, these data suggest that the PD-1 pathway plays an important role in the regulation of terminal differentiation and apoptosis of activated Tregs.

Since other inhibitory molecules such as CTLA-4 and LAG-3 are directly involved in the suppressive function of Tregs[54,55], it was important to examine the immune suppressive function of PD-1 deficient Tregs. These experiments showed that IL-2-expanded PD-$1^{-/-}$ Tregs exhibit normal levels of suppressive activity, indicating that PD-1 does not directly

affect Treg function. Unlike effector T cells, where PD-1 expression is associated with T cell dysfunction and exhaustion, PD-1 expression in Tregs promotes the survival of these cells in inflammatory environments. Thus systemic PD-1 blockade acts to both enhance the function of effector T cells and limit the survival of Tregs. These results are consistent with previous reports in murine models of chronic viral infection where combined therapy with IL-2 plus anti-PD-L1 antibody synergistically enhanced virus-specific CD8 T cell responses and decreased viral load even though Treg numbers transiently increased.[56]

Examination of Tregs in patients receiving IL-2 therapy provided additional evidence that PD-1 plays an important role in Treg homeostasis. In previous clinical studies, daily administration of low-dose IL-2 rapidly expanded circulating Tregs without increasing effector T cells.[28,29] Increased numbers of circulating Treg persisted for the entire duration of low dose IL-2 treatment. Notably, Treg proliferation dramatically increased in the first week after starting IL-2 but returned to baseline levels by the second week of treatment.[30,40] In the current study, analysis of cryopreserved cells from patients enrolled on this trial revealed that PD-1 expression also increased on Tregs during IL-2 therapy. Whereas Treg proliferation peaked 1 week after starting IL-2, PD-1 expression increased early but did not peak until week 4. Tregs isolated during IL-2 therapy exhibited limited proliferation in vitro in response to CD3/CD28 stimulation, but Treg proliferation increased dramatically when PD-1 blockade was added to CD3/CD28 stimulation. Consistent with results in our murine model, rapidly proliferating human Tregs also expressed high levels of Annexin-V after PD-1 blockade.

The clinical relevance of these findings is suggested by the observation that objective improvement of chronic GVHD was more evident in those patients that expressed higher levels of PD-1 on Treg during IL-2 therapy. Since our study included a relatively small number of patients, larger scale studies are needed to identify the impact of PD-1 expression on Treg on clinical response to IL-2 therapy. Clarification of the clinical significance of Treg PD-1 expression as a new biomarker will allow the development of new strategies for modulating Treg homeostasis after transplantation and potentially for developing new ways of preventing or treating chronic GVHD.

Taken together, these results demonstrate that the PD-1 pathway plays a critical role in the regulation of CD4 Tregs. This is most evident when Treg expansion in vivo is promoted by the exogenous administration of low dose IL-2, but likely also occurs in the setting of chronic inflammation and endogenous activation of Tregs. In the absence of PD-1, IL-2 induces rapid proliferation and Treg expansion, but these cells also undergo terminal differentiation and become highly susceptible to apoptosis. This results in depletion of the Treg pool. In the presence of PD-1, IL-2 induced Treg proliferation is less intense but Treg do not undergo terminal differentiation. As a result Treg are less susceptible to apoptosis and expansion of the Treg pool continues as long as exogenous IL-2 is administered.

The mechanisms that regulate the expression of PD-1 by Tregs during IL-2 therapy remain to be clarified. Functional deficiency of PD-1 has been reported in a variety of human autoimmune diseases including SLE, rheumatoid arthritis, type 1 diabetes and multiple sclerosis and recent studies have suggested that single-nucleotide polymorphisms (SNPs) in

human PD-1 gene are associated with autoimmune diseases.[57,58] Further analysis of genetic polymorphisms and PD-1 function in patients receiving IL-2 may elucidate additional mechanisms that regulate PD-1 activity in Tregs and may predict clinical response to IL-2 therapy.

Although our studies were conducted to develop approaches to promote immune tolerance and reverse symptoms of GVHD, these findings are also relevant to the role of Treg in chronic viral infection and tumor immunity. It is now well established that activation of the PD-1 pathway causes dysfunction and exhaustion of effector T cells and prevents antigen-specific elimination of target cells. PD-1 blockade reverses the suppression of effector T cells, allowing effector cells to eliminate target cells in vivo.[46,59] Our results suggest that activation of the PD-1 pathway also promotes expansion of Tregs to further suppress effector T cells. In patients with cancer, PD-1 inhibition of Tregs may provide an additional mechanism whereby PD-1 blockade promotes effective tumor immunity. Our studies suggest that in the context of PD-1 blockade, administration of low dose IL-2 will promote the terminal differentiation of Treg and prevent prolonged Treg expansion. Since IL-2 can also promote the expansion of activated tumor-specific effector T cells in the tumor microenvironment, administration of low dose IL-2 in combination with PD-1 blockade may provide synergistic anti-tumor immunity through their combined effects on CD4 Tregs as well as effector T cells.

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Authorship contributions

TA designed and performed experiments and wrote the paper. JK designed and supervised the clinical trial and clinical data collection and edited the paper. HTK designed the clinical trial, performed statistical analysis for the crinical trial and edited the paper. YM, TY, YK, MI, MN and YS performed experiments and edited the paper. HY provided mAbs for the study and supervised the laboratory studies. YM and MT supervised the laboratory studies and edited the paper. JR designed the clinical trial, supervised the laboratory studies and edited the paper. KM designed and supervised the research and edited the paper.

Disclosure of Conflicts of interest

The authors declare no potential conflicts of interest.

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Table1. Clinical characteristics of patients who received IL-2 (n=14)

Characteristic	Number
M/F	5/9
Diagnosis	
AML	5
CLL	5
CML	1
HL	1
MDS	1
NHL	1
Conditioning regimen	
Myeloablative	7
Nonmyeloablative	7
Stem cell source	
PBSCs	13
BM and PBSCs	1
Donor type	
Matched-related	4
Matched-unrelated	7
Mismatched-unrelated	3
Acute GVHD prophylaxiss	
Sirolimus-containing	9
No Sirolimus-containing	5
Acute Grade 2-4 GVHD	5
Days to chronic GVHD, median (range)	269 (130-483)
Immunosuppressive therapy at start of IL-2 treatment	
Prednisone	13
Sirolimus	6
Tacrolimus	5
MMF	10

AML, Acute Myeloid Leukemia; CLL, Chronic Lymphocytic Leukemia; CML, Chronic Myelogenous Leukemia; HL, Hodgkin Lymphoma; ALL, Acute Lymphoblastic Leukemia; MDS, Myelodyplastic Syndrome; NHL, Non-Hodgkin Lymphoma; PBSC, Peripheral Blood Stem Cells; BM, Bone Marrow; MMF, Mycophenolate mofetil

Figure Legends

Fig. 1. Selective expansion of CD4 Tregs in a murine model of low dose IL-2 therapy.

(**A**) Representative lymphocyte gates for identification of CD4 and CD8 T cell subsets. Within the CD4 T cell gate, Tregs are identified as $CD4^+CD25^+Foxp3^+$ cells and Tcons are identified as $CD4^+CD25^-Foxp3^-$ cells. (**B**) IL-2 dose-dependent phosphorylation of Stat5 in T cell subsets. Left panel: Spleen cells (5×10^5 per each well) were cultured for 30 minutes in various concentrations of recombinant IL-2. Right panel: Wild type C57BL/6 mice received single doses of recombinant IL-2 and spleen cells were harvested after 30 minutes. The level of intracellular pStat5 was determined by flow cytometry. (**C-D**) Wild type C57BL/6 mice received control vehicle, 5,000 or 20,000 IU recombinant IL-2 once daily for 14 days and spleen cells were analyzed on day 15. (**C**) Representative flow cytometry histograms for identification of $Ki\text{-}67^+$ proliferating cells in CD8 T cells, Tcons and Tregs. Percentage of $Ki\text{-}67^+$ cells is shown for each histogram. (**D**) IL-2 dose dependent increase of $Ki\text{-}67^+$ proliferating cells in each T cell subset. (**E-G**) Wild type C57BL/6 mice received control vehicle or 5,000 IU recombinant IL-2 subcutaneously once daily for 14 days and spleen cells were analyzed on day 15. (**E**) Representative panel gated on CD4 T cells identifying CD4 Tregs (red box) in mice treated with vehicle control or IL-2. (**F**) Frequency of $CD4^+CD25^+Foxp3^+$ Tregs. (**G**) Number of CD8 T cells (left), Tcons (center) and Tregs (right). (**H-I**) In vitro Treg suppression assay. Tcons labeled with CellTrace^{TM}Violet from wild type C57BL/6 were cultured at 1:1 ratio with Tregs isolated from vehicle or IL-2 treated mice in the presence of CD3/28 stimulation for 3 days. (**H**) Representative flow cytometry histograms measuring Tcon proliferation in the presence or absence of Tregs. Percentage of divided Tcons is shown for each histogram. (**I**) Percentage of divided Tcons at various

Tcon:Treg cell ratios. Reponder Tcons (1×10^5 per each well) were cultured with various numbers of suppressor Tregs. n = 4 mice per group per experiment. Data are representative of two (**H-I**) or three (**A-G**) independent experiments and expressed as means +/- SEM. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

Fig. 2. Phenotypic changes in murine Tregs after IL-2 therapy.

Wild type C57BL/6 mice received control vehicle or 5,000 IU human recombinant IL-2 subcutaneously once daily for 14 days and spleen cells were analyzed on day 15. (**A**) Representative panels identify $CD44^{low}CD62L^{high}$ naïve, $CD44^{high}CD62L^{high}$ central memory and $CD44^{high}CD62L^{low}$ effector memory subsets within CD8 T cells, Tcons and Tregs. (**B**) Percentage of each subset in CD8 T cells (left), Tcons (center) and Tregs (right) after in vivo treatment with control vehicle or IL-2. (**C**) Representative flow cytometry histograms identifying $PD\text{-}1^+$ cells in each T cell subset after treatment with control vehicle or IL-2. (**D**) Percentage of $PD\text{-}1^+$ cells in CD8 T cells (left), Tcons (center) and Tregs (right) after treatment with control vehicle or IL-2. (**E**) Representative flow cytometry histograms detecting expression of CTLA-4, LAG-3, Tim-3 and PD-1 on CD8 T cells, Tcons and Tregs. n = 4 mice per group per experiment. Data are representative of three independent experiments and expressed as means +/- SEM. ***P<0.001 and ****P<0.0001. N : Naïve, CM : Central Memory, EM : Effector Memory.

Fig. 3. Effects of combined IL-2 therapy and PD-1 blockade on Treg expansion in vivo.

Wild type C57/B6 mice received vehicle (plus isotype antibody), IL-2 (plus isotype antibody), anti–PD-1 antibody (plus vehicle control) or IL-2 + anti–PD-1 antibody. 250 μg anti–PD-1

antibody was administrated intraperitoneally twice weekly for a total of 4 injections beginning on the first day of IL-2. IL-2–treated groups received 5,000 IU IL-2 once daily for 14 days. Peripheral blood cells were collected and analyzed at day 0, 4, 8, 11, and 15. (**A**) Increase of %Ki-67^{+} proliferating Tregs from the baseline level of each group during therapy. (**B**) Increase of %Tregs during therapy from the baseline level of each group. n = 4 mice per group per experiment. Data are representative of two independent experiments and expressed as means +/- SEM. *P<0.05.

Fig. 4. PD-1 deletion alters Treg homeostasis during IL-2 therapy.

C57BL/6 PD-1$^{-/-}$ or C57BL/6 wild type (WT) mice were treated with control vehicle or 5,000 IU IL-2 once daily for 4 weeks. (**A**) Effect of IL-2 therapy on frequency of Tregs during treatment. Increase of %Tregs during therapy from the baseline level of each group. (**B-H**) Spleen cells were analyzed after 1 week of IL-2 therapy. (**B**) pSTAT5 expression in Tregs. (**C**) Representative flow cytometry histograms detecting Ki-67^{+} proliferating Tregs. Percentage of Ki-67^{+} Tregs is shown for each histogram. (**D**) Percentage of Ki-67^{+} proliferating Tregs in PD-1$^{-/-}$ and PD-1^{wt} mice. (**E**) Representative histogram identifying CD4 Tregs in PD-1$^{-/-}$ and PD-1^{wt} mice receiving control vehicle or IL-2. (**F**) Frequency of CD4^{+}CD25^{+}Foxp3^{+} Tregs in spleen (% of CD4 T cells). (**G**) Number of CD4^{+}CD25^{+}Foxp3^{+} Tregs in spleen. (**H-L**) Spleen cells were analyzed after 2 weeks daily IL-2 therapy. (**H**) Representative histograms identify CD44^{low}CD62L^{high} naïve, CD44^{high}CD62L^{high} central memory and CD44^{high}CD62L^{low} effector memory Treg subsets after IL-2 therapy. (**I**) Percentage of each Treg subset after IL-2 therapy. (**J**) Percentage of Annexin-V^{+} apoptotic cells in each Treg subset after IL-2 therapy. (**K**) The ratio of Bcl-2 expression (MFI) in Treg of IL-2 treated mice / Bcl-2 expression (MFI) in Treg of control vehicle treated mice. (**L**) The ratio of Fas expression (MFI) in Treg of IL-2 treated mice / Fas expression (MFI) in Treg of

control vehicle treated mice. n = 4 mice per group per experiment. Data are representative of two (**A**) or three (**B-L**) independent experiments and expressed as means +/- SEM. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

Fig. 5. Selective increase of PD-1 expression on CD45RA- activated-memory Tregs in patients with chronic GVHD receiving low dose IL-2.

(A) Representative flow cytometry histograms used to define CD4 T cell subsets. (B) Percentage of PD-1+ cells in Tcon (left) and Treg (right) subsets during IL-2 therapy (median values). *P<0.05, **P<0.01, CD45RA- activated-memory Tregs at versus baseline, Wilcoxon signed rank test.

Fig. 6. PD-1 blockade enhances IL-2 induced proliferation of expanded human Tregs and promotes apoptosis.

Purified $CD45RA^+$ naïve Tregs and Tcons labeled with CFSE were stimulated with IL-2, CD3/28 beads and anti-PD-L1 antibody for 4 days. (**A**) Representative flow cytometry histograms used to quantify CFSE dilution and identify $PD\text{-}1^+$ cells. (**B**) Representative flow cytometry histograms used to quantify CFSE dilution and identify Annexin-V^+ cells. (**C**) Percentage of Annexin-V^+ apoptotic cells within Tcon and Treg populations stimulated with IL-2, CD3/28 beads and anti PD-L1 antibody for 4 days. Data are obtained from one experiment.

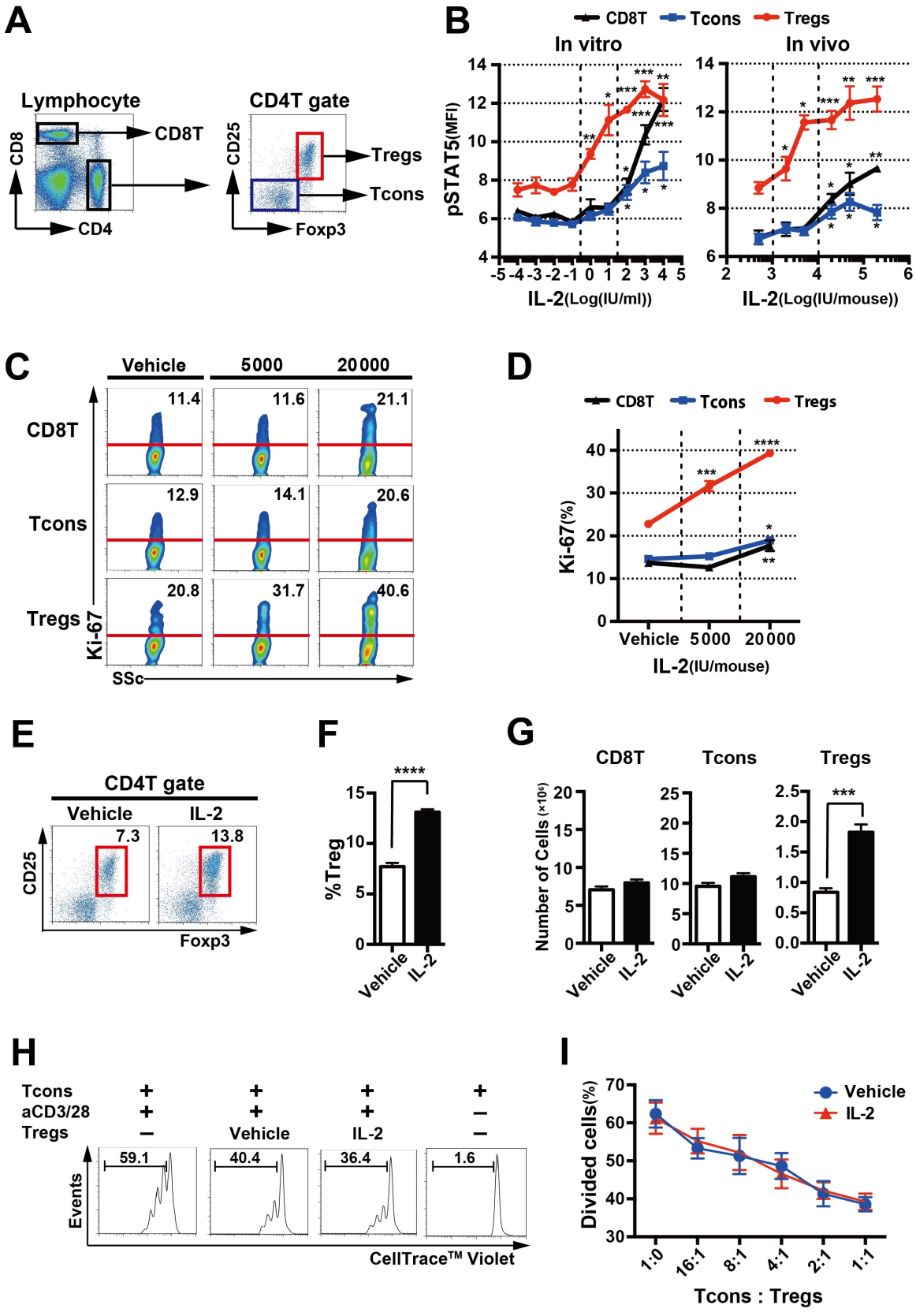
Fig. 7. Comparison of PD-1 expression in clinical responders and non-responders during low dose IL-2 therapy.

(**A**) Scatter plot of $\%PD\text{-}1^+CD45RA^-$ -Tregs and $\%PD\text{-}1^+CD45RA^-$ -Tcons in non-responders

and responders at week 2 during IL-2 therapy. (**B**) Ratio of Treg-%PD-1^{+}/Tcon-%PD-1 in non-responders (left) and responders (right) before and 2 weeks after starting IL-2 therapy. The ratio is significantly increased in clinical responders 2 weeks after IL-2 administration (P = 0.03, Wilcoxon signed rank test). Median values are shown in red.

Figures:

Fig. 1.



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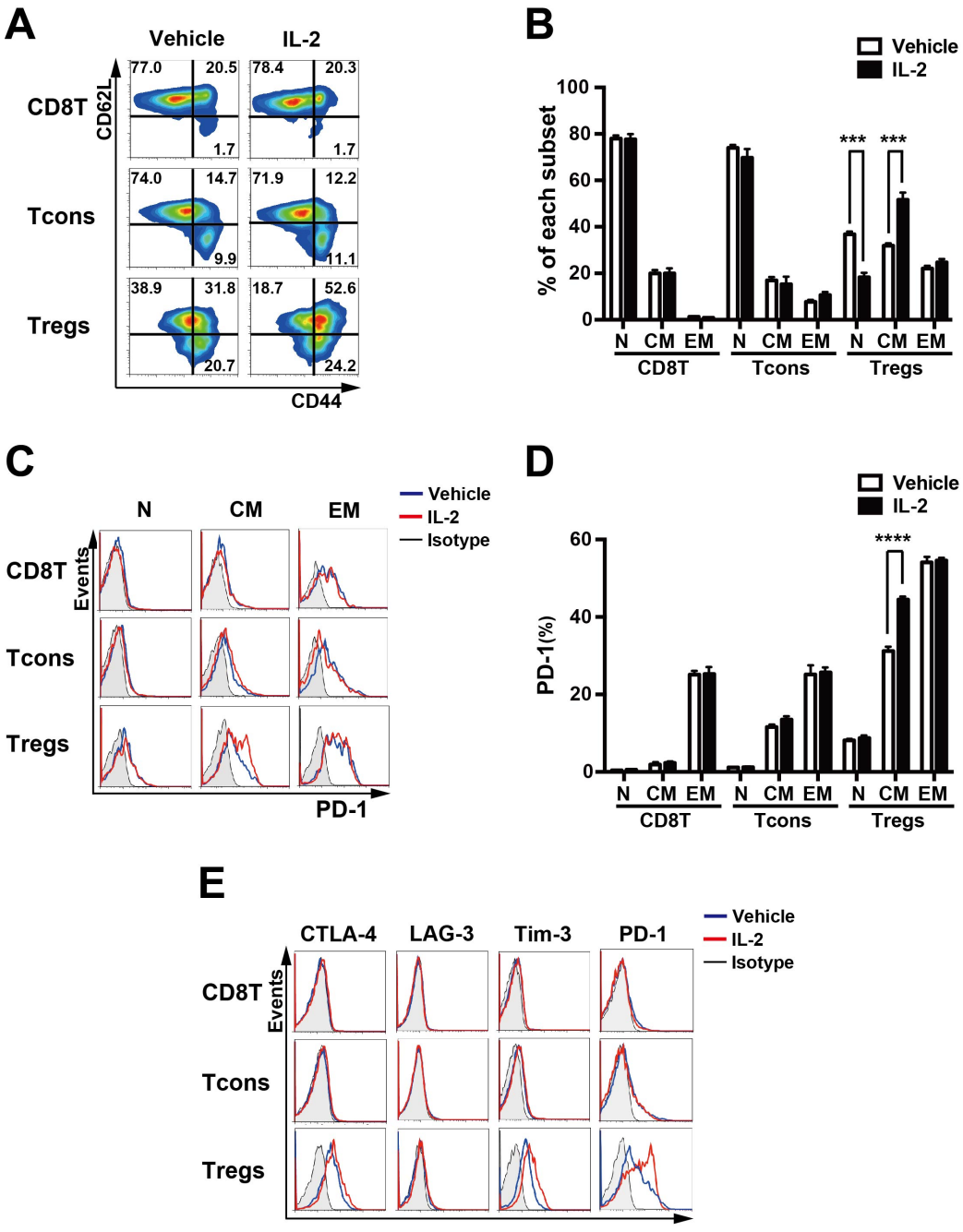
A
Vehicle
IL-2
CD8T
Tcons
Tregs
CD62L
CD44
77.0
20.5
1.7
78.4
20.3
1.7
74.0
14.7
9.9
71.9
12.2
11.1
38.9
31.8
20.7
18.7
52.6
24.2
B
Vehicle
IL-2
% of each subset
***
***
N CM EM N CM EM N CM EM
CD8T
Tcons
Tregs
C
N
CM
EM
Vehicle
IL-2
Isotype
CD8T
Tcons
Tregs
Events
PD-1
D
Vehicle
IL-2
PD-1(%)
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CD8T
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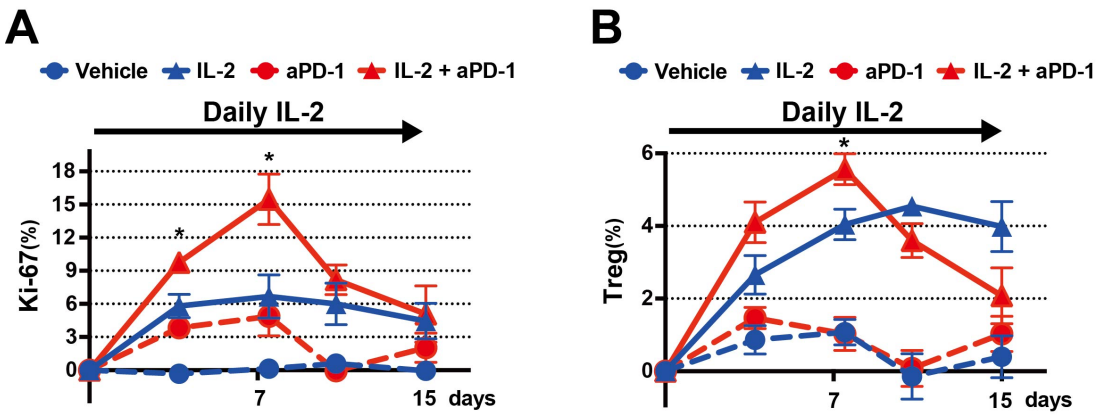
Events

Fig. 3.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Vehicle
IL-2
aPD-1
IL-2 + aPD-1
Daily IL-2
Ki-67(%)
18
15
12
9
6
3
0
*
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7
15 days
B
Vehicle
IL-2
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Daily IL-2
Treg(%)
6
4
2
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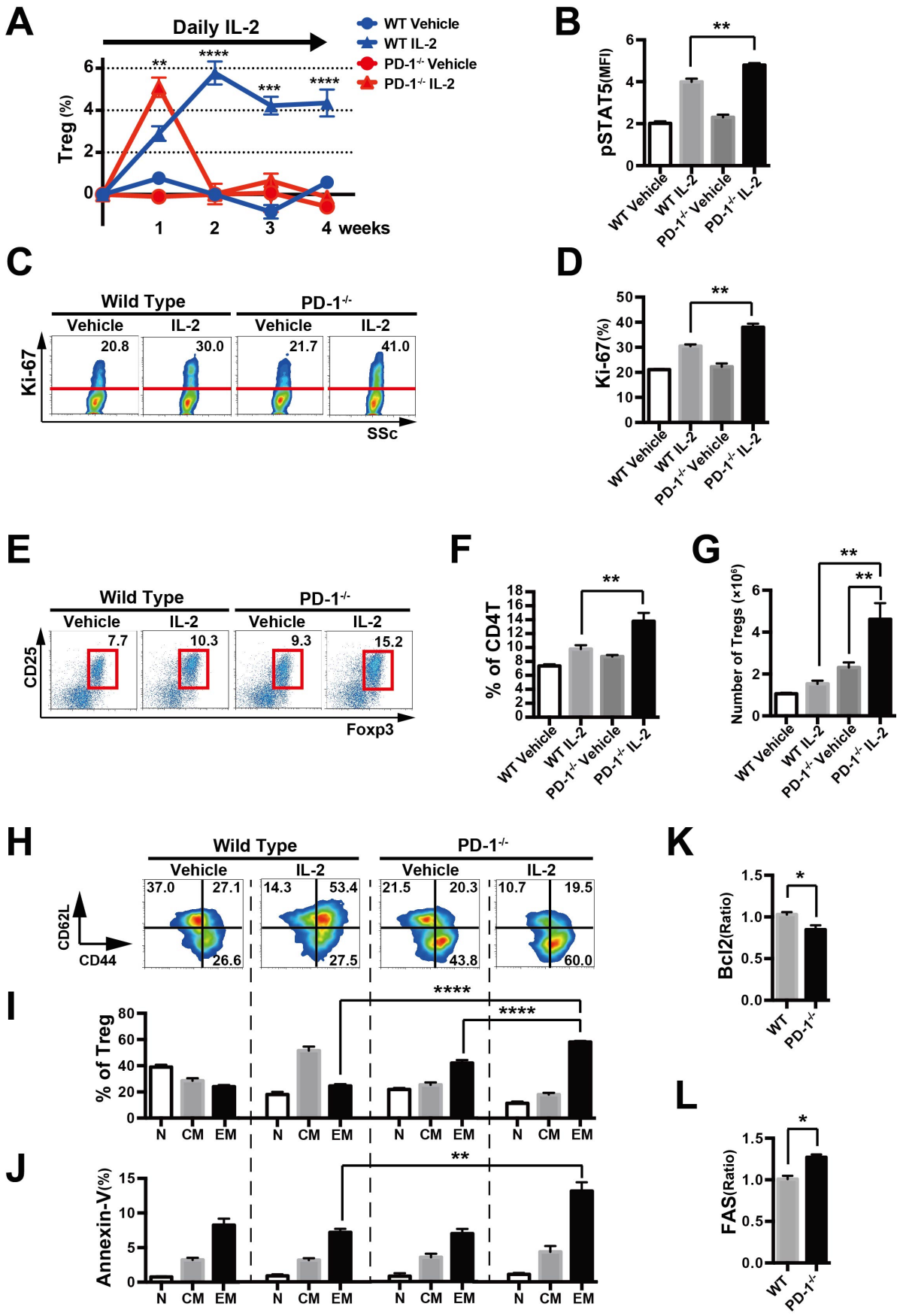
7
15 days

Fig. 4.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WT Vehicle
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PD-1-/- Vehicle
PD-1-/- IL-2
Treg (%)
1
2
3
4 weeks
B
pSTAT5(MFI)
C
Wild Type
PD-1-/-
Vehicle
IL-2
20.8
30.0
21.7
41.0
Ki-67
SSc
D
Ki-67(%)
E
CD25
Foxp3
7.7
10.3
9.3
15.2
F
% of CD4T
G
Number of Tregs (×10⁶)
H
CD62L
CD44
37.0
27.1
26.6
14.3
53.4
27.5
21.5
20.3
43.8
10.7
19.5
60.0
I
% of Treg
N
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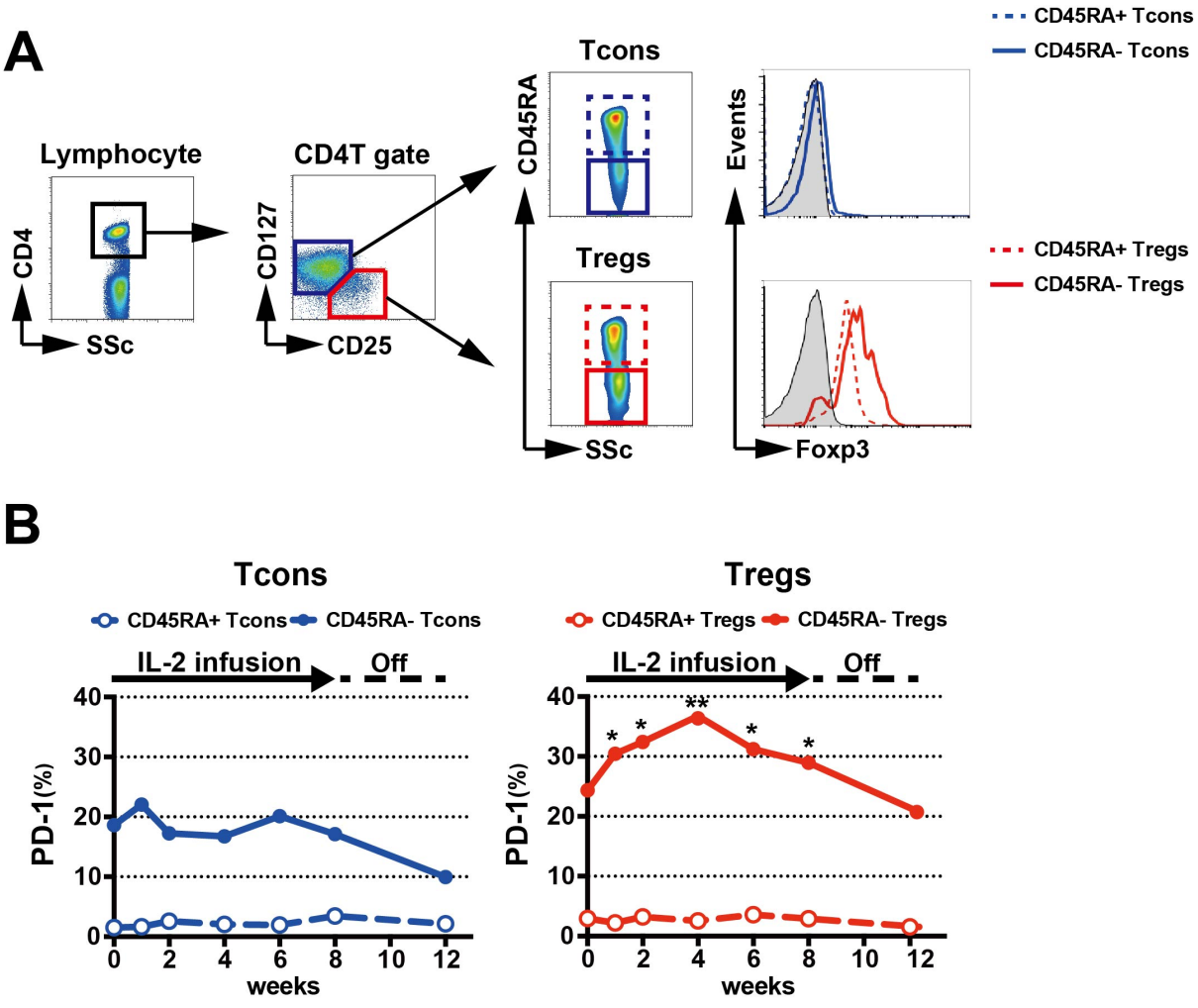
EM
J
Annexin-V(%)
K
Bcl2(Ratio)
WT
PD-1-/-
L
FAS(Ratio)

Fig. 5.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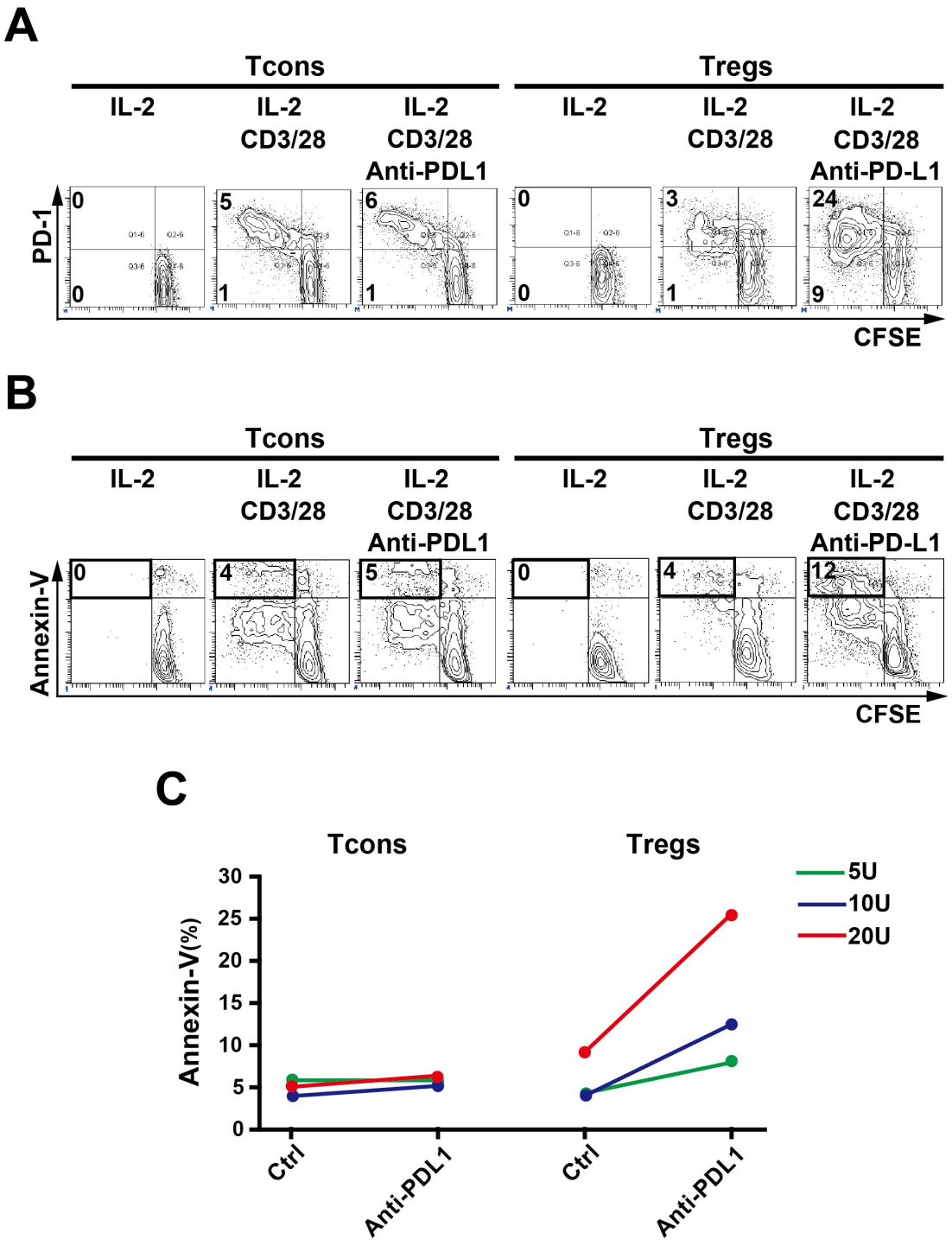
CD45RA- Tcons
CD45RA+ Tregs
CD45RA- Tregs
B
Tcons
CD45RA+ Tcons
CD45RA- Tcons
IL-2 infusion
Off
PD-1(%)
weeks
Tregs
CD45RA+ Tregs
CD45RA- Tregs
IL-2 infusion
Off
PD-1(%)
weeks

Fig. 6.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Tcons
Tregs
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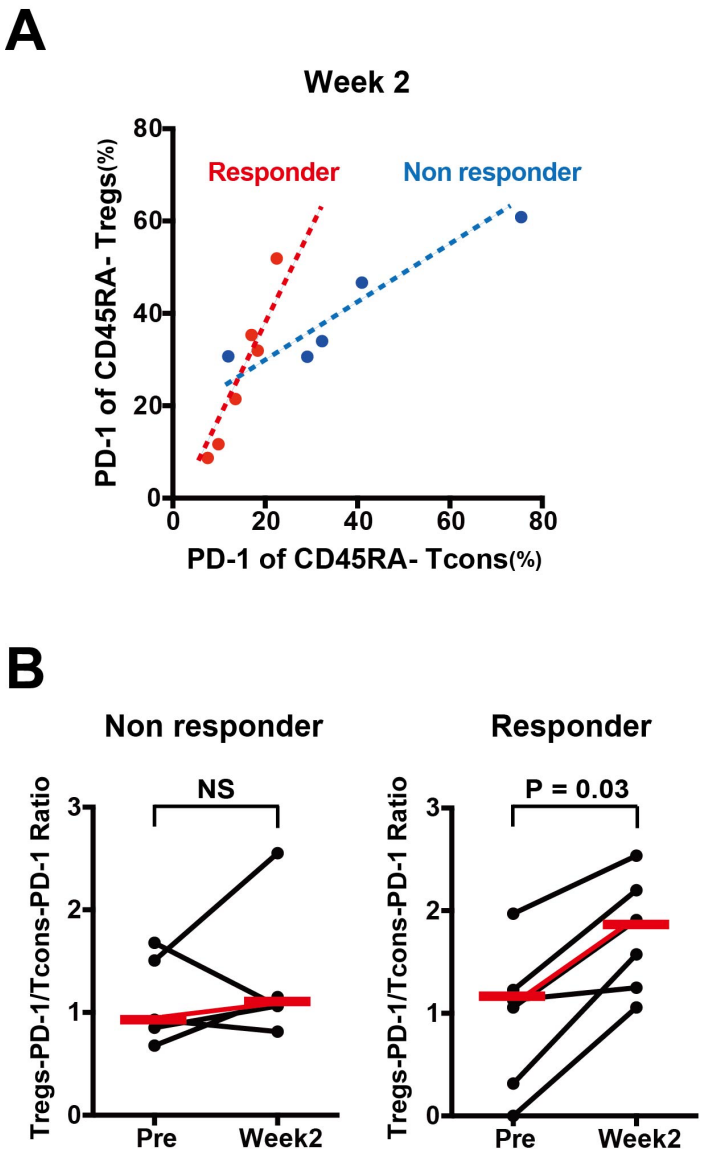
IL-2 CD3/28 Anti-PDL1
IL-2
IL-2 CD3/28
IL-2 CD3/28 Anti-PD-L1
PD-1
CFSE
B
Annexin-V
C
Annexin-V(%)
5U
10U
20U
Ctrl
Anti-PDL1

Fig. 7.


A
Week 2
Responder
Non responder
PD-1 of CD45RA- Tregs(%)
PD-1 of CD45RA- Tcons(%)
0
20
40
60
80
B
Non responder
Responder
NS
P = 0.03
Tregs-PD-1/Tcons-PD-1 Ratio
Pre
Week2
0
1
2
3