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New Insights on OX40 in the Control of T Cell Immunity and Immune Tolerance In Vivo

Xiang Xiao,* Weihua Gong,* Gulcin Demirci,* Wentao Liu,* Silvia Spoerl,* Xiufeng Chu,* D. Keith Bishop,† Laurence A. Turka,* and Xian C. Li*‡

OX40 is a T cell costimulatory molecule that belongs to the TNFR superfamily. In the absence of immune activation, OX40 is selectively expressed by Foxp3+ regulatory T cells (Tregs), but not by resting conventional T cells. The exact role of OX40 in Treg homeostasis and function remains incompletely defined. In this study, we demonstrate that OX40 engagement in vivo in naive mice induces initial expansion of Foxp3+ Tregs, but the expanded Tregs have poor suppressive function and exhibit features of exhaustion. We also show that OX40 enables the activation of the Akt and Stat5 pathways in Tregs, resulting in transient proliferation of Tregs and reduced levels of Foxp3 expression. This creates a state of relative IL-2 deficiency in naive mice that further impacts Tregs. This exhausted Treg phenotype can be prevented by exogenous IL-2, as both OX40 and IL-2 agonists drive further expansion of Tregs in vivo. Importantly, Tregs expanded by both OX40 and IL-2 agonists are potent suppressor cells, and in a heart transplant model, they promote long-term allograft survival. Our data reveal a novel role for OX40 in promoting immune tolerance and may have important clinical implications. *The Journal of Immunology, 2012, 188: 000–000.

OX40-deficient Tregs and wt Tregs are comparable in suppression of T effector cells (5). However, Tregs lacking OX40 are much less effective than are wt Tregs in suppressing T effector cells in vivo in a colitis model (9, 10). Of particular interest is the finding that OX40-deficient Treg fail to get to the inflamed gut where they are needed the most to control inflammation (10). A recent study suggests that OX40 signaling to Tregs contributes to the functional fitness of Tregs (9), but the mechanisms and implications are unclear. We and others reported that stimulation of OX40 on Tregs interferes with their regulatory functions (3, 5, 11), whereas other laboratories argued that OX40 engagement expands Tregs and that the expanded Tregs can act as potent suppressor cells when the cytokine milieu is right (12, 13). Furthermore, in selected tumor models, OX40 ligation has been shown to promote anti-tumor immunity by disabling Tregs (14), but other data suggest that OX40 ligation induces initial Treg expansion, but the OX40-expanded Tregs then undergo apoptosis, especially in the presence of cyclophosphamide (15), thus facilitating tumor rejection. A key message from these studies is that the impact of OX40 on Tregs is likely to be complex and that OX40 may employ a multiplicity of mechanisms to control different aspects of Tregs. This is a significant issue that warrants further clarification.

With better defined tools, reagents, and models, we sought to resolve the issue of the role of OX40 in Foxp3+ Treg homeostasis and function in vivo. We found that OX40 engagement in naive hosts indeed expands Tregs, but OX40-expanded Tregs readily undergo “exhaustion” and function poorly as suppressor cells. This phenotype is due to a relative IL-2 deficiency in vivo and can be reversed by exogenous IL-2. In fact, OX40 ligation plus an agonist IL-2/anti–IL-2 mAb complex together induce robust Treg...
proliferation in naive hosts. Importantly, Tregs expanded by both OX40 and IL-2 are potent regulatory cells, and they induce long-term allograft survival in a heart transplant model. Thus, OX40 can potentially drive a potent immune regulatory response in vivo.

Materials and Methods

Animals

C57BL/6 (B6; H-2b), C57BL/10ScSn (H-2d), Balb/c (H-2b), Rag-1−/− (H-2b), and IL-2 knockout (KO) (H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Generation of OX40−/− and OX40Ltg mice, all of which are on the B6 background, has been previously reported (16, 17). Foxp3−/−GFP reporter mice (Foxp3gfp) on the B6 background were created as previously reported (18). OX40−/−Foxp3gfp, CD45.1+Foxp3gfp, and CD45.1+OX40Ltg reporter mice were obtained from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases. All animals were bred in specific pathogen-free conditions.

Reagents

All Mabs used for cell surface staining were obtained from BD Pharmingen or eBioscience (San Diego, CA). An agonist anti-OX40 mAb (clone OX86, IgG2a) and a blocking anti-CD45 mAb (clone MR1, hamster IgG1) were used. BALB/c (H-2d), B6-CD45.1, BALB/c (H-2d), and C57BL/6 (B6; H-2b), and Rag-1−/− (H-2b), and IL-2 knockout (KO) (H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Generation of OX40−/− and OX40Ltg mice, all of which are on the B6 background, has been previously reported (16, 17). Foxp3−/−GFP reporter mice (Foxp3gfp) on the B6 background were created as previously reported (18). OX40−/−Foxp3gfp, CD45.1+Foxp3gfp, and CD45.1+OX40Ltg reporter mice were generated by crossing the Foxp3gfp reporter mice with OX40−/− mice or CD45.1+OX40Ltg mice and then selected by FACS or PCR-based genotyping. OX40Ltg/IL-2KO mice were generated by crossing the OX40Ltg and IL-2KO mice. Animal care and use conformed to the guidelines established by the Animal Care Committee at Harvard Medical School (Boston, MA).

Flow cytometry

Cells prepared from the spleen, lymph nodes, the liver, or the lungs were stained with fluorochrome-conjugated Abs on ice for 20 min, washed twice in PBS/BSA, and fixed in 1% paraformaldehyde prior to FACS analysis. For intracellular staining, cells were first permeabilized with BD Cytofix/Cytoperm solution, followed by staining with fluorochrome-conjugated Abs. All samples were acquired using the FACSCalibur or an LSRII (BD Biosciences, Mountain View, CA). Data analysis was performed using FlowJo 7.5 software (Tree Star, Ashland, OR), as we reported before (21).

Cell sorting and adoptive cell transfer

All sorting was performed using the MoFlo high-speed cell sorter (DakoCytomation, Ft. Collins, CO). For sorting Foxp3+ Tregs, cells were pooled from the spleen and lymph nodes of Foxp3gfp reporter mice and briefly labeled with CyChrome-anti-CD4 and, GFP+ cells in the CD4+ population were identified, electronically gated, and then selectively sorted. The purity of sorted cells using this method is usually >98% (5).

For adoptive cell transfer, naive syngeneic B6 mice, OX40Ltg mice, and OX40Ltg/IL-2KO mice (without the gfp reporter gene) were used as hosts. The sorted GFP(Foxp3)+ Tregs were adoptively transferred into the host mice via the tail vein (1 × 106/ml) and stimulated with anti-OX40 mAb and syngeneic APCs (1 × 106/ml). The cell-depleted spleen cells were used as APCs. They were briefly treated with mitomycin C before each experiment. CD45.1+ responder T cell proliferation with or without Tregs was assessed by flow cytometry 3 d later based on dilution of the CFSE dye (22).

Cell proliferation assay

Cell proliferation was determined using tritium uptake assays. FACs-sorted CD4+Foxp3+ Tregs from Foxp3gfp reporter mice were plated into 96-well tissue culture plates (1 × 104/well) and stimulated with anti-CD3 (1 μg/ml) plus equal numbers of syngeneic B6 APCs or OX40Ltg APCs in the presence or absence of rIL-2 at 37˚C for 3 d. For the last 8 h of culture, cells were pulsed with 1 μCi [3H]thymidine deoxyribose per well (Amersham, Boston, MA), and [3H]thymidine deoxyribose incorporation was determined using a Betaplate scintillation counter (PerkinElmer, Wellesley, MA). Data were presented as mean cpm of triplicate assays.

BrdU labeling

CD4+Foxp3+ Tregs sorted from CD45.1+Foxp3gfp reporter mice were passively transferred into OX40Ltg mice (CD45.2). The host mice were given a single i.p. injection of BrdU at 1.5 mg/ml in Dulbecco’s PBS 2 wk after cell transfer, and 12 h later the host mice were sacrificed and spleen cells prepared. Detection for BrdU was performed using an APC BrdU flow kit according to the manufacturer’s instruction (BD Biosciences) and then assessed by flow cytometry. BrdU+ T cells among CD4+CD45.1+ Foxp3+ Tregs were plotted and shown.

Heterotopic heart transplantation

Donor BALB/c hearts were grafted into the peritoneal cavity of recipient B6 mice as previously reported (23). In this model, the heart graft was anastomosed to the great vessels of the host abdomen and perfused with the recipient’s blood. The heart graft resumes contractions immediately after transplantation, and graft survival was monitored by abdominal palpation. Treatment of recipient mice consisted of OX46 at 0.25 mg i.p. and 0.3 μg IL-2 i.p. for 3 consecutive days, starting on day −7 relative to heart transplantation, which was performed on day 0. Some recipients received a single dose of anti-CD154 mAb (clone MR1; 0.25 mg i.p.) at the time of heart grafting. Graft survival was presented in the Kaplan–Meier plot.

Tissue histopathology

Tissue samples were prepared from host mice, fixed in 10% formalin, and embedded in paraffin. Serial tissue sections (5 μm) were cut and mounted on Superfrost slides (Fisher Scientific, Pittsburgh, PA), fixed in methanol, and stained with H&E for identification of tissue damage and cellular infiltration.

Statistics

Statistical difference was determined with an unpaired Student t test with GraphPad Prism software (GraphPad Software, La Jolla, CA), and allo-graft survival was compared using the log-rank test. A P value of <0.05 was considered significant.

Results

OX40 ligation in vivo drastically expands the pool of Foxp3+ Tregs in naive mice

In naive mice, OX40 is predominantly expressed by CD4+Foxp3+ Tregs, but not by resting Tconv (5). By taking advantage of an OX40L transgenic model and an agonist anti-OX40 mAb (OX86), we examined the impact of persistent versus transient OX40 triggering on Tregs on their expansion in vivo. As shown in Fig. 2A, wt B6 mice and OX40KO mice had comparable numbers of Foxp3+ Tregs (~30% of total CD4+ T cells). Remarkably, ~30% of CD4+ T cells in the spleen of OX40Ltg mice were Foxp3+ Tregs. This represents an ~3-fold increase in Tregs over the wt B6 mice. Moreover, Foxp3+ Tregs in OX40Ltg mice were also present...
in large numbers in the extralymphoid organs, including the liver and the lungs, both in relative percentage and in absolute cell numbers (Fig. 1A, 1B). This is in stark contrast to Foxp3+ Tregs in wt B6 mice where Tregs are primarily in the lymphoid organs (Fig. 1A). Overall, as measured by the absolute cell number, OX40Ltg mice had ∼6-fold more Foxp3+ Tregs than did the wt B6, OX40KO, and OX40Ltg mice. The data presented are means ± of five experiments. C, Tissue samples were prepared from wt B6, OX40−/−, and OX40Ltg mice at ∼12 wk age and examined by H&E staining. Images shown are representative of three to five animals in each group. Original magnification ×40. *p < 0.05.

Because the OX40L transgene is driven by an actin promoter (16), OX40L is ubiquitously expressed in vivo. A concern is that findings in OX40Ltg mice may not be physiological. To address this concern, we injected naive wt B6 mice with an agonist anti-OX40 mAb (i.e., OX86) to transiently engage the OX40 receptor in vivo. We varied the doses of OX86 injected to mimic differences in the strength of OX40 stimulation. We then examined changes in Foxp3+ Tregs in the treated mice within a time frame of 3 wk. As compared with control Ab-treated mice, mice treated with OX86 (0.25 mg i.p. for 3 consecutive days) showed a robust expansion of Foxp3+ Tregs, and kinetic analysis revealed that Foxp3+ Tregs started to expand on day 4, reached a plateau on day 9, and then declined over time (Fig. 2A). In the host spleen, ∼30% of CD4+ T cells in OX86-treated mice were Foxp3+ Tregs (Fig. 2B). This again represents a 3-fold increase over control Ab-treated mice. In the extralymphoid sites (e.g., the lungs), Foxp3+ Tregs also increased substantially after OX86 treatment (Fig. 2B), and the absolute number of Tregs increased from 5,533 in control mice to 121,667 in OX86-treated mice 7 d later (∼24 fold increase) (mean of three mice in each group). Note that OX86 also expanded memory CD4+ T cells (CD4+CD44high) by ∼2-fold, and a higher dose of OX86 and a longer treatment period (0.25 mg i.p. for 7 consecutive days) further expanded memory CD4+ T cells (Fig. 2C, 2D).

OX40 ligation on Tregs downregulates Foxp3 expression and induces tissue inflammation in naive hosts

A noticeable feature of OX86-expanded Tregs, as compared with control Tregs, is that OX40-expanded Tregs express much lower levels of Foxp3, as shown by the mean fluorescence intensity (Figs. 2B, 3A). A similar pattern was observed for the expression of CD25 (Fig. 3A). Changes in other Treg-associated surface markers (i.e., CTLA-4, GITR, CD39, CD73) were not remarkable between

FIGURE 1. Comparison of CD4+GFP(Foxp3+) Tregs and CD4+GFP(Foxp3)− Tconv in wt B6, OX40KO, and OX40Ltg mice. A, Cells were prepared from age-matched wt B6, OX40KO, and OX40Ltg mice carrying the Foxp3gfp reporter gene (∼8 wk old) and analyzed by FACS. Data shown are the relative percentages of CD4+GFP(Foxp3)+ T cells among total CD4+ T cells gated. Representative plots of 1 of at least 10 experiments are shown. B, The absolute number of CD4+GFP (Foxp3)+ Tregs in the spleen (SPL), peripheral lymph nodes (LN), liver, and the lungs of age-matched wt B6, OX40KO, and OX40Ltg mice. The data presented are means ± of SD of five experiments. C, Tissue samples were prepared from wt B6, OX40−/−, and OX40Ltg mice at ∼12 wk age and examined by H&E staining. Images shown are representative of three to five animals in each group. Original magnification ×40. *p < 0.05.
control Ab and OX86-treated Tregs (Fig. 3A, lower panel). Interestingly, as compared with the control Ab-treated Tregs, Foxp3+ Tregs expanded by OX86 markedly upregulated the expression of PD-1 on the cell surface (Fig. 3A, upper panel), a marker of T cell exhaustion in other cell types (25).

To determine the function of Tregs expanded by OX86 in vivo, we FACs sorted Foxp3+ Tregs from the spleen of control IgG- and OX86-treated mice (2 wk after treatment) and compared their effects in suppressing proliferation of CD4+Foxp3+ T cells in vitro. As shown in Fig. 3B, the suppressive function of Foxp3+ Tregs from OX86-treated mice was markedly impaired as compared with control Tregs. This finding prompted us to examine whether OX86 would trigger autoimmune-like features in naive hosts. As shown in Fig. 3C, treatment of naive B6 mice with OX86 induced extensive inflammatory infiltrates in the lungs, consisting of predominantly mononuclear leukocytes. A higher dose of OX86 or a prolonged period of treatment triggered even more severe pathology (data not shown). This pathology is reminiscent of that previously reported in OX40Ltg mice (24). Thus, OX40 stimulation appears to destabilize Foxp3+ Tregs in naive mice, which may contribute to the autoimmune pathology in vivo.

Foxp3+ Tregs in wt and OX40KO mice respond differently to IL-2

IL-2 is central to Treg homeostasis and function (1). To determine how Foxp3+ Tregs, with or without OX40, would respond to IL-2 in vivo, we complexed IL-2 to an anti–IL-2 mAb (19) and injected this IL-2/Ab complex into age-matched wt B6 and OX40KO Foxp3gfp reporter mice. We titrated down the IL-2/Ab complex from 1 to 0.1 μg relative to the IL-2 mass, and changes in Foxp3+ Tregs in the blood of treated mice were examined at different time points. As shown in Fig. 4A, IL-2/Ab at 0.1 μg (for 3 consecutive days) failed to induce Treg expansion in wt and OX40KO mice, and Tregs in these mice remained constant over a 7-d period. However, at 0.3 μg IL-2/Ab injected, Tregs in wt mice expanded vigorously, and at day 5 after IL-2/Ab injection (3 d after the last dose of IL-2/Ab), Tregs in wt mice were doubled whereas those in OX40KO mice did not show any expansion (Fig. 4A). Interestingly, at higher doses of IL-2/Ab (i.e., 1 μg IL-2), Foxp3+ Tregs in both wt mice and OX40KO mice expanded at comparable levels, and in this setting Tregs accounted for as much as 40% of CD4+ T cells in the blood (Fig. 4B). We noticed that high doses of IL-2/Ab also induced substantial expansion of memory CD8+ T cells and NK cells (Fig. 4C), cell types that are known to express IL-2R, and therefore are responsive to excess IL-2 (26). Thus, OX40-deficient Tregs appear to have a clear disadvantage in response to IL-2, which corroborates a recent report in an adoptive transfer model (9). These data uncover a potential crosstalk between IL-2 and OX40 in the control of Treg homeostasis in vivo.

A key role for IL-2 in OX40-triggered expansion of Foxp3+ Tregs in vivo

To determine whether OX40 directly affects Tregs or indirectly affects Tregs by promoting IL-2 from non-Tregs, we performed a series of adoptive cell transfer experiments. We FACS sorted...
Foxp3+ Tregs from wt Foxp3gfp reporter mice and adoptively transferred them into syngeneic B6, OX40Ltg, and OX40Ltg/IL-2KO mice (without the eGFP reporter gene), and examined the behavior of transferred GFP-tagged Tregs 2 wk later. As shown in Fig. 5A, the transferred Tregs were readily detected in OX40Ltg hosts, but not in wt B6 hosts. BrdU labeling revealed vigorous proliferation of transferred Tregs in the OX40Ltg hosts (Fig. 5B). Moreover, the transferred Tregs also accumulated at both lymphoid and nonlymphoid sites (Fig. 5A). In this setting, the number of Foxp3(GFP)+ Tregs recovered from each OX40Ltg host was ~5 × 10^6 (Fig. 5C), which is ~5-fold more than the number of Tregs initially transferred (1 × 10^6 per host), suggesting an extensive proliferation of transferred Tregs in OX40Ltg hosts. Remarkably, such an expansion of transferred Tregs was not observed in OX40Ltg/IL-2KO hosts (Fig. 5A), demonstrating an indispensable role for IL-2 in OX40-mediated Treg expansion. Additionally, OX40KO Tregs completely failed to expand in OX40Ltg hosts upon cell transfer. Thus, it seems that OX40 triggering on Tregs renders them highly responsive to basal levels of IL-2 (in naive hosts), and both drive Treg expansion. The increase in Tregs is unlikely due to conversion, as transferring CD4+ CD45.1+ T cells (non-Tregs) to OX40Ltg hosts did not show any conversion of non-Tregs to Foxp3+ Tregs in the OX40Ltg mice (Fig. 5A).

To further explore the interplay of OX40 and IL-2 in Treg expansion in vivo and the direct role of OX40, we transferred GFP-tagged wt Tregs (CD45.1+) into OX40KO-Foxp3gfp reporter mice (CD45.2+). The host mice were treated with OX86 (0.25 mg i.p. for 3 d) and/or IL-2/Ab (0.1 μg i.p. for 3 d). Expansion of both host and transferred Tregs in the same hosts was examined 7 d later. Both the relative percentage and the absolute Treg numbers are shown. *p < 0.05.
usually treated or untreated controls, this T effector/Foxp3+ Treg ratio is gives rise to a T effector/Foxp3+ Treg ratio of ∼8-fold, as assessed by the absolute cell number, and bothOX86 and IL-2/Ab resulted in further expansion of transferred Tregs (∼15-fold), proving evidence thatOX40 directly acts on Tregs to facilitate their expansion.

As IL-2 is limited in naïve mice, it is possible that when Tregs are stimulated throughOX40, the availability of IL-2 in vivo may determine Treg expansion versus Treg exhaustion. To test this possibility, we treated naïve B6 mice withOX86 with or without the IL-2/Ab complex, using doses that preferentially stimulated Foxp3+ Tregs (0.3 μg IL-2/Ab, 0.25 mgOX86 for 3 consecutive days), and Treg expansion in vivo was monitored. As shown in Fig. 6A, bothOX86 and IL-2/Ab triggered impressive expansion of Tregs in naïve B6 hosts, and 7 d after the treatment, ∼50% of the CD4+ T cells in the host spleen were Foxp3+ Tregs, which gives rise to a T effector/Foxp3+ Treg ratio of ∼1:1. In control Ab-treated or untreated controls, this T effector/Foxp3+ Treg ratio is usually ∼10:1 (Fig. 6B). This increase in Tregs inOX86 and IL-2/Ab–treated mice persisted for ∼8 d, followed by declines in the absence of further treatment. Importantly, as compared with Tregs stimulated withOX86 alone, Tregs expanded with bothOX86 and IL-2/Ab showed increased CD25 and Foxp3 expression, with levels that are comparable to that of natural Tregs, and CD25 is often 2-fold higher than that of unmanipulated Tregs (Fig. 6B).

The doses ofOX86 and IL-2/Ab used in this setting induced minimal expansion of other cell types besidesFoxp3+ Tregs (Fig. 6C). These data further support the notion thatOX40 acts together withIL-2 to mediate Treg expansion in vivo in naïve hosts.

**OX40 ligation permits Foxp3+ Treg expansion by altering IL-2 signaling**

To probe the mechanisms by whichOX86 and IL-2/Ab complex mediate vigorous expansion of Tregs in vivo, we examined the signaling pathways known to be activated byIL-2 andOX40 in other cell types. When FACS-sortedFoxp3+ Tregs are stimulated with anti-CD3/APCs in vitro, phosphorylation ofSTAT5 and Akt was not detected within the first hour (Fig. 7A) or 12 h later (data not shown). Addition of IL-2 at concentrations that normally stimulate proliferation of T effector cells (≤1ng/ml) triggered prominent phosphorylation ofSTAT5 in Tregs, but not the activation ofAkt (Fig. 7A), and Tregs failed to proliferate under such conditions (partial or incomplete IL-2 signaling) (Fig. 7B), which is consistent with our published reports (27, 28). Stimulation ofOX40 on Tregs alone induced modestSTAT5 and Akt activation, but Tregs also failed to proliferate (Fig. 7B). However, stimulation of Tregs withOX40 plus IL-2 induced robust proliferation of Tregs (Fig. 7B), and this was associated with strong phosphorylation of bothSTAT5 and Akt (Fig. 7A). This pattern of Akt and STAT5 activation can last for up to 48 h, suggesting that bothOX40 ligation and IL-2 fully activate the signaling pathways necessary for Treg proliferation.

To ascertain the role ofAkt in Treg expansion in vivo, we treated naïve Foxp3gfp reporter mice withOX86 and IL-2/Ab complex. A cohort of mice was additionally treated with rapamycin to block the activation of mammalian target ofrapamycin (mTOR) (3 mg/kg/day for 3 d), a critical signaling molecule in the PI3/Akt pathway (29), and then compared the expansion ofFoxp3+ Tregs in vivo. As shown in Fig. 7C, rapamycin markedly inhibited theOX40/IL-2–induced Treg expansion in vivo. As compared withOX86/IL-2–treated mice, rapamycin reduced Treg expansion by ∼50%. A similar reduction was observed with absolute Treg numbers in the treated mice (Fig. 7D). These data indicate that bothOX40 and IL-2/Ab together overcome Treg exhaustion and stimulate vigorous proliferation ofFoxp3+ Tregs, both in vitro and in vivo.

**OX40 ligation and IL-2 together expand functional Foxp3+ Tregs that are immunosuppressive**

We further examined the functional status ofFoxp3+ Tregs expanded byOX86 with or without IL-2/Ab complex in vitro (30). As shown in Fig. 8A, anti-CD3/APCs stimulated a vigorous proliferation of CD4+Foxp3+ responder T cells, as shown by CFSE dilutions. Such a proliferation was inhibited by wt Foxp3+ Tregs from naïveFoxp3gfp control mice. Again, Tregs fromOX86–treated mice exhibited impaired suppressor functions, which is consistent with that observed in Fig. 3B. Interestingly, Tregs from mice treated with bothOX86 and IL-2/Ab complex showed strong suppressive functions that is comparable to that of unmanipulated Tregs. Thus, IL-2/Ab complex prevented the functional impairment ofOX40 expanded Tregs in vivo.

We also examined the histology of the lungs inOX86-treated naïve B6 mice with or without IL-2/Ab complex. As shown in Fig. 8B, and in contrast to mice treated withOX86 alone, which developed prominent interstitial inflammatory infiltrates, mice...
treated with both OX86 and IL-2/Ab complex exhibited normal lung histology, with minimal or no cellular infiltration, suggesting that OX86/IL-2–expanded Tregs are functional Tregs, so that even if certain cytopathic non-Tregs are stimulated (Fig. 6C), they are effectively controlled by the expanded Tregs.

To further test the potency of OX86 and IL-2/Ab–expanded Tregs in vivo under stringent conditions, we transplanted fully MHC mismatched heart allografts to recipient mice that were treated with OX86 and IL-2/Ab complex. We chose a different approach in this model. B6 recipients were first treated with OX86 (0.25 mg i.p.) and the IL-2 complex (0.3 mg i.p.), and 7 d later when Treg expansion peaked (Fig. 6A), mice were then transplanted with the heart allografts. Some recipients were given a single subtherapeutic dose of anti-CD154 (clone MR1, 0.25 mg i.p.) at the time of transplant, and graft survival was then determined. As shown in Fig. 8C, control mice rejected the heart allografts within 10 d (median survival time [MST] 8 d, n = 5). The IL-2/Ab complex or a single dose of MR1 prolonged graft survival to ~18 d, and further graft prolongation was observed with combined IL-2/Ab complex and MR1 (MST 28 d). Graft survival was markedly prolonged in recipients pretreated with OX86 and IL-2/Ab complex. In fact, two of the seven heart transplants survived indefinitely (>100 d). Treatment of mice with both OX86/IL-2/Ab complex and MR1 uniformly induced long-term graft survival (MST > 60 d). Thus, Foxp3+ Tregs expanded with OX86 and IL-2/Ab are potent regulatory cells capable of promoting long-term transplant survival.
**Discussion**

In the present study, we found that OX40 ligation exerts a profound effect on Foxp3$^+$ Tregs in vivo in naive mice, but the outcome of this effect is heavily influenced by IL-2. Clearly, in naive hosts where IL-2 is limited, stimulation of OX40 on Tregs drives Tregs to “exhaustion,” as they have reduced levels of Foxp3 and CD25 and function poorly as suppressor cells. Such Tregs also fail to undergo further expansion in vivo, even if given prolonged OX40 stimulation. Moreover, the OX40-expanded Tregs upregulated PD-1, a marker that is often associated with T cell exhaustion (25). We also showed that OX40 ligation on Tregs along with exogenous IL-2 induced further expansion of Tregs, and Tregs expanded under such conditions often achieve a 1:1 ratio to non-Tregs in the host spleen. Importantly, such Foxp3$^+$ Tregs are functional Tregs; they readily promote the survival of fully MHC-mismatched heart allografts, demonstrating the therapeutic efficacy of Foxp3$^+$ Tregs expanded by both OX86 and IL-2/Ab complex in vivo.

Our data show several novel insights on the complexity of OX40 in T cell-mediated immunity and immune tolerance. By all counts, Foxp3$^+$ Tregs must outcompete T effector cells for IL-2 to properly expand or function. The involvement of OX40 in this response implements additional controls over Tregs in vivo, especially under inflammatory conditions or during ongoing immune activation. First, if Tregs fail to engage OX40L in situ at the site of tissue inflammation or lack OX40 expression on the cell surface, they will have a competitive disadvantage in responding to IL-2. This may help explain why OX40KO Tregs fail to control wt T effector cell-induced pathology in a colitis model (10). Second, stimulation of OX40 on Foxp3$^+$ Tregs increases the demand for IL-2, but Tregs themselves cannot produce IL-2. Thus, when IL-2 becomes limited, Foxp3$^+$ Tregs are pushed to a state of exhaustion, allowing activation of cytotoxic T effector/memory cells (24). A key point from our study is that such an exhausted Treg phenotype can be prevented by exogenous IL-2 in vivo. Finally, the timing of OX40 ligation and the status of immune activation at the time of OX40 ligation can have striking effects on the fate of Foxp3$^+$ Tregs. Considering that Tregs constitutively express OX40 and CD25, they certainly have a competitive advantage to respond to OX40 and IL-2 in the absence of immune activation, which explains the selective expansion of Foxp3$^+$ Tregs in naive mice by stimulating OX40 and IL-2R signaling. However, OX40 ligation at the time of immune activation (e.g., transplantation) also stimulates T effector cells and promotes allograft rejection (23). In this setting, activated T effector cells acquire the expression of OX40, and OX40 costimulation supports proliferation of T effector cells, resulting in T effector cells that are numerically superior over Tregs in the rejection response (31).

Our data highlight the OX40/OX40L pathway has the potential to drive opposing outcomes in the T cell response. This is an important point in therapeutic targeting of OX40 in certain settings. The IL-2/Ab complex itself is known to drive Treg expansion in vivo, and when combined with rapamycin, the expanded Tregs ameliorated ongoing experimental autoimmune encephalomyelitis and prevented islet allograft rejection (19). However, the OX40 and IL-2 protocol does have certain advantages over the IL-2/Ab alone. For example, in the presence of OX40 ligation, a similar degree of Treg expansion in vivo can be achieved by using a 3-fold less IL-2/Ab complex (Fig. 4) (19). This suggests that the off-target effects of the IL-2/Ab complex can be minimized. It is well known that NK cells and CD8$^+$ memory T cells constitutively express the intermediate affinity IL-2R (i.e., the βγ complex), and therefore they are also responsive to IL-2 (26). Indeed, we found that with high doses of IL-2/Ab complex, NK cells and memory CD8$^+$ T cells, in addition to the Tregs, are noticeably expanded (Fig. 4C). In the transplant setting, this will limit the efficacy of Tregs, as NK cells and CD8$^+$ memory T cells are potent effector cells in rejection (32, 33). Along the same line, high doses of OX86 or prolonged OX40 stimulation can also stimulate memory CD4$^+$ T cells in addition to Foxp3$^+$ Tregs, and the inclusion of the IL-2/Ab complex allowed a marked reduction of the OX86 mAb. Thus, the combination of both reagents allowed a much better selectivity in expansion of Tregs in vivo.

Our data reinforce the notion that Foxp3$^+$ Tregs are not anergic. Instead, they demand signals from multiple cell surface receptor signals to proliferate, expand, and function. Unlike freshly activated Tconv, Tregs respond poorly to IL-2 despite bearing the high-affinity IL-2R (the IL-2Rαβγ trimer) (28). At a molecular level, IL-2 fails to trigger the activation of the PI3/Akt pathway in Tregs, though STAT5 is prominently phosphorylated (27). Thus, IL-2 signaling in Tregs is only partial or incomplete. This defect is attributed to the high levels of PTEN or increased SOCS-1 expression in Tregs (27, 34). Indeed, PTEN-deficient Tregs proliferated vigorously to IL-2, and in this setting, IL-2 activates both the STAT5 and the PI3/Akt pathway (27). Our data suggest that OX40 ligation appears to be instrumental in the activation of the Akt pathway in Tregs. In fact, OX40 ligation has been reported to trigger Akt activation in other cell types (35, 36). A key role for the Akt/mTOR pathway in Treg expansion in vivo is evident, as treatment of host mice with rapamycin, which blocks mTOR activation (37), inhibited OX86/IL-2-induced proliferation of Tregs (Fig. 7C). This is a surprising finding, considering the notion that rapamycin has been shown to inhibit T effector cells but spare or even expand Tregs (38–40). However, most of these studies are performed in vitro involving cultures of unfractonated T cells or in vivo in immunodeficient mice (41). In fact, a recent study using immune-replete mice demonstrated that rapamycin indeed prevents the expansion of both Tregs and T effector cells (42). A cautionary note is that the impact of rapamycin on Tregs is likely to be conditional or dependent on the models and the context of Treg activation. Nonetheless, OX40 can effectively partner with IL-2 in driving Treg expansion in vivo with minimal effects on other cell types. This finding may help explain a long-standing paradox that Tregs are anergic in vitro but expand vigorously in vivo (43), as the in vivo environment may allow Tregs to readily access OX40L$^+$ APCs.

There are other receptors on Tregs that can activate the Akt/mTOR pathway (e.g., CD28), which have the potential to collaborate with IL-2 in driving expansion of Tregs (44, 45). As CD28 is ubiquitously expressed by Tregs and T effector cells, targeting such molecules for the purpose of Treg expansion in vivo is challenging (46). Interestingly, OX40 knockout Tregs show reduced response to IL-2, especially when IL-2 levels are low. This corroborates an earlier report (9) and suggests that OX40 in fact plays an indispensable role in regulating how Tregs respond to IL-2. A puzzling matter is that Tregs develop relatively normally in OX40 knockout mice, except for a slight reduction in Treg numbers in the thymus of neonatal mice (47). One possibility is that there might be other mechanisms or molecular pathways that compensate for the absence of OX40, but such pathways await further identification.

In summary, we provide evidence that OX40 can have striking effects on the T cell responses through its impact on Foxp3$^+$ Tregs, and depending on the availability of IL-2 and the status of immune activation, OX40 can stimulate both an effector and a regulatory response. As blocking OX40 costimulation (e.g., tolerance induction) or engaging OX40 (e.g., cancer therapy) can potentially...
affect both Tregs and T effector cells, and Tregs have been shown to exhibit considerable plasticity (48), a challenge is to develop clinically applicable protocols that selectively target both OX40 and IL-2 pathways to promote or inhibit desired immune responses. Additionally, the issue of pre-existing memory T cells, which are numerous in large animal models and humans, in the expansion and function of Tregs in response to OX40 and IL-2 warrants further investigation.

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Disclosures

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