## Distinct Roles for the OX40-OX40 Ligand Interaction in Regulatory and Nonregulatory T Cells

Ikuo Takeda, Shoji Ine, Nigel Killeen, Lishomwa C. Ndhlouv, Kazuko Murata, Susumu Satomi, Kazuo Sugamura and Naoto Ishii

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Distinct Roles for the OX40-OX40 Ligand Interaction in Regulatory and Nonregulatory T Cells

Ikuo Takeda,* Shoji Ine,* Nigel Killeen,‡ Lishomwa C. Ndhluvu,* Kazuko Murata,* Susumu Satomi,† Kazuo Sugamura,* and Naoto Ishii*2*

The OX40 (CD134) molecule is induced primarily during T cell activation and, as we show in this study, is also expressed on CD25+CD4+ regulatory T (Treg) cells. A necessary role for OX40 in the development and homeostasis of Treg cells can be inferred from the reduced numbers of the cells present in the spleens of OX40-deficient mice, and their elevated numbers in the spleens of mice that overexpress the OX40 ligand (OX40L). The homeostatic proliferation of Treg cells following transfer into lymphopenic mice was also found to be potentiated by the OX40-OX40L interaction. Suppression of T cell responses by Treg cells was significantly impaired in the absence of OX40, indicating that, in addition to its homeostatic functions, OX40 contributes to efficient Treg-mediated suppression. However, despite this, we found that CD25+CD4+ T cells became insensitive to Treg-mediated suppression when they were exposed to OX40L-expressing cells, or when they were treated with an agonistic OX40-specific mAb. OX40 signaling could also abrogate the disease-preventing activity of Treg cells in an experimental model of inflammatory bowel disease. Thus, although the data reveal important roles for OX40 signaling in Treg cell development, homeostasis, and suppressive activity, they also show that OX40 signals can oppose Treg-mediated suppression when they are delivered directly to Ag-engaged naive T cells. The Journal of Immunology, 2004, 172: 3580–3589.

A mong the several types of regulatory T (Treg) cells that have been described, CD25+CD4+ Treg cells are of particular interest, because they feature prominently in multiple experimental autoimmune settings (1–6). For example, the elimination of these cells from normal naive mice is sufficient to induce autoimmune disease. Disease of this sort, or the disease induced by transfer of purified CD25+CD4+ T cells into nude mice, can be prevented simply by providing the mice with NOD mice, and this susceptibility could be readily correlated with a reduced number of Treg cells in the affected mice. Strikingly, the transfer of Treg cells from normal NOD mice to CD28-deficient mice prevented diabetes. Although these observations underscore the importance of CD4+CD25+ Treg cells in diabetes, they also make clear an important requirement for B7/CD28 costimulation for Treg cell differentiation and homeostasis. IL-2 has been implicated in Treg function through related types of observations. Treg cells appear to require IL-2 for their development and expansion, and reconstitution of Treg cells can prevent the spontaneous autoimmune disease development seen in mice that have defects in IL-2 responses (11–16). Thus, both CD28 and IL-2 signals are important for Treg development and function.

CD25+CD4+ Treg cells are anergic to stimulation through the T cell Ag receptor (17–19). Exogenous IL-2 or treatment with anti-CD28 mAb can, however, overcome their anergic state and interfere with their capacity to mediate suppression (17, 18). The suppressive activity of CD25+CD4+ Treg cells is also abolished by treatment with Abs specific for CTLA-4 or glucocorticoid-induced TNFR, both of which the cells express constitutively (20–23). Such observations make clear that several molecules and signaling pathways are involved in the regulation of CD25+CD4+ Treg cell function, while leaving open the mechanisms underlying their suppressive activity.

OX40 (CD134) and its ligand, OX40 ligand (OX40L), are members of the TNFR and TNF superfamily, respectively (24–27). OX40 is expressed on activated CD4+ T cells and on some CD8+ T cells (24, 26–28), whereas OX40L is expressed on APCs such as activated B cells (29), dendritic cells (30, 31), microglia (32), and endothelial cells (33). Using OX40- or OX40L-deficient mice, we and others have demonstrated that the interaction of OX40L expressed on APC with OX40 expressed on activated CD4+ T cells is critically involved in CD4+ T cell activation (34–37). Similarly, several reports have shown that ligation of OX40 on CD4+ T cells induces an increase in clonal expansion and cytokine production of CD4+ T cells, leading to enhancement of memory CD4+ T cell...
development (38–40). Bansal-Pakala et al. (41) recently reported that OX40 crossinglink can provoke Ag-specific proliferation and cytokine production by CD4+ T cells that are anergic in the absence of the OX40 signal, indicating that OX40 signaling can overcome CD4+ T cell tolerance. Consistent with this, the expression of OX40 and/or OX40L has been demonstrated in the tissues of several immune disorders such as graft-vs-host disease (42), proliferative lupus nephritis (43), rheumatoid arthritis (44), human inflammatory bowel disease (IBD) (45, 46), and human inflammatory muscle diseases (47), and in thymomas of patients with myasthenia gravis (48). Despite these suggestive observations, a clear link between the OX40-OX40L interaction and the development of these immune disorder-associated diseases remains to be established.

We have recently reported that OX40L-transgenic (OX40L-TgL) mice, which express OX40L on T cells, spontaneously developed IBD-like colitis when the mice had been backcrossed onto the C57BL/6, but not the BALB/c background (49). Consistent with the involvement of OX40 in IBD, Powrie and colleagues (50) reported that blockade of the OX40-OX40L interaction inhibited the development of IBD, using an adoptive transfer model system in which the cotransfer of CD25+CD4+ Treg cells prevented disease (20, 50). Recent gene expression analyses for molecules specific for CD25+CD4+ Treg cells have revealed high expression of OX40 in the Treg cells compared with CD25+CD4+ T cells (19, 23). Furthermore, recent reports demonstrated that stimulation through glucocorticoid-induced TNFR, which is structurally similar to OX40, abrogates the suppressive activity of Treg cells (22, 23). On the basis of these observations, we have looked for associations between the OX40-OX40L interaction, Treg cell function, and IBD pathogenesis. The present study documents multiple roles for OX40 signals in the regulation of Treg cell function and experimental IBD.

Materials and Methods

Mice
OX40L-TgL, OX40L- (pCXN2) transgenic (OX40L-TgC), OX40L-deficient (OX40L-knockout (KO)), and OX40-deficient (OX40-KO) mice have been described previously (34, 37, 49, 51). OX40L-TgL mice, which express OX40L mainly on T cells, were constructed by using the proximal promoter from the mouse icκ gene (49, 51). OX40L-TgC mice, which express OX40L ubiquitously in various tissues, were constructed by using the chicken β-actin promoter (51). Recombination-activating gene (RAG)2-deficient (RAG2-KO) mice on the C57BL/6 background were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan) and were intercrossed with OX40L-TgC and OX40L-KO mice to generate RAG2-KO/OX40L-TgC (RAG2TgC) and RAG2/OX40L double-deficient (RAG2LDKO) mice, respectively. Ly5.1-C57BL/6 mice were kindly provided by Dr. A. Kume (Jichi Medical School, Tochigi, Japan). CD28-deficient (CD28-KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Wild-type C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). All of the mice used were backcrossed at least 12 times onto the C57BL/6 strain. Age- and sex-matched wild-type littermates of the OX40L-Tg mice were used as control mice. All mice were bred and maintained under specific pathogen-free conditions in the Institute for Animal Experimentation, Tohoku University Graduate School of Medicine.

Abs and agents
The following Abs were used for flow-cytometric experiments, the purification of cells, and in vitro cell cultures: biotin-conjugated anti-CD25 (7D4), FITC-conjugated anti-CD25 (7D4), PE-conjugated anti-CD4 (H129.19), FITC-conjugated streptavidin (purchased from BD PharMingen (San Diego, CA)), anti-CD4 (10 μg/ml; Sigma-Aldrich, St. Louis, MO), and anti-CD3 antibody (10 μg/ml; BD PharMingen), anti-IL-2 (10 μg/ml; Sigma-Aldrich, St. Louis, MO), and anti-CD3 mAb (10 μg/ml; 145-2C11). OX40 mAb, an agonistic anti-mouse OX40 mAb, was obtained from the European Cell Culture Collection (Wiltshire, U.K.). MOP34 mAb, an inhibitory mouse anti-OX40L mAb, was described previously (37).

Flow cytometry
Cells were incubated with the indicated mAbs for 20 min at 4°C. The cells were then washed with PBS and analyzed with a FACS Calibur flow cytometer (BD Biosciences, Mountain View, CA). The analyses were conducted using the CellQuest program (BD Biosciences).

Purification of CD25+CD4+ and CD25−CD4+ T cells
Splenocytes were incubated with biotinylated anti-CD25 mAb at 4°C for 20 min. The cells were then washed twice with chilled PBS, incubated with anti-biotin MicroBeads (Miltenyi Biotec, Gladbach, Germany), and separated using an AutoMACS cell sorter (Miltenyi Biotec). Alternatively, to estimate OX40 expression on Treg cells, splenocytes were incubated with
OX40 signals are related to Treg cell suppressive activity, and regulate Treg cell development, and homeostasis. A. Splenic CD25⁺ CD4⁺ T cells derived from wild-type, OX40-KO, and OX40L-Tg mice were incubated with biotinylated anti-OX40 mAb or biotinylated anti-OX40L mAb in combination with CD25-FITC and CD4-PE, followed by streptavidin-allophycocyanin, and subjected to FACS analysis. Filled histograms represent control staining. Wild-type CD25⁺ CD4⁺ T cells (5 × 10⁴) were stimulated with anti-CD3 mAb (10 μg/ml) in the presence of wild-type APCs (2 × 10⁵) and CD25⁺ CD4⁺ T cells from wild-type, OX40-KO (B), or OX40L-TgL (C) mice at the indicated ratio. [³²P]Thymidine incorporation during the last 8 h of the 3-day cultures was measured as an indicator of cell proliferation and is expressed as the mean (±SD) of triplicate (Figure legend continues).
Table I.  CD25<sup>+</sup> CD4<sup>+</sup> T cell number in the thymi and spleens of OX40-KO or OX40L-Tg mice<sup>a</sup>

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<th>Thymus (×10&lt;sup&gt;6&lt;/sup&gt;)</th>
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<td>16–18 days old</td>
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<tr>
<td>WT (n = 6)</td>
<td>2.71 ± 1.34</td>
<td>4.46 ± 2.18</td>
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<td>OX40L-TgL (n = 4)</td>
<td>3.19 ± 0.68</td>
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<td>12–13 wk old</td>
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<td>OX40L-TgC (n = 5)</td>
<td>4.41 ± 2.13</td>
<td>7.53 ± 2.07</td>
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<tr>
<td>OK-40-KO (n = 5)</td>
<td>1.35 ± 0.64</td>
<td>2.02 ± 0.42</td>
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<sup>a</sup>Results are presented as the mean ± SD. The significance of the data was evaluated by Student’s t test (*, p < 0.05; **, p < 0.01). WT, Wild type.

FITC anti-CD25 mAb followed by anti-FITC MicroBeads (Miltenyi Biotec), and separated using the AutoMACS machine. To purify CD25<sup>+</sup> CD4<sup>+</sup> T cells from lymphopenic recipient mice into which CD4<sup>+</sup> T cells were previously transferred, negative selection for CD4<sup>+</sup> T cells with the recipient splenocytes were performed by using CD4<sup>+</sup> T cell isolation kit (Miltenyi Biotec). Following this, CD25<sup>+</sup> and CD25<sup>–</sup> cells were separated from the purified CD4<sup>+</sup> T cells fragment as described above by using an AutoMACS cell sorter. The CD25<sup>+</sup> fraction was typically >90% pure CD25<sup>+</sup> CD4<sup>+</sup> T cells (see Fig. 1A). Alternatively, the CD25<sup>+</sup> fraction from recipient mice that were previously transferred with CD25<sup>+</sup> CD4<sup>+</sup> T cells, were typically >80% pure CD25<sup>+</sup> CD4<sup>+</sup> T cells (data not shown). CD25<sup>+</sup> fractions were incubated with CD4 MicroBeads (Miltenyi Biotec) and were then purified using the AutoMACS machine. The CD4<sup>+</sup> fraction was typically ≥99% pure CD25<sup>+</sup> CD4<sup>+</sup> T cells (see Fig. 1A).

**CFSE labeling**

Purified CD25<sup>+</sup> CD4<sup>+</sup> T cells were labeled with CFSE (Molecular Probes, Eugene, OR) by incubation with 2.5 µM CFSE in protein-free PBS for 10 min at 37°C and 1 min with 10-fold volume of RPMI 1640 medium containing 10% FCS. Cells were then washed twice with PBS.

**In vitro T cell response**

Purified CD25<sup>+</sup> CD4<sup>+</sup> T cells were stimulated with 10 µg/ml soluble anti-CD3 mAb in the presence of APCs for 3 or 4 days. Purified CD25<sup>+</sup> CD4<sup>+</sup> T cells were added to the culture at the indicated dose. The APCs used were irradiated (3000 rad) splenocytes taken from naive wild-type, OX40L-TgC, or OX40-KO mice. The cultured cells were assayed for [3H]Thymidine uptake at day 3 of culture. Suppressive activity of Treg cells was defined as the following: ([count in the absence of Treg cells] – [count in the presence of Treg cells])/([count in the absence of Treg cells] × 100%). Alternatively, the cultured cells including CFSE-labeled CD25<sup>+</sup> CD4<sup>+</sup> T cells were assayed for CFSE intensity at day 4 of culture.

**Experimental procedure for IBD development**

Single-cell suspensions were prepared from the spleens of 4- to 7-wk-old OX40-KO or wild-type mice and pooled. CD25<sup>+</sup> CD4<sup>+</sup> and CD25<sup>–</sup> CD4<sup>+</sup> T cells were purified from the pooled cells using the AutoMACS system as described above. Purified CD25<sup>+</sup> CD4<sup>+</sup> T cells (5 × 10<sup>6</sup>) in the presence or absence of CD25<sup>+</sup> CD4<sup>+</sup> T cells (1 × 10<sup>6</sup>) were i.v. injected into Rag2-KO, RAG40L-DKO, or RAG-TgC mice. Between 2 and 5 wk after the transfer, the recipient mice were sacrificed, and their colons were extracted for histopathological analysis using 5-µm paraffin-embedded sections stained with H&E. From the sections, colitis severity scores were estimated in a blinded fashion as previously described (52). In brief, scoring was as follows: grade 0, normal; grade 1, mild epithelial hyperplasia; grade 2, pronounced hyperplasia and significant inflammatory infiltrates; grade 3, severe hyperplasia and infiltration with significant decrease in goblet cells; and grade 4, severe hyperplasia, severe transmural inflammation, ulceration, crypt abscesses, and substantial depletion of goblet cells. The body weights of the recipient mice were monitored after transfer.

**FIGURE 3.** Constitutive OX40-OX40L interactions abrogate Treg cell anergy. A, Splenic CD25<sup>+</sup> CD4<sup>+</sup> T cells (5 × 10<sup>6</sup>) were stimulated with anti-CD3 mAb (10 µg/ml) plus agonistic anti-OX40 mAb (10 µg/ml) for 3 days in the presence of wild-type APCs (2 × 10<sup>6</sup>). Alternatively, wild-type APCs were replaced with APCs derived from OX40L-TgC mice. [3H]Thymidine incorporation during the last 8 h of the 3-day cultures was measured as an indicator of cell proliferation and is expressed as the mean (±SD) of triplicate cultures. B, Splenic CD25<sup>+</sup> CD4<sup>+</sup> T cells (5 × 10<sup>6</sup>) derived from wild-type (□), OX40L-TgC (□), or OX40L-TgL (□) mice were examined for their proliferative responses to CD3 stimulation in the presence of wild-type APCs (2 × 10<sup>6</sup>). Inhibitory anti-OX40L mAb (MGP34; 10 µg/ml) was added into the culture as an indicator. [3H]Thymidine incorporation during the last 8 h of the 3-day cultures was measured as an indicator of cell proliferation and is expressed as the mean (±SD) of triplicate cultures. These results were representative of three independent experiments.

*a*Results were presented as the mean ± SD. The significance of the data was evaluated by Student’s t test (*, p < 0.05; **, p < 0.01). WT, Wild type.
FIGURE 4. Legend continues
Results
Expression of OX40 on CD25−CD4+ and CD25−CD4+ T cells
mRNA profiling experiments have shown that the OX40 gene is highly expressed in CD25+CD4+ T cells (19, 23). To confirm this observation at the level of protein expression, we first examined the expression of the OX40 molecule on Treg cells by flow cytometry. OX40 could be readily detected on splenic Treg cells, but not on CD25−CD4+ T cells isolated from naive C57BL/6 mice (Fig. 1A). Similar results were obtained from the analysis of thymic CD25+CD4+ single-positive T cells (Fig. 1A). CD3 stimulation up-regulates the expression of OX40 on purified Treg cells (by 3-fold) and on freshly isolated conventional CD25−CD4+ T cells compared with that on unstimulated Treg and conventional T cells, respectively (Fig. 1B).

To confirm that the induced expression of OX40 on CD3-stimulated CD25−CD4+ T cells can be maintained even in the presence of Treg cells, we performed a coculture experiment using congenic mice. Stimulation with anti-CD3 was sufficient to retain the OX40 positivity of the CD25−CD4+ T cells even in the presence of Treg cells (Fig. 1C).

OX40-OX40L interactions regulate Treg cell development, homeostasis, and suppressive activity
To determine whether OX40 signals are involved in the normal differentiation, expansion, and/or survival of Treg cells, we examined the thymuses and spleens of OX40-KO mice and of mice that overexpress OX40L in a lymphoid-restricted (OX40L-TgL) or more general fashion (OX40L-TgC). The expression profile of OX40 and OX40L in the Treg fraction of these mice was first evaluated. Treg cells from wild-type and OX40L-TgL/TgC mice were positive for OX40 expression; however, transgene-derived OX40L appears to reduce the expression OX40 on Treg cells (Fig. 2A). In contrast, wild-type and OX40-KO Treg cells did not express OX40L (Fig. 2A). Although the number of CD25−CD4+ Treg cells appeared to be normal in the thymuses of mice lacking OX40, there were increased numbers of thymic Treg cells in OX40L-Tg mice (Table I). Treg cell numbers were also markedly increased in the spleens of OX40L-Tg mice, whereas we noted a significant decrease in the representation of Treg cells in the spleens of young, but not old, OX40-KO mice (Table I). These complementary observations on mice in which OX40 signaling is either augmented or absent point to an important role for the OX40-OX40L interaction in the normal development or homeostasis of Treg cells.

We next attempted to confirm the suppressive activity of the CD25−CD4+ T cell population in OX40-KO and OX40L-Tg mice, and also investigated whether OX40 is required for Treg-dependent suppression of T cell mitogenesis. For this purpose, we...
purified CD25⁺CD4⁺ Treg cells from OX40-KO and OX40L-TgL mice, and then examined their capacity to inhibit anti-CD3-induced proliferation of wild-type CD25⁺CD4⁺ T cells. As shown in Fig. 2B, OX40-deficient Treg cells were less effective at suppressing T cell proliferation than their wild-type counterparts. In contrast, Treg cells from OX40L-TgL mice were more effective suppressors than those from wild-type mice (Fig. 2C). These results suggest that the suppressive function of Treg cells may be potentiated by OX40-OX40L interactions.

As an alternative means for examining the involvement of OX40 in Treg cell homeostasis, we established a system in which Treg cells were transferred into RAG2-KO mice that expressed different levels of OX40L. Two novel types of RAG2-KO recipients were used in these experiments: mice that were homozygous for the OX40L-null mutation and thus expressed no OX40L (R/40L-DKO), and mice that expressed OX40L in most tissues from a transgene regulated by the chicken β-actin promoter (RAG/TgC). As shown in Fig. 2D, 11 days or 3 wk after transfer, CD25⁺CD4⁺ Treg cells were markedly decreased in number in the spleens of recipients that lacked OX40L compared with RAG2-KO controls. In contrast, the OX40L-transgenic recipients harbored significantly more Treg cells in their spleens than the control recipients (Fig. 2D). The functional phenotype of the Treg cells present in the recipient mice was confirmed to be suppressive in nature (Fig. 2E). These transfer data therefore reinforce the notion that OX40 signals are important for homeostatic proliferation of Treg cells and emphasize the potential importance of the OX40-OX40L interaction for the postthymic expansion of Treg cell numbers.

FIGURE 6. In vivo OX40 stimulation prevents Treg cell-derived regulatory function in a model of IBD. A. Purified splenic CD25⁺CD4⁺ T cells (purity, >99%; 5 × 10⁵) from wild-type mice were injected into the tail veins of three groups of RAG2-deficient mice (RAG2-KO, RAG/TgC, and R/40L-DKO mice; n = 6 in each group). B, CD25⁺CD4⁺ T cells (5 × 10⁵) and CD25⁻CD4⁺ T cells (purity, >90%; 1 × 10⁵) were cotransfused into the same types of recipient mice. Body weights of the RAG2-deficient recipient mice in the three groups were monitored and are represented as the average body weight index: (body weight at the indicated days)/(body weight at day 0) ± SD. H&E-stained sections of the colon of the RAG2-KO (C and D) and RAG/TgC mice (E and F) 30 days after cotransfer of CD25⁺CD4⁺ T and CD25⁻CD4⁺ Treg cells (C and E) or transfer of CD25⁻CD4⁺ T cells alone (D and F) are shown. The arrowhead in F indicates a crypt abscess. G, Colitis severity scores in the three groups of RAG2-deficient (RAG2-KO, RAG/TgC, and R/40L-DKO) mice that received wild-type CD25⁺CD4⁺ Treg cells (▲), wild-type CD25⁻CD4⁺ T cells (○), or wild-type CD25⁺CD4⁺ T cells in combination with wild-type CD25⁻CD4⁺ Treg cells (●) were determined 30 days after transfer. The data are pooled from two independent experiments. H, Colitis severity scores in the two groups of RAG2-deficient (RAG2-KO and RAG/TgC) mice transfused with OX40-deficient CD25⁺CD4⁺ T cells (●) or OX40-deficient CD25⁻CD4⁺ T cells in combination with wild-type CD25⁺CD4⁺ Treg cells (○) were determined 35 days after transfer.

FIGURE 7. In vivo OX40 stimulation promotes early expansion of transferred CD25⁺CD4⁺ T cells in the presence of Treg cells. Three groups of RAG2-deficient mice (RAG2-KO, RAG/TgC, and R/40L-DKO mice; n = 4 or 5 in each group) were i.v. transfused with wild-type CD25⁺CD4⁺ T cells (5 × 10⁵) in the absence (square) or presence of CD25⁻CD4⁺ T cells (1 × 10⁵) (□). Nine and 25 days after transfer, the number of CD25⁺CD4⁺ T cells in the spleens of the recipient mice was calculated by a microscopic cell count and flow cytometry. Results are expressed as the mean ± SD. The significance of the data was evaluated by Student’s t test (*, p < 0.05; **, p < 0.01).
OX40-OX40L interactions regulate Treg cell anergy and proliferation

Fresh ex vivo Treg cells are normally anergic to TCR stimulation in vitro (17, 18). To test whether OX40 signaling could counteract Treg cell anergy, we stimulated purified wild-type Treg cells with anti-CD3 while also incubating them with either an agonistic anti-CD40 mAb, or APCs derived from OX40L-TgC mice to provide enhanced OX40 stimulation. As shown in Fig. 3A, both of these treatments significantly promoted Treg cell proliferation. Similarly, we noted that Treg cells from OX40L-TgL or -TgC mice proliferated more vigorously than wild-type Treg cells when stimulated with anti-CD3 mAb (Fig. 3B). The increased proliferation of OX40L-Tg Treg cells was dependent on the interaction between OX40 and the ligand as it could be suppressed by the addition of an inhibitory anti-CD40L mAb (MGP34) (Fig. 3B).

OX40 signals counteract the suppression of CD25^+CD4^+ T cells by Treg cells

Paradoxically, although OX40 potentiates Treg cell suppressive activity, in multiple experimental settings, we found that OX40 signaling potently counteracted Treg-mediated suppression. Treg cells failed to suppress the proliferation of CD25^+CD4^+ T cells that had been stimulated with anti-CD3 in the presence of OX40L-TgC APCs (Fig. 4, A, rows 3 and 8, and B). Cell division experiments confirmed this conclusion (Fig. 4C, ii and v). In a similar, but less pronounced fashion than observed with the OX40L-transgenic APCs, we found that agonistic anti-CD40 mAb could also counteract the suppression mediated by Treg cells (Fig. 4, A and C).

OX40 expression on Treg cells appears to be important for the optimal suppressive effect (Fig. 4D, compare rows 2 and 4) as also depicted in Fig. 2B. To verify this, we conducted four separate experiments and found a statistical difference in the suppressive activity between wild-type (86.0 ± 6.09%) and OX40-deficient Treg cells (70.7 ± 6.53%) (mean ± SD of the four experiments; p < 0.02, Student’s t test; see Materials and Methods). Because OX40-deficient Treg cells still retain some suppressive function as shown in Fig. 2B, although significantly less than wild-type Treg cells, it was next important to determine whether the effect of OX40 signaling in target CD25^+CD4^+ T cells counteracts Treg cell-mediated suppression. Surprisingly, we found that in the presence or absence of OX40 signaling into the Treg population, the Treg-mediated suppression of CD25^+CD4^+ T cells could be inhibited by agonistic anti-CD40 mAb (Fig. 4D, compare rows 4 and 5). Furthermore, to rule out the possibility that the enhanced OX40 signals may affect the APCs used (because recent reports have detected OX40 expression on APCs (51, 53)), we also tested whether the agonistic anti-CD40 mAb could overcome Treg-mediated suppression of OX40KO or wild-type Treg cells in the presence of OX40KO APCs. Even when using OX40-deficient Treg cells and APCs, both of which were unresponsive to OX40 stimulation, stimulation with anti-CD40 mAb counteracted the suppression mediated by OX40KO-Tg T cells (Fig. 4D, compare rows 9 and 10). Cumulatively, these experiments showed clearly that despite the enhancing effect of OX40 signaling in Treg cell suppressive function, the relevant target of the OX40 signal in counteracting Treg-mediated suppression was the CD25^+CD4^+ T cells rather than the Treg cells. In addition, because CD3 stimulation induces negligible proliferative responses if the CD25^-CD4^+ cell population lacks OX40 expression (Fig. 4D, rows 11–14), OX40 signals appear to be essential for proliferation of CD25^-CD4^+ T cells in this experimental system.

OX40 signaling overcomes Treg-mediated suppression in a CD28- but not IL-2-dependent fashion

Reminiscent of the effect of OX40 signaling just described, IL-2 or CD28 stimulation can also block the suppressive activity of Treg cells (17, 18). We therefore examined the effect of exogenous IL-2 or anti-CD28 mAb on OX40 expression on Treg and CD25^+CD4^+ T cells. As shown in Fig. 5A, compared with anti-CD3 stimulation alone, neither treatment had any marked effect on the expression of OX40 on Treg cells. In separate experiments using cells from CD28-KO mice, we found that OX40 signaling could only partially overcome Treg-mediated suppression if the responding CD25^+CD4^+ T cells did not express CD28 (Fig. 5B). In contrast, related experiments using anti-IL-2 failed to reveal a requirement for IL-2 in the OX40 effect. Thus, CD28, but not IL-2, is required for OX40 signaling to overcome Treg-mediated suppression.

OX40 signaling abrogates Treg-mediated suppression in vivo

To study further the in vivo significance of the OX40-OX40L interaction for Treg cell function, we made use of an experimental IBD model system in which Treg cells suppress disease caused by CD25^-CD4^+ T cells transferred into RAG2-KO mice. Three groups of RAG2-deficient mice (RAG2-KO, RAG1/TgC, and R40L-DKO mice) were injected i.v. with wild-type or OX40-deficient CD25^-CD4^+ T cells in the presence or absence of CD4^-CD25^- Treg cells. As shown in Fig. 6, A–D and G, the cotransfer of Treg cells with CD25^-CD4^+ T cells into RAG2-KO mice strongly suppressed weight loss and histological manifestations of disease. In contrast, all RAG1/TgC mice reconstituted with wild-type CD25^-CD4^+ T cells developed severe IBD even when they received wild-type Treg cells (Fig. 6, A, B, and E–G). These observations are consistent with the in vitro results demonstrating that OX40 stimulation counteracts the capacity of Treg cells to suppress T cell responses. RAG2-KO and RAG1/TgC mice reconstituted with Treg cells alone never developed colitis (Fig. 6G). Strikingly, R40L-DKO mice reconstituted with CD4^-CD25^- T cells showed no symptoms of IBD, even when Treg cells were not cotransferred (Fig. 6, A, B, and G). We also found that OX40-deficient CD25^-CD4^+ T cells failed to induce IBD in the absence of Treg cells when they were transferred into either RAG2-KO or RAG1/TgC mice (Fig. 6H).

CD4^- T cell numbers in the spleens of the recipient mice were also examined following the transfer of wild-type CD4^-CD25^- T cells in the presence or absence of Treg cells. In this model system, the reduction in splenic CD4^- T cell number correlates well with the suppression of IBD onset. Nine days after transfer, the presence of Treg cells significantly reduced CD4^- T cell number in the spleens of recipient RAG2-KO but not RAG1/TgC mice (Fig. 7). In contrast, CD4^- T cell numbers remained low in the R40L-DKO mice even when Treg cells were not cotransferred (Fig. 7). The differences in splenic CD4^- T cell number among the three groups of RAG2-deficient mice were not evident 25 days after transfer (Fig. 7).

Taken together, the above results reveal an essential role for OX40 signaling in CD25^-CD4^+ T cells for IBD development in this experimental model system. Consistent with our in vitro observations, we found that Treg cells or a lack of OX40 signaling could suppress the early proliferation and pathogenesis of transferred CD25^-CD4^+ T cells, whereas constitutive expression of OX40L potentiated disease development.

Discussion

In this study, we show that OX40 signaling impacts Treg cell function in three prominent ways. First, abnormal OX40 signaling
disturbed the development of Treg cells, and affected their outgrowth following transfer into lymphopenic recipients. Thus, OX40 is important for the development and homeostasis of Treg cells. Second, the OX40-OX40L interaction contributed to the efficient Treg-mediated suppression of T cell responses in vitro. Finally, OX40 signals counteracted Treg-mediated suppression when they were delivered to the responding nonregulatory T cell population. Thus, the OX40-OX40L interaction affects both regulatory and nonregulatory T cells to influence suppression, susceptibility to suppression, and clonal expansion.

Our findings about the significance of the OX40-OX40L interaction are reminiscent of previous observations concerning CD28 and IL-2. Treg cells develop in much reduced numbers in the absence of the CD28-B7 interaction, and this deficit in Treg cellularity renders mice susceptible to spontaneous autoimmune disease (10). Similarly, the suppressive capacity of Treg cells is markedly impaired in the absence of IL-2 signaling, and mice that lack IL-2 or its receptor are again prone to spontaneous autoimmune disease (11–16). Although the deficiency of OX40 or OX40L does not induce obvious autoimmunity on the C57BL/6 background, the data presented here suggest that, like CD28 deficiency, the OX40-related defects in Treg cell development and function will enhance disease susceptibility on autoimmune-prone backgrounds such as NOD.

Dendritic cells can lose expression of OX40L when they are exposed to Treg cells in vivo during IBD development (50). Similarly, we have shown that OX40-bearing cells induce a marked reduction in OX40L expression on other cells in an OX40-OX40L interaction-dependent fashion (54). In this context, we note that CD25⁺ CD4⁺ T cells lacking OX40 signals made responses that were typically of a similar magnitude to those made by wild-type CD25⁺ CD4⁺ T cells cultured in the presence of Treg cells (Figs. 4, 6, and 7). Thus, one mechanism by which Treg cells may induce a suppressive effect could involve depriving dendritic cells of OX40L and thus rendering them incapable of properly activating T cells. Additional experiments are required to test this hypothesis and to reconcile it with previously published data indicating that Treg-mediated suppression may not depend on a T cell:APC interaction (55).

Whereas deficient signaling by CD28, IL-2, or OX40 may potentiate spontaneous autoimmune diseases such as IBD and diabetes, experimental autoimmune encephalomyelitis (EAE; an autoimmune disease induced by immunization with an autoantigen) appears to require efficient OX40 signaling and is markedly less severe in its absence (32, 56). The mechanisms by which OX40/OX40L signals contribute to EAE have not been clearly defined, although decreased disease in OX40L KO mice could be correlated with impaired recall responses to the myelin oligodendrocyte glycoprotein immunogen (56). On the basis of this, it seems reasonable that EAE is enhanced by OX40/OX40L signaling because of an effect on the clonal expansion of myelin oligodendrocyte glycoprotein-reactive effector and/or memory T cell populations, and not because of an effect on Treg cells.

Despite the role of OX40 in efficient Treg-mediated suppression, we found that OX40 signaling could restore proliferative responses to T cells that would otherwise be inhibited by Treg cells. However, as just suggested for EAE, the relevant target of the OX40 signal in this context was clearly the responding T cell population and not the Treg cells. Inhibition of Treg-mediated suppression by OX40 signaling required OX40 and CD28 expression on the responding T cells, but did not require IL-2. However, both IL-2 and anti-CD28 can render T cells insensitive to suppression by Treg cells through mechanisms that may be related to those engaged by the OX40 signal. These data show that OX40 signaling can either oppose or enhance Treg-mediated suppression dependent on whether the signaling is received by the Treg cell itself or the target cell with which it interacts.

Seventy to 80% of the CD25⁺ CD4⁺ T cells used in our IBD experiments had a CD45RBhigh phenotype (data not shown). In this, and the disease suppression mediated by transferred CD25⁺ CD4⁺ CD45RBhigh Treg cells, our studies resemble the related IBD studies performed by Powrie and colleagues. One recent study from Powrie’s group (50) showed that a blocking anti-OX40 mAb could suppress IBD development mediated by CD4⁺ CD25⁺ CD45RBhigh T cells transferred into scid mice. In addition to the IBD studies described here that also implicate OX40 in the development of IBD following T cell transfer, we have previously reported that OX40L-Tg mice spontaneously develop an IBD-like syndrome (49). Thus, there are now several convergent observations suggesting an important role for OX40 signaling in the development of IBD.

The pathogenesis of human and murine IBD depends on intestinal bacterial flora (57, 58). Interestingly, LPS stimulation induces OX40L expression on APCs (59) and could thereby generate cells capable of counteracting Treg-mediated suppression by the mechanisms made clear in this paper. Consistent with this, a recent study showed that LPS-stimulated dendritic cells can indeed interfere with Treg-mediated suppression (60). Thus, it is possible that intestinal bacteria may induce OX40L expression on APCs or other resident cells in the gut and in this way lead to IBD development by interference with Treg cell function. However, it remains to be determined whether this is the prominent mechanism by which OX40 contributes to IBD pathogenesis, and whether this mechanism might also be important in other autoimmune syndromes.

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References


