Cutting Edge: OX40 Inhibits TGF-β- and Antigen-Driven Conversion of Naive CD4 T Cells into CD25⁺Foxp3⁺ T cells

Takanori So and Michael Croft

*J Immunol* 2007; 179:1427-1430; doi: 10.4049/jimmunol.179.3.1427

http://www.jimmunol.org/content/179/3/1427

References

This article cites 24 articles, 14 of which you can access for free at: http://www.jimmunol.org/content/179/3/1427.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Cutting Edge: OX40 Inhibits TGF-β- and Antigen-Driven Conversion of Naive CD4 T Cells into CD25+Foxp3+ T cells

Takanori So and Michael Croft2

Naive CD4 T cells can develop into regulatory T cells (Treg)3 play a critical role in immunity. Two major populations of Treg, namely natural and adaptive cells, arise in the thymus and the periphery, respectively. Treg express the lineage-specific marker Foxp3, which controls their development and function (1–3). Adaptive Foxp3+ T cells can develop from naive CD25-Foxp3- CD4 T cells in response to Ag and driven by TGF-β (4–6). Signals supporting Foxp3 induction by TGF-β arise from engagement of the TCR and costimulation from CD28 and IL-2R (4–9), but the role of other costimulatory signals is not clear.

OX40 (CD134), a member of the TNFR superfamily, is a costimulatory receptor that, unlike CD28, is not constitutively expressed on naive T cells but is induced within 12–24 h after Ag recognition (10). OX40 activates PI3K, Akt, NF-κB, and NFAT pathways that support clonal expansion, survival, memory generation, and Th1/Th2 development (11–14). Thus, OX40 acts as a critical positive regulatory element as supported by data in models of inflammation and autoimmunity where preventing OX40 from interacting with its ligand, OX40L, can strongly inhibit immune responses (11, 15).

Whether the action of OX40 is only through regulating the generation and activity of effector T cells is not clear. In this report, we now show that OX40 signals strongly antagonize the TGF-β- and Ag-mediated conversion of naive CD25−Foxp3− CD4 T cells into CD25+Foxp3+ CD4 cells.

Materials and Methods

Mice and reagents

Wild-type AND (Tg(TcrAND)53Hed), AND × ox40−/− (mff4−/−), and AND × cd28−/− (cd28tm1Mak) TCR-transgenic mice (B6V/Jae11) were on a B10.BR-H2k H2-T18a/SgN (The Jackson Laboratory) background (12). All experiments were in compliance with guidelines of the American Association for the Accreditation of Laboratory Animal Care.

Purification of T cells and APCs

Naive CD25− CD4 T cells were purified from spleen and lymph nodes by passing over nylon columns with complement lysis using Abs to CD8 (clone 3.155), CD25 (clones PC61 and 7D4), macrophage (clone M1/70), NK cells (clone PK136), and dendritic cells (clone 3D1), followed by a Percoll gradient. APCs were depleted of T cells with complement and Abs to Thy1.2 (clones F7D5 and HO.13.4), CD4 (clone RA3.6B2), macrophage (clone M1/70), NK cells (clone PK136), and dendritic cells (clone 3D11), followed by a Percoll gradient. APCs were depleted of T cells with complement and Abs to Thy1.2 (clones F7D5 and HO.13.4), CD4 (clone RL172.4), and CD8 (clone 3.155) and irradiated with 3,000 rad. A fibroblast DCEK cell line transfected with both I-Ek and OX40L was also used (10).

T cell cultures

Cells were cultured in RPMI 1640 medium (Invitrogen Life Technologies) with penicillin, streptomycin, glutamine, HEPES, 2-ME, and 7% FCS (Omega Scientific). Naive CD25− CD4 T cells were plated at 5 × 105 cells/ml with 2 × 104 APC per milliliter and 0.1–1 μM molybdenum cytochrome c (MCC) peptide (MCC−88−103) for 4 days. Anti-OX40 (clone OX86; 10 μg/ml; R&D Systems) and OX40L (DCEK cell line transfected with both I-Ek and OX40L) were added (10). These cells lack CD80, CD86, ICAM-1, VCAM-1, VLA-4, α1β1, heat stable Ag, and CD48 and were treated with mitomycin C (10 μg/ml; Sigma-Aldrich).

FACS

After Fc block with the 2.4G2 mAb, cells were stained with anti-CD4 (RM4–5) and anti-CD25 (clone PC61) with or without TNF-α (clone 53G6-7.1; PeproTech) and biotinylated TGF-β1 (R&D Systems) for 4 h. After washing, cells were stained with anti-CD8 (clone 53–5.7; eBioscience) and PE-conjugated streptavidin (eBioscience) for 30 min at 4°C with the FACSCalibur (BD Biosciences) and FlowJo (Tree Star) software.

Real-time RT-PCR

Quantitative RT-PCR was performed by using SYBR Green I dye and a Stratagene MX4000 instrument. Total RNA was extracted using TRIzol (Invitrogen).
agonistic Ab suppressed the ratio of Foxp3
with this, further ligation of OX40 on wild-type T cells with an
Naive CD4
number of Foxp3
gated CD4 populations after 4 days (flow
and intracellular Foxp3 were evaluated on
presence or absence of anti-OX40. CD25
APC and various doses of MCC in the
genic mice were cultured with T-depleted

   5-CTGGTGAAAAGGACCTCTG-3; reverse primer, 5-TGAAGTACT
   5-GCTGATCATGGCTGGGTTGT-3) and murine HPRT (forward primer,
   5-GGCCCTTCTCCAGGACAGA-3; reverse primer, 5-TGAAGTACT
   CATTATAGTCAAGGGCA-3).

Results and Discussion
OX40 costimulation controls conversion of naive CD4 T cells into
CD25 Foxp3 CD4 cells
To investigate the differentiation of CD25−Foxp3− CD4 T cells into Foxp3+ T cells, we cultured naive cells from wild-type
or OX40−/− AND TCR transgenic mice with T-depleted splenocytes and varying doses of MCC88−103 peptide without
any exogenous source of TGF-β (Fig. 1). After 4 days, the primed T cells were stained for the expression of CD25 and Foxp3. Coexpression of both CD25 and Foxp3 was strongly
seen when wild-type T cells were cultured with lower rather than higher doses of Ag (Fig. 1, top row), supporting previous results regarding the induction of Foxp3 (5, 16, 17). Importantly,
the appearance of Foxp3+ cells was more evident when naive T cells could not express Foxp3 (Fig. 1, bottom row). In line with this, further ligation of OX40 on wild-type T cells with an
agonistic Ab suppressed the ratio of Foxp3+ to Foxp3− T cells (Fig. 1, middle row). The effect of anti-OX40 was specific for Foxp3 in that OX40 signals enhanced CD25 expression.

Furthermore, anti-OX40 inhibited the absolute number of Foxp3+CD4+ T cells that were induced, and greater numbers resulted when OX40 was absent (Fig. 1, right bottom graph),
indicating that OX40 signals did not simply enhance the outgrowth of Foxp3 effector T cells. The T cells that received OX40 signals with lower doses of Ag and displayed poor Foxp3 expression
differentiated preferentially into Th2 cytokine-producing effectors (data not shown), as previously reported (14). Collectively, these results demonstrate that OX40 engagement on recently activated
naive CD25− CD4 T cells can suppress the Ag-driven induction of Foxp3. In contrast, OX40 signaling to already differentiated pre-existing natural CD25+Foxp3+ Treg purified from wild-type
AND mice did not show any effect on intracellular Foxp3 expression (data not shown), implying that OX40 specifically antagonizes the extracellular stimuli that support initial Foxp3 induction.

OX40 inhibits TGF-β-mediated Foxp3 induction
TGF-β promotes Foxp3 in CD4 T cells (4, 5, 7). Hence, the development of the CD25−Foxp3− phenotype at lower Ag
doses might have resulted from endogenous TGF-β production by CD4 T cells (18) or APC (19). In line with this, a TGF-β-neutralizing Ab strongly suppressed differentiation into
CD25+Foxp3+ cells (Fig. 2A). This implied that OX40 might act either by suppressing TGF-β production or by inhibiting
TGF-βR signals. In support of the latter, OX40 engagement
antagonized the induction of Foxp3 driven by exogenous TGF-β (Fig. 2, B and C). This was most evident at lower doses of TGF-β, although OX40 still suppressed to an extent the
development of Foxp3+ cells at saturating concentrations. Anti-
OX40 also strongly inhibited sustained and maximal Foxp3 mRNA induction, either driven by endogenous TGF-β or exogenous TGF-β (Fig. 2D).

With high doses of exogenous TGF-β, the cells that differentiated into CD25+Foxp3+ cells were equivalent in their suppressive capacity compared with those derived in the absence of OX40 signals (data not shown). Additionally, the resultant cell populations that still contained a high ratio of Foxp3+ to Foxp3− cells did not display significant production of Th2/Th1 cytokines (data not shown). This indicates that the balance of TGF-βR vs OX40 signals dictates the ultimate ratio of Foxp3+ Treg to effector
T cells and the overall amount of effectors activity, with a high level of TGF-β being able to overcome the action of OX40.

OX40/OX40L interactions oppose CD28 and IL-2R signals that promote Foxp3
To contrast with CD28 costimulation, which acts earlier than
OX40 during naive T cell activation because CD28 is constitutively expressed (20), CD4 T cells from CD28−/− AND mice were examined. The phenotype in the absence of CD28
was opposite to that in the absence of OX40, with Foxp3 being suppressed rather than enhanced (Fig. 3A). The lack of Foxp3 was largely due to defective IL-2 production in the absence of CD28 in that exogenous IL-2 restored the number of Foxp3+ cells generated, confirming recent results (9). In line with a divergent activity of IL-2R vs OX40 signals, exogenous IL-2 partially prevented anti-OX40 from suppressing the induction of Foxp3+ T cells (Fig. 3B). Furthermore, anti-CD28 stimulation of naive T
cells did not inhibit the generation of Foxp3+ cells, contrasting

FIGURE 1. Conversion of naive CD25− Foxp3− CD4 T cells into CD25+ Foxp3+ T cells is suppressed by OX40. Naive CD4+ (CD25−Foxp3−) T cells from wild-type (top and middle rows) or OX40−/− (bottom row) AND TCR transgenic mice were cultured with T-depleted APC and various doses of MCC in the presence or absence of anti-OX40. CD25 and intracellular Foxp3 were evaluated on gated CD4 populations after 4 days (flow plots) and after 2 days for analysis of the number of Foxp3+ CD4+ T cells generated (line graph). All data are representative of three experiments. Upper right, expression of Foxp3 and CD25 in naive T cells before stimulation and with isotype controls.
FIGURE 2. OX40 inhibits Foxp3 expression induced by endogenous and exogenous TGF-β. Naive CD4 T cells from wild-type AND mice were cultured with APC and 0.25 μM (A) or 0.5 μM (B–D) MCC in the presence or absence of anti-TGF-β (αTGF-β), varying doses of exogenous TGF-β, or anti-OX40 (αOX40). Expression of CD25 and Foxp3 in CD4 T cells was evaluated at day 4 by flow cytometry (A and B), at day 2 for the enumeration of Foxp3+/CD4+ T cells (C), or from day 1 to day 3 by PCR for Foxp3 mRNA (D). Data are representative of at least two experiments. Cont IgG, Control IgG.

FIGURE 3. Differential roles of OX40 and CD28 in the induction of Foxp3. Naive CD4 T cells from wild-type (A, top row) and B or CD28−/− (A, bottom row) AND mice were cultured with APC and 0.1 μM (A) or 0.5 μM (B) MCC, in the presence or absence of IL-2, TGF-β (1 ng/ml in A; 0.4 ng/ml in B), anti-CD28 (αCD28), or anti-OX40 (αOX40). Expression of CD25 and Foxp3 was evaluated at day 4 (A) or day 3.5 for enumeration of Foxp3+/CD4+ T cells (B). Data are representative of two experiments.

with anti-OX40 (Fig. 3B). This suggests that OX40 delivers quantitatively or qualitatively distinct signals to CD28 and IL-2R that allow suppression of TGF-βR activity for Foxp3, and it also correlates with the finding that CD28 but not OX40 strongly controls the initial production of IL-2 by naive T cells (20).

Lastly, to evaluate more clearly the inhibitory role of OX40/OX40L interactions, we established a two-step T cell culture system with IEk, OX40L interactions, we established a two-step T cell culture system with IEk-derived APC expressing only OX40L. Naive T cells were cultured as before with low-dose Ag for 18 h. These activated cells were then repurified for secondary culture with OX40L-expressing APC in the presence or absence of TGF-β. At 18 h before restimulation, wild-type and OX40−/− T cells expressed equivalent CD25 and TGF-βRII and no Foxp3 (Fig. 4A). After restimulation with Ag and OX40L, wild-type T cells did not develop into Foxp3+ cells without the addition of TGF-β (Fig. 4, B and C). Moreover, even when TGF-β was added only a small fraction of T cells became Foxp3+, implying that OX40L specifically suppressed the induction of Foxp3. This was shown with T cells lacking OX40. Without exogenous TGF-β, a fraction of OX40−/− T cells became Foxp3, comparable to wild-type cells cultured with TGF-β. More strikingly, TGF-β strongly promoted Foxp3+ cells when OX40L could not engage OX40. This data directly correlated with the levels of Foxp3 mRNA expressed in wild-type vs OX40−/− T cells (Fig. 4D). OX40 can promote the secretion of IL-2 and IL-4 (10, 14), but these cytokines did not account for the action of OX40/OX40L in suppressing Foxp3 because blocking their receptor signals did not restore the induction of CD25+Foxp3+ cells (Fig. 4E). Rather, similar to the data in Fig. 3B, exogenous IL-2 partially overcame the inhibitory effect of OX40 on Foxp3, although it did not restore Foxp3 induction to the level seen in the absence of OX40 (Fig. 4E).

In conclusion, we show that OX40 signaling can inhibit TGF-βR-mediated conversion of naive CD4 T cells into CD25+Foxp3+ Treg cells. CD28 signaling and IL-2R signaling form a feedback loop to synergize with TGF-β to promote Foxp3 expression, whereas OX40, when it is induced and can be ligated by OX40L, antagonizes sustained and maximal Foxp3 mRNA expression and thereby suppresses the development of Ag-specific Foxp3+ Treg. OX40 has previously been shown to regulate the generation of high frequencies of effector T cells, in part through targeting the cell division process in responding naive T cells and in part through allowing activated T cells to survive for long periods of time (12, 13, 20, 21). Furthermore, OX40 triggering, either directly on natural CD25+ Treg or on effector T cells that are being regulated, can abrogate or overcome suppressive activity, which would also enhance the ability of naive T cells to expand into effector cells (22, 23).
studies add further weight to the significance of the OX40/OX40L interaction along with the recent demonstration in human CD4 cells that OX40 can suppress the development of IL-10-secreting populations with suppressive activity (24).

Acknowledgments
We thank Xiaohong Tang and YanFei Adams for technical assistance.

Disclosures
The authors have no financial conflict of interest.

References

Figure 4. OX40/OX40L interactions antagonize TGF-β induction of Foxp3. Naive CD4+ T cells from wild-type or OX40−/− mice were cultured with APC and 0.75 μM MCC for 18 h. Negatively selected CD4 T cells were then repleted with DEX/ribose fibroblasts that expressed TGF-β and OX40L. T cells were stimulated with MCC for an additional 3 days in the presence or absence of exogenous TGF-β (0.4 ng/ml), or anti-IL-2R (αIL-2R), anti-IL-4 (αIL-4), or IL-2. A, CD25 vs Foxp3 and TGF-βRII expression on wild-type or OX40−/− CD4 T cells at 18 h before restimulation. B, CD25 and Foxp3 expression on gated CD4 cells after 3 days of secondary culture with varying doses of Ag. C, Total numbers of Foxp3+ CD4+ T cells generated 38 h after restimulation. D, Kinetics of Foxp3 mRNA expression after secondary stimulation with 1 μM Ag. E, CD25 and Foxp3 expression on gated CD4 cells after 3 days of culture with 1 μM Ag. Data are representative of two experiments.