OX40/OX40L Costimulation Affects Induction of Foxp3$^+$ Regulatory T Cells in Part by Expanding Memory T Cells In Vivo[1]

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OX40 is a member of the TNFR superfamily and has potent T cell costimulatory activities. OX40 also inhibits the induction of Foxp3$^+$ regulatory T cells (Tregs) from T effector cells, but the precise mechanism of such inhibition remains unknown. In the present study, we found that CD4$^+$ T effector cells from OX40 ligand-transgenic (OX40Ltg) mice are highly resistant to TGF-β mediated induction of Foxp3$^+$ Tregs, whereas wild-type B6 and OX40 knockout CD4$^+$ T effector cells can be readily converted to Foxp3$^+$ T cells. We also found that CD4$^+$ T effector cells from OX40Ltg mice are heterogeneous and contain a large population of CD44^{high}CD62L$^-$ memory T cells. Analysis of purified OX40Ltg naive and memory CD4$^+$ T effector cells showed that memory CD4$^+$ T cells not only resist the induction of Foxp3$^+$ T cells but also actively suppress the conversion of naive CD4$^+$ T effector cells to Foxp3$^+$ Tregs. This suppression is mediated by the production of IFN-γ by memory T cells but not by cell-cell contact and also involves the induction of T-bet. Importantly, memory CD4$^+$ T cells have a broad impact on the induction of Foxp3$^+$ Tregs regardless of their origins and Ag specificities. Our data suggest that one of the mechanisms by which OX40 inhibits the induction of Foxp3$^+$ Tregs is by inducing memory T cells in vivo. This finding may have important clinical implications in tolerance induction to transplanted tissues. *The Journal of Immunology,* **2008, 181: 3193–3201.**

Naive CD4$^+$ T effector cells (i.e., conventional resting T cells) have the capability to differentiate into functionally different T cell subsets upon activation, which is controlled to a large extent by the expression of distinct transcription factors and by the different cytokine milieus in which T cells are stimulated (1). It is well known that an IL-12-rich environment along with activation of the STAT4 and T-bet transcription factors mediate Th1 differentiation, whereas IL-4 and the activation of STAT6 and GATA-3 promote Th2 differentiation (2, 3). Moreover, CD4$^+$ T effector cells can also differentiate into tissue-destructive Th17 cells and immunosuppressive Foxp3$^+$ regulatory T cells (Tregs)[3] (4). Specifically, TGF-β turns on Foxp3 gene expression, which programs activated T effector cells to become Foxp3$^+$ Tregs. However, in the presence of inflammatory cytokines, especially IL-1, IL-6, or TNF-α, TGF-β rather induces the expression of RORγt, which mediates Th17 differentiation (5). Th17 cells can induce tissue inflammation by producing powerful inflammatory cytokines and by recruiting other leukocytes to inflammatory sites (6, 7). Because the activation of different T cell subsets impacts the nature of an immune response and, in the case of immune-mediated diseases, affects the nature of the disease process as well as the treatment strategies, understanding precisely the molecular pathways that regulate subset specification and the development of the means to effectively modulate such pathways is of great scientific and clinical interest.

OX40 is a T cell costimulatory molecule that belongs to the TNFR superfamily (8, 9). It is believed that OX40 expression is confined to activated T effector cells and that the engagement of OX40 delivers a costimulatory signal that mediates survival, proliferation, and differentiation of activated T cells (9). In some models, OX40 costimulation preferentially induces a Th2 type of immune response (10–12), while in other models OX40 signaling supports both Th1 and Th2 responses (13–15). Studies using mice genetically deficient for OX40 or the OX40 ligand (OX40L) have also demonstrated that OX40 costimulation has a profound effect on the generation of memory T cells, especially memory CD4$^+$ T cells (16, 17). In fact, OX40-deficient mice fail to develop effector memory T cells in the periphery, even though the primary T cell response is relatively normal in those mice (17, 18). Thus, OX40 seems to have an obligatory and nonredundant role in the generation of memory T cells, most likely by supporting cell survival via the up-regulation of cell survival genes (19, 20). Interestingly, Foxp3$^+$ Tregs constitutively express OX40 on the cell surface and OX40 costimulation has been shown to abrogate their suppressor functions (21, 22), which contrasts sharply to its costimulatory effect on T effector cells. In certain models, OX40 imparted signals also inhibit the induction of new Foxp3$^+$ Tregs from activated T effector cells (22, 23), suggesting that OX40 may act as a potent negative regulator of Foxp3$^+$ Tregs in the periphery. Because the manipulation of Foxp3$^+$ Tregs is an important and clinically relevant issue in the treatment of T cell-mediated cytopathic conditions, more studies are warranted to further unravel the role

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[3] Abbreviations used in this paper: Treg, regulatory T cell; B6, C57BL/6; foxp3gfp, Foxp3-GFP knockin mice on B6 background; KO, knockout; OX40L, OX40 ligand; OX40Ltg, OX40L transgenic; OX40KO, OX40 knockout; SN, supernatant; Wt, wild type.

of OX40 in the development of a regulatory type of immune response, especially in the context of its costimulatory effects on other T effector cells.

In the present study, we used OX40 knockout (KO) mice and OX40L transgenic (OX40Ltg) mice in which the Foxp3$^+$ Tregs are color coded by enhanced GFP to critically examine the role of OX40 costimulation in the induction of new CD4$^+$Foxp3$^+$ Tregs from T effector cells. We found that, unlike wild-type (Wt) C57BL/6 (B6) and OX40-deficient T cells, CD4$^+$ T effector cells from OX40Ltg mice are highly resistant to TGF-β-mediated induction of Foxp3$^+$ Tregs. The failure to induce CD4$^+$Foxp3$^+$ T cells from OX40Ltg T effector cells is due to the presence of large numbers of memory CD4$^+$ T cells, which actively suppress the conversion of naive CD4$^+$ T cells to Foxp3$^+$ Tregs by producing copious amount of IFN-γ.

Materials and Methods

Animals

Foxp3-GFP knockin mice on the B6 background (foxp3gfp) were created by introducing the bicistronic enhanced GFP reporter gene into the endogenous Foxp3 locus as previously reported (4). Generation of OX40$^{-/-}$ and OX40Ltg mice has already been described (24, 25). OX40$^{-/-}$ foxp3gfp mice and OX40Ltg-foxp3gfp mice were generated by crossing foxp3gfp mice with OX40$^{-/-}$ mice or OX40Ltg mice and selected by PCR-based genotyping (26). Both Wt and OX40Ltg-foxp3gfp mice were also crossed with the congenic CD45.1 mice. BALB/c, C57BL/6 (CD45.1 or CD45.2), IFN-$\gamma^{-/-}$, IFN-γR$^{-/-}$, and T-bet$^{-/-}$ mice were purchased from The Jackson Laboratory. Rag$^{-/-}$ DO11.10 TCR transgenic mice were obtained from Taconic Farms. The TEa CD4$^+$ TCR-transgenic mice were provided by Dr. R. Noelle (Dartmouth University Medical School, Lebanon, NH) (27). Both TEa mice and OT II mice were crossed with our foxp3gfp mice. Animal use and care conformed to the guidelines established by the Animal Care Committee at Harvard Medical School in Boston, MA.

Abs and cytokines

The following anti-mouse mAbs used for cell surface and intracellular staining were purchased from eBioscience and BD Pharmingen: PE-Cy5-anti-CD4 (clone GK1.5), PE-anti-CD25 (clone PC61), PE-anti-OX40L (clone RM134L), PE-anti-CD45.1 (clone A20), PE-anti-IFN-γ, FITC-anti-IFN-γ (clone XMG1.2), PE-anti-IL-4, FITC-anti-IL-4 (clone 11B11), PE-anti-Foxp3 (clone FJK-16s), FITC-anti-Foxp3 (clone FJK-16s), PE-CY7-anti-CD44 (clone IM7), Pacific Blue-anti-CD62L (clone MEL-14), and isotype control Abs.

The following mAbs used for neutralization assays were obtained from eBioscience: anti-IFN-γ (clone XMG1.2), anti-IL-1α (clone B122), anti-IL-1β (clone ALF-161), anti-IL-2 (clone JES6.1A12), anti-IL-4 (clone 11B11), anti-IL-6 (clone MP5–20F3), anti-IL-10 (clone JES5-2A5), and anti-IL-12/23 (clone C17.8). The anti-IL-18 (clone 93-10C) and anti-IL-21 (clone AF594) neutralizing Abs were obtained from R&D Systems. Recombinant mouse IL-4, IL-6, and IL-12 and recombinant human TGF-β1 were also purchased from R&D Systems.

Cell staining and flow cytometry

Spleen and lymph nodes were harvested from donor mice and single cell suspensions were prepared. Cells were resuspended in PBS and 0.5% BSA solution and then incubated with anti-CD16/32 Ab (clone 2.4G2; BD Pharmingen) to block Fc receptor-mediated nonspecific binding. Cells were washed and further stained with fluorochrome-conjugated Abs against specific cell surface markers on ice for 20 min. After the staining, cells were washed twice in PBS/BSA and fixed in 1% paraformaldehyde before FACS analysis.

For intracellular Foxp3 staining, cells were stained with fluorochrome-conjugated specific mAbs against surface markers first and then were fixed, permeabilized, and stained with PE-anti-Foxp3 mAb according to the manufacturer's protocol (eBioscience). For intracellular IFN-γ and IL-4 staining, cells were briefly stimulated with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (550 ng/ml; Sigma-Aldrich) in the presence of GolgiStop (BD Pharmingen) for an additional 4 h. Cells were stained with mAbs against the proper cell surface markers, fixed in 2% paraformaldehyde, permeabilized with 0.5% saponin, and then labeled with either FITC-anti-IFN-γ or PE-anti-IL-4. For all cell stainings, isotype-matched control Abs were included as controls.

Samples were acquired using the FACScan or LSR II flow cytometer (BD Biosciences), data analysis was performed using CellQuest software (BD Biosciences) or FlowJo software (Tree Star).

Cell sorting

FACSAria (BD Biosciences) was used for all cell sorting (22). Briefly, CD4$^+$Foxp3(GFP)$^-$ T effector cells were sorted from Wt foxp3gfp mice, OX40$^{-/-}$, and OX40Ltg-foxp3gfp mice by selectively gating onto the GFP$^-$ fraction in the CD4$^+$ T cell population. The sorted CD4$^+$ Foxp3(GFP)$^-$ T effector cells were then used for in vitro conversion assays. To obtain naive and memory CD4$^+$ T cells, spleen and lymph node cells were prepared from donor mice in PBS plus 0.5% BSA solution. The resulting cell suspension was stained with PE-CY5-anti-CD4, PE-Cy7-anti-CD44, and Pacific Blue-anti-CD62L in PBS plus 0.5% BSA for 20 min at 4°C. The CD44^{low}CD62L$^+$ cells (naive) and the CD44^{high}CD62L$^-$ cells (effector memory) within the CD4$^+$ subset were electronically gated and selectively sorted. In some experiments, CD4$^+$ T effector cells from IFN-$\gamma^{-/-}$, IFN-γR$^{-/-}$, and T-bet$^{-/-}$ mice were sorted based on CD25 expression (CD4$^+$CD25$^-$ T cells) and used for the Treg conversion assays (22). The purity of cells sorted by this method is typically >99% (22).

Conversion of CD4$^+$Foxp3$^-$ T effector cells to Foxp3$^+$ Tregs in vitro

FACS-sorted CD4$^+$ T effector cells, naive CD4$^+$ T cells, or memory CD4$^+$ T cells were stimulated in vitro (1×10^5 cells/well) with anti-CD3 (2 μg/ml; clone 2C11, BD Pharmingen) plus an equal number of APCs in the presence or absence of recombinant TGF-β1 (3 ng/ml; R&D Systems) for 3–5 days. APCs were prepared from congenic mice by depleting T cells from total spleen cells with anti-CD3-conjugated MACS beads (Miltenyi Biotec) and then briefly treated with mitomycin C at 50 μg/ml (Sigma-Aldrich) at 37°C for 20 min. Induction of Foxp3$^+$ Tregs in the CD4$^+$ fraction was analyzed by FACS based on the expression of GFP or intracellular staining for the Foxp3 protein (22). In some experiments, the naive and memory CD4$^+$ T cells were mixed at different ratios in the coculture assays or separated using the Transwell system where the naive T cells and memory T cells were separated by a 0.4-μm semipermeable membrane (Corning Costar). Cells were stimulated with anti-CD3 plus APCs in both compartments in the presence or absence of TGF-β1, and induction of Foxp3$^+$ Tregs was determined 3–5 days later by FACS.

Generation of Ag-specific memory CD4$^+$ T cells

TEa mice or OTII mice were immunized with their cognate Ags prepared in complete adjuvant. Four to 6 wk later, mice were killed, spleen and lymph node cells were prepared, and cells were briefly stained with PE-CY5-anti-CD4, PE-Cy7-anti-CD44, and Pacific Blue-anti-CD62L in PBS plus 0.5% BSA solution. CD44^{hig}CD62L$^-$ cells (effector memory) within the CD4$^+$GFP$^-$ subset were selectively sorted and used as Ag-specific memory cells for all the experiments.

In some experiments, the sorted CD4$^+$ memory T cells were stimulated with cognate Ag plus APCs or anti-CD3 plus APCs for 3 days, and in some cultures TGF-β was added. The culture supernatant (SN) was collected and used in the Treg conversion experiments.

Real-time PCR

Total cellular RNA was extracted using the RNeasy mini kit (Qiagen) and reverse transcribed into cDNA with the ABI PRISM TaqMan reverse transcription method. Expression of genes of interest and of GAPDH control was assessed in simplex RT-PCR with FAM and VIC probes (Applied Biosystems). All TaqMan primers and probes were purchased from Applied Biosystems. Transcript levels of target genes were calculated according to the $2^{-\Delta\Delta Ct}$ method as supplied by the manufacturer (ABI PRISM 7700 user bulletin; Applied Biosystems) and expressed as arbitrary units (26).

Statistics

Data were compared using the Student t test, and a $p < 0.05$ was considered significant.

Results

CD4$^+$Foxp3$^-$ T effector cells from OX40Ltg mice are highly resistant to the induction of Foxp3$^+$ Tregs

To examine the role of OX40 signaling in the induction of Foxp3$^+$ Tregs, we FACS sorted CD4$^+$GFP(Foxp3$^-$ T effector cells from Wt foxp3gfp, OX40KO, and OX40Ltg-foxp3gfp mice, stimulated

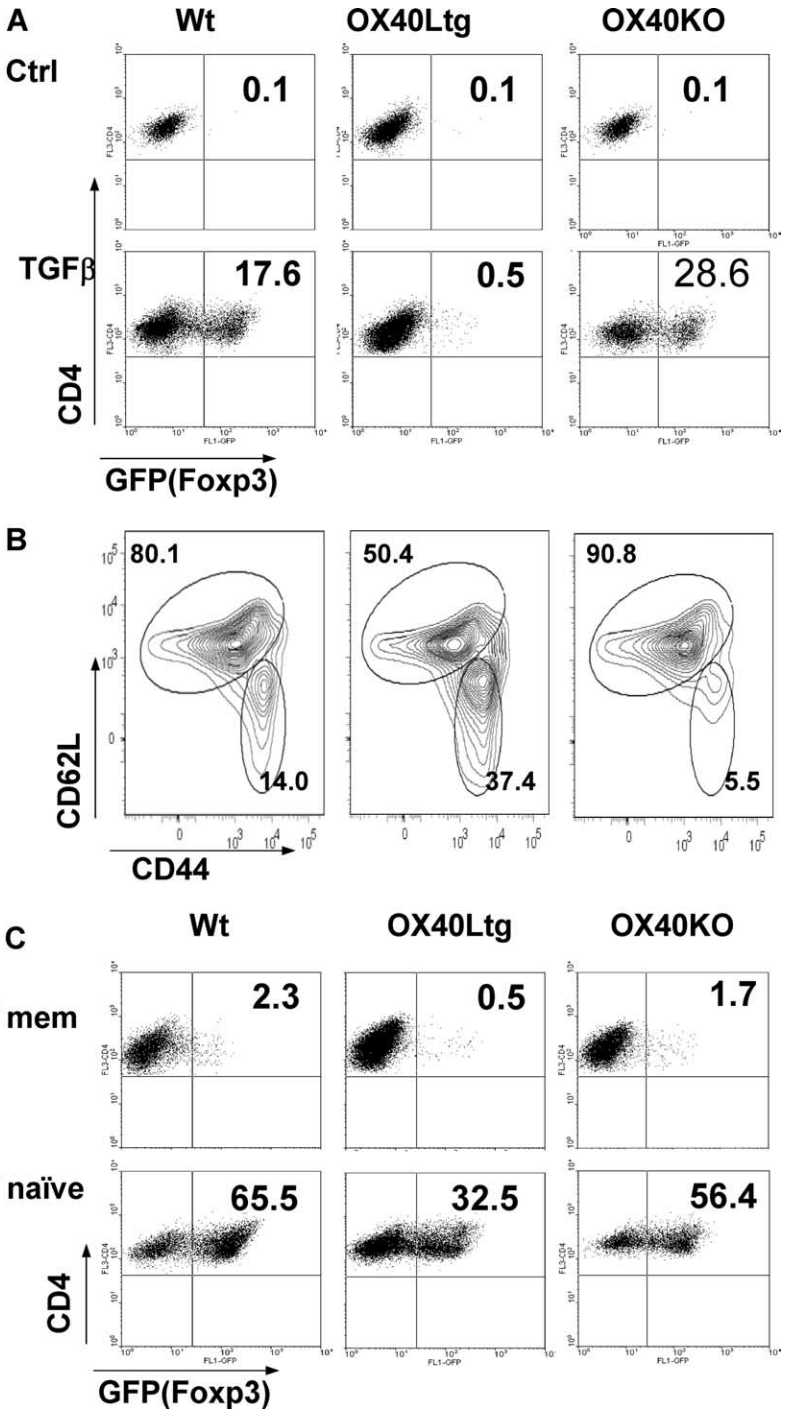

FIGURE 1. Induction of $CD4^+Foxp3(GFP)^+$ Tregs from Wt B6, $OX40^{-/-}$, and OX40Ltg T effector cells. *A*, $CD4^+$ $Foxp3(GFP)^-$ T effector cells were FACS sorted from Wt, $OX40^{-/-}$, and OX40Ltg-foxp3gfp mice and stimulated in vitro with anti-CD3 plus Wt APCs in the presence or absence of TGF-β for 4 days. Induction of $Foxp3(GFP)^+$ Tregs was determined by FACS. Representative data from one of four experiments are shown. Ctrl, Control. *B*, Spleen cells from Wt, $OX40^{-/-}$, and OX40Ltg-foxp3gfp mice were phenotyped based on the expression of CD44 and CD62L. The plot shown are cells gated on $CD4^+Foxp3(GFP)^-$ T effector cells. One of four individual experiments is shown. *C*, $CD4^+Foxp3(GFP)^-$ T effector cells were sorted into $CD44^{low}CD62L^+$ naive T cells and $CD44^{high}CD62L^-$ memory T cells (T mem), the sorted T cells were stimulated with anti-CD3 and APCs along with TGF-β, and the induction of $Foxp3(GFP)^+$ T cells was analyzed simultaneously. Data shown are representative data of three experiments.

them in vitro with anti-CD3 plus syngeneic APCs in the presence or absence of TGF-β for different time points, and analyzed the induction of $GFP(Foxp3)^+$ Tregs by flow cytometry. As shown in Fig. 1*A*, a significant fraction of Wt $CD4^+$ T effector cells could be readily converted to $Foxp3(GFP)^+$ T cells (~17%) 4 days after the culture. Similarly, OX40KO $CD4^+$ T effector cells were also readily converted to $Foxp3(GFP)^+$ T cells (~28%), although at a slightly higher rate than the Wt controls. To our surprise, OX40Ltg $CD4^+GFP(Foxp3)^-$ T effector cells are completely resistant to TGF-β-induced conversion, and very few T cells (<0.5%) were converted to $Foxp3(GFP)^+$ T cells under identical culture conditions, which is in striking contrast to Wt and OX40KO T effector cells.

To investigate the possible reasons for the resistance of TGF-β induced Foxp3 induction, we first analyzed the phenotype of $CD4^+Foxp3(GFP)^-$ T effector cells from Wt, OX40KO, and OX40Ltg mice. As shown in Fig. 1*B*, the $CD4^+Foxp3(GFP)^-$ T effector cells are in fact highly heterogeneous and consist of both naive ($CD44^{low}CD62L^{high}$) and effector memory ($CD44^{high}CD62L^-$) T cells. Interestingly, the memory fraction in OX40Ltg $CD4^+$ T effector cells was increased by almost 3-fold as compared with the Wt controls. In contrast, such $CD44^{high}CD62L^-$ memory cells were largely absent in OX40KO $CD4^+$ T cells, confirming a key role for the OX40/OX40L pathway in the generation of effector memory $CD4^+$ T cells (16). We then sorted the $CD4^+Foxp3(GFP)^-$ T effector cells into naive ($CD44^{low}CD62L^{high}$) and memory ($CD44^{high}CD62L^-$)

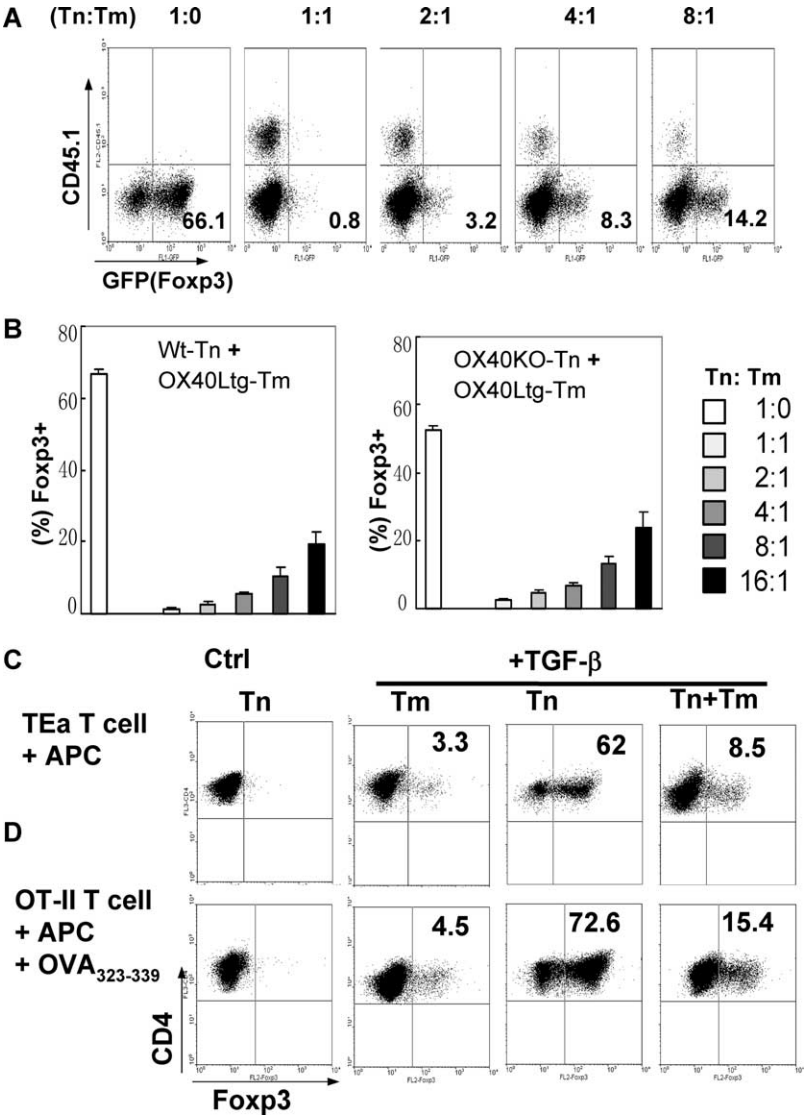

FIGURE 2. Memory CD4$^+$ T cells (Tm) inhibit the conversion of naive CD4$^+$ T effector cells (Tn) to Foxp3$^+$ Tregs. *A*, Naive Wt CD4$^+$Foxp3(GFP)$^-$ T effector cells (CD45.2) were mixed with OX40Ltg memory CD4$^+$ T cells (CD45.1) at different ratios and stimulated in the same culture with anti-CD3 plus APCs in the presence of TGF-β. Induction of Foxp3$^+$ Tregs from naive and memory T cells in the same cultures was analyzed by FACS 4 days later. Naive Wt CD4$^+$ T effector cells stimulated in the absence of OX40Ltg memory T cells were included as a positive control. Representative data of three individual experiments are shown. *B*, Conversion of CD4$^+$ T effector cells to Foxp3$^+$ Tregs in the presence or absence of CD4$^+$ memory T cells. Data shown are mean $\pm$ SD of three experiments. *C*, Naive CD4$^+$ T effector cells (CFSE labeled) from TEa-foxp3gfp mice were stimulated with their cognate Ags with or without TGF-β for 4 days. In some cultures, sorted memory CD4$^+$ T cells or a mixture of naive and memory CD4$^+$ T cells were stimulated under identical conditions. Induction of Foxp3$^+$ T cells in naive CD4$^+$ T cells (CFSE$^+$) or memory CD4$^+$ T cells (CFSE$^-$) was determined by intracellular Foxp3 staining. Representative data of three individual experiments are shown. Ctrl, Control. *D*, Naive CD4$^+$ T effector cells (CFSE labeled) from OTII foxp3gfp mice were stimulated with OVA peptide (0.5 μg/ml) plus APCs with or without TGF-β for 4 days. In some cultures, sorted memory CD4$^+$ T cells or a mixture of naive and memory CD4$^+$ T cells were stimulated under identical conditions. Induction of Foxp3$^+$ T cells in naive CD4$^+$(CFSE$^+$) and memory CD4$^+$(CFSE$^-$) T cells was determined by intracellular Foxp3 staining. Representative data of three individual experiments are shown.

cells and then compared their responsiveness to TGF-β-mediated induction of Foxp3$^+$ T cells. As shown in Fig. 1*C*, memory CD4$^+$ T cells, regardless of their origins, could not be converted to Foxp3$^+$ T cells. In stark contrast, naive OX40Ltg CD4$^+$ T cells, similar to naive Wt CD4$^+$ T cells, could be readily converted to GFP(Foxp3)$^+$ T cells. Under such culture conditions, ~65% of naive Wt CD4$^+$ T cells became Foxp3(GFP)$^+$ 4 days later, whereas ~32% of naive OX40Ltg CD4$^+$ T cells were Foxp3(GFP)$^+$. Because OX40Ltg T effector cells expanded more vigorously than the Wt controls, the absolute number of Foxp3(GFP)$^+$ T cells in both cultures was largely comparable. This finding suggests that memory T cells may not only resist TGF-β-mediated conversion but also interfere with the conversion of naive CD4$^+$ T cells into Foxp3$^+$ Tregs.

CD44^{high}CD62L$^-$ memory T cells inhibit the induction of Foxp3$^+$ Tregs from CD4$^+$ T effector cells

The striking difference between bulk and purified naive OX40Ltg CD4$^+$ T effector cells in regard to the induction of Foxp3$^+$ T cells prompted us to examine whether memory T cells actively inhibit the conversion of naive CD4$^+$ T cells to Foxp3$^+$ Tregs. For this purpose, we FACS sorted naive CD4$^+$GFP(Foxp3)$^-$ T effector cells from Wt congenic CD45.2 mice and mixed them with memory CD4$^+$ T cells from OX40Ltg mice (CD45.1) at different ratios. This cell mixture was stimulated with anti-CD3 plus APCs in the presence of TGF-β and induction of Foxp3$^+$ T cells was examined 4 days later. In these experiments, the naive CD4$^+$ T cells and memory CD4$^+$ T cells are stimulated in the same culture and, therefore, confounding issues regarding Foxp3 induction can be excluded. As shown in Fig. 2*A*, naive CD4$^+$ T cells (CD45.1$^-$), but not memory CD4$^+$ T cells (CD45.1$^+$), could be converted to Foxp3$^+$ T cells in the same cultures. Moreover, the presence of memory CD4$^+$ T cells in the cultures inhibited the conversion of naive CD4$^+$ T cells to Foxp3$^+$ T cells in a dose-dependent fashion and, at a 1:1 ratio, the inhibition of Foxp3 induction by memory T cells was >95%, which was highly significant. To exclude the possible concerns of OX40L expression on memory CD4$^+$ T cells from OX40Ltg mice, we repeated the same coculture experiments using naive CD4$^+$ T effector cells sorted from OX40$^{-/-}$ foxp3gfp mice as responding cells. As shown in Fig. 2*B*, OX40Ltg memory CD4$^+$ T cells also inhibited the conversion of OX40-deficient T effector cells to Foxp3$^+$ Tregs, suggesting it is unlikely that OX40L expression on OX40Ltg memory T cells affects the inhibitory effects of memory T cells in this model.

To determine whether the effect of memory T cells on the induction of Foxp3$^+$ T cells is unique to the OX40Ltg model or is a general feature of all memory T cells, we used two TCR transgenic models to address this issue. The first is the TEa mice in which the CD4$^+$ T cells express a transgenic TCR that recognizes

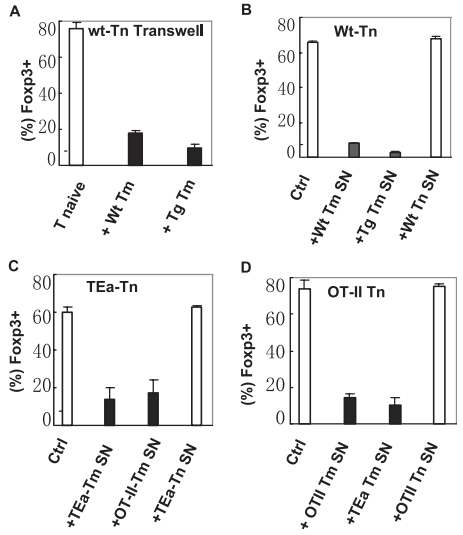

FIGURE 3. Suppression of Foxp3$^+$ T cells by memory T cells (Tm) is mediated by soluble factors but not by cell-cell contact. *A*, Naive CD4$^+$ T effector cells (Tn) from Wt foxp3gfp mice and memory CD4$^+$ T cells from Wt and OX40Ltg-foxp3gfp mice were separated in the Transwell cultures by a semipermeable membrane. Cells in both chambers were stimulated with anti-CD3 and APCs plus TGF-β, and induction of Foxp3$^+$ T cells was determined and compared with that naive CD4$^+$ T cells stimulated in the absence of memory CD4$^+$ T cells. Data shown are mean ± SD of three experiments. *B*, Naive CD4$^+$ T effector cells from Wt foxp3gfp mice and were stimulated with anti-CD3 plus APCs in the presence of TGF-β. In those cultures, SNs from anti-CD3/APC-stimulated Wt and OX40Ltg memory CD4$^+$ T cells or naive CD4$^+$ T cells were added (50 μl of SN in a total culture volume of 200 μl), and their effects on the induction of Foxp3$^+$ Tregs were shown. Data shown are mean ± SD of three experiments. Ctrl, Control. *C*, CD4$^+$ T effector cells from TEa mice were stimulated with cognate Ags plus TGF-β. In those cultures, SNs from TEa memory T cells or OTII memory T cells simulated with their cognate Ags plus APCs were added (50 μl), and the effects on the induction of Foxp3$^+$ Tregs were determined and shown. Data shown are mean ± SD of three experiments. *D*, CD4$^+$ T effector cells from OTII mice were stimulated with OVA peptide/APCs plus TGF-β. In those cultures, SNs from TEa memory T cells or OTII memory T cells simulated with their cognate Ags plus APCs were added (50 μl), and the effects on the induction of Foxp3$^+$ Tregs were determined and shown. Data shown are mean ± SD of three experiments.

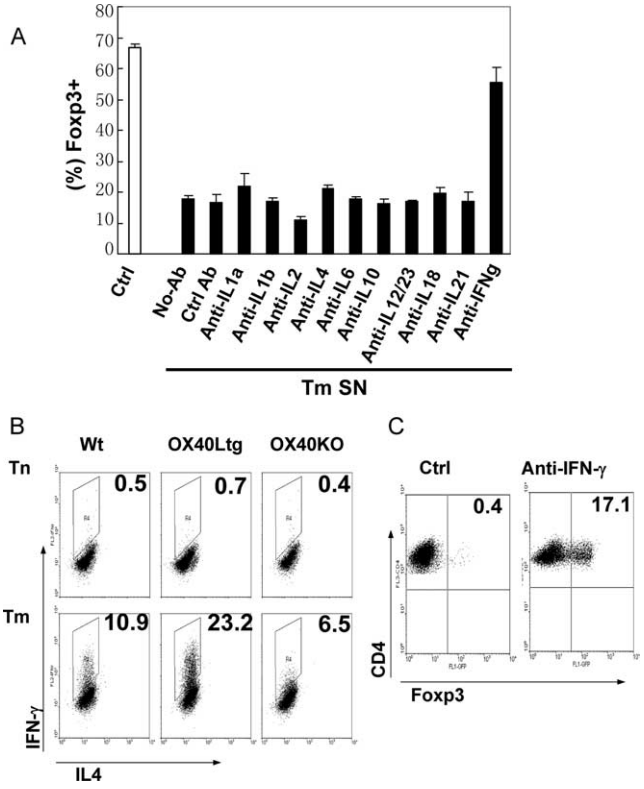

FIGURE 4. Memory T cell (Tm)-derived IFN-γ is involved in suppression of Foxp3+ Treg induction. *A*, Wt naive CD4$^+$GFP(Foxp3)$^-$ T effector cells (Tn) were stimulated with anti-CD3/APCs and TGF-β. In these cultures, SNs from stimulated OX40Ltg CD4$^+$ memory T cells were added (50 μl) along with neutralizing mAbs against various cytokines (20 μg/ml). Data shown are conversion of CD4$^+$ T effector cells to GFP(Foxp3)$^+$ Tregs. Representative data of four individual experiments are shown. Ctrl, Control. *B*, Freshly prepared naive and memory CD4$^+$ T cells from Wt foxp3gfp, OX40$^{-/-}$, and OX40Ltg-foxp3gfp mice were briefly stimulated with PMA/ionomycin, and the expression of IFN-γ and IL-4 was analyzed by intracellular staining. Data shown are representative data from three independent experiments. *C*, CD4$^+$GFP(Foxp3)$^-$ T effector cells (consisting of both naive and memory cells) were sorted from OX40Ltg mice and stimulated with ant-CD3/APCs plus TGF-β. Induction of Foxp3$^+$ T cells with or without a neutralizing anti-IFN-γ mAb (20 μg/ml) was determined 4 days later. Data shown are one of four independent experiments.

I-E^d alloantigens presented in the context of I-A^b and, therefore, respond vigorously to (C57BL/6 × BALB/c)F1 APCs (27). The second is the OTII mouse model where the CD4$^+$ T cells express a TCR transgene that recognizes the OVA peptide (28). Ag-specific memory T cells were generated by immunizing mice with corresponding cognate Ags and then FACS sorting based on the expression of CD44 and CD62L. As shown in Fig. 2, *C* and *D*, when naive CD4$^+$ T cells from either TEa mice or OTII mice were stimulated with APC plus cognate Ags in the absence of TGF-β, the activated CD4$^+$ T cells remained Foxp3$^-$. However, the addition of TGF-β in the cultures readily converted a significant fraction of naive CD4$^+$ T effector cells to Foxp3$^+$ cells (~60 to 70%). Once again, TGF-β failed to convert memory CD4$^+$ T cells to Foxp3$^+$ T cells under identical culture conditions. Furthermore, in cultures in which naive and memory CD4$^+$ T cells were mixed at 1:1 ratio, the induction of Foxp3$^+$ T cells from naive CD4$^+$ effector cells was markedly inhibited. This finding suggests that the inhibition of CD4$^+$ T effector cells to convert to Foxp3$^+$ Tregs is a general feature of memory T cells.

Suppression of Foxp3$^+$ Treg induction by memory T cells is mediated by soluble factors not by cell-cell contact

To examine the mechanisms by which memory T cells inhibit the conversion of naive CD4$^+$ T cells to Foxp3$^+$ cells, we first examined whether such inhibition requires cell-cell contact. For this purpose, we performed the same in vitro conversion assay using Transwell cultures in which OX40Ltg memory CD4$^+$ T cells were placed in the upper wells and sorted naive Wt CD4$^+$ T cells in the lower wells. Once again, cells were stimulated with anti-CD3 plus syngeneic APCs in the presence of TGF-β. As shown in Fig. 3*A*, naive CD4$^+$ T effector cells from Wt B6 mice were readily converted to Foxp3$^+$ T cells, and such conversions were strongly inhibited by the addition of memory CD4$^+$ T cells. Interestingly, either Wt or OX40Ltg memory CD4$^+$ T cells were equally potent in the inhibition of Foxp3$^+$ T cell conversion, suggesting it is unlikely that cell-cell contact is critical to the inhibition of Foxp3 induction. We then stimulated the sorted memory CD4$^+$ T cells with anti-CD3/APCs and TGF-β, collected the culture SN, and tested whether the culture SN could replace memory T cells in

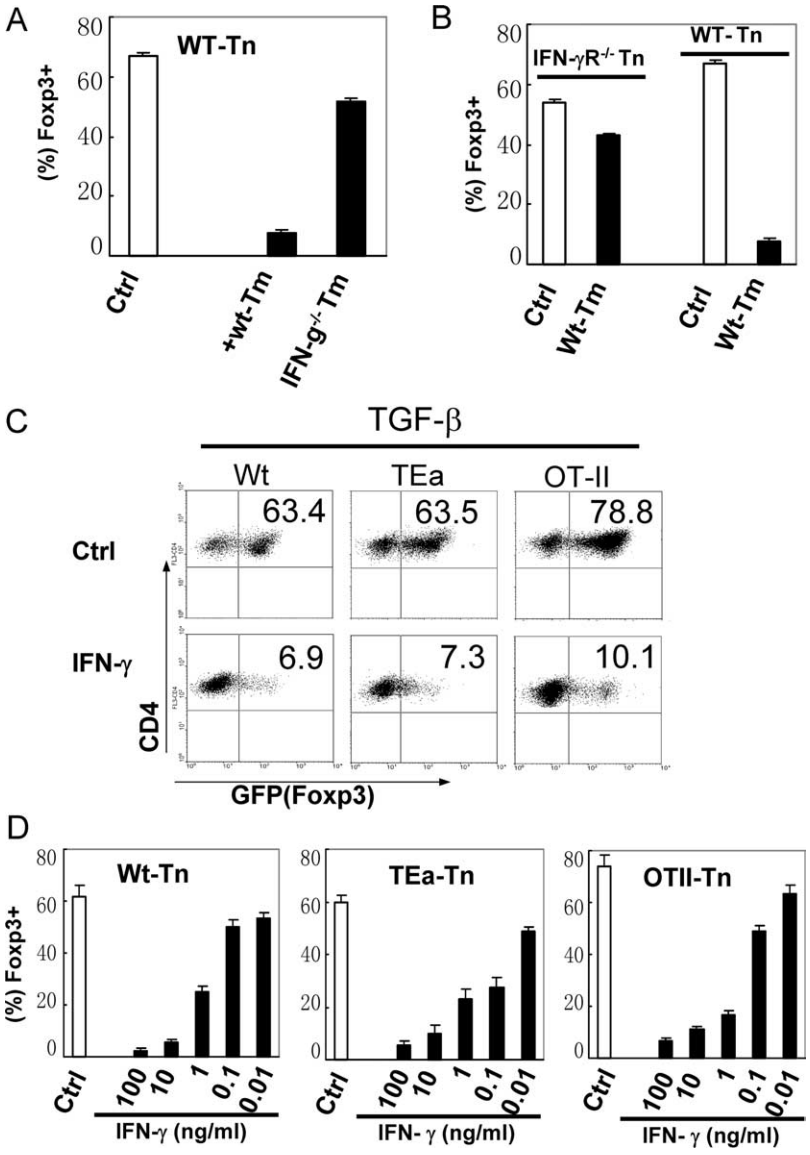

FIGURE 5. IFN-γ inhibits the conversion of naive CD4$^+$ T effector cells (Tn) to Foxp3$^+$ Tregs. *A*, Wt CD4$^+$GFP(Foxp3)$^-$ T effector cells (CD45.1$^+$) were stimulated with anti-CD3/APCs in the presence of TGF-β, CD4$^+$ memory T cells (Tm) sorted from Wt B6 mice (wt-Tm) and IFN-γ KO mice (IFN-g$^{-/-}$ Tm) were added at equal numbers in the cultures, and the induction of GFP(Foxp3)$^+$ Tregs in CD45.1$^+$CD4$^+$ cells was determined and shown. Data shown are mean $\pm$ SD of three independent experiments. Ctrl, Control. *B*, Naive CD4$^+$GFP$^-$ (or CD25$^-$) T effector cells from Wt B6 mice (Wt-Tn) and IFN-γR KO mice (IFN-γR$^{-/-}$ Tn) were stimulated with anti-CD3/APCs in the presence of TGF-β. In these cultures, equal numbers of CD4$^+$ memory T cells sorted from Wt B6 mice (CD45.1$^+$) were added and the induction of Foxp3$^+$ T cells in CD4$^+$CD45.2$^+$ T cells was determined 4 days later. Data shown are mean $\pm$ SD of three independent experiments. *C*, Naive CD4$^+$GFP(Foxp3)$^-$ T effector cells were FACS sorted from Wt B6, TEa, and OT-II mice, and the sorted CD4$^+$ T effector cells were stimulated with anti-CD3/APCs or the cognate Ags plus APCs. In some cultures, exogenous IFN-γ was added at 10 ng/ml, and the induction of GFP(Foxp3)$^+$ Tregs was determined 4 days later by FACS. Data shown are from one of four independent experiments. *D*, The conversion experiments were set up as described above in Fig. 5*C*, but different concentrations of IFN-γ ranging from 0.01 ng/ml to 100 ng/ml were added into the cultures. Induction of Foxp3$^+$ T cells among total number of CD4$^+$ T cells with or without exogenous IFN-γ was shown. Data shown are mean $\pm$ SD of three individual experiments.

suppressing the induction of Foxp3$^+$ T cells. As shown in Fig. 3*B*, culture SNs from either Wt memory cells or OX40Ltg memory cells strongly inhibited the conversion of naive CD4$^+$ T cells to Foxp3$^+$ Tregs. In contrast, the SN from cultured naive CD4$^+$ T cells had no effect on the conversion. Additional experiments showed that when Ag-specific memory CD4$^+$ T cells from either TEa or OT-II mice were stimulated with their cognate Ags plus APCs, the culture SNs also inhibited the conversion of naive CD4$^+$ T cells to Ag-specific Foxp3$^+$ Tregs and that such inhibition did not show any Ag specificity (Figs. 3, *C* and *D*), supporting the involvement of soluble factor(s) in this model.

IFN-γ is a key cytokine produced by memory T cells that inhibits the induction of Foxp3$^+$ Tregs

To determine the identity of soluble factor(s) in the culture SN involved in the inhibition of Foxp3$^+$ Treg induction, we repeated the conversion experiments in which the sorted naive CD4$^+$ T cells were stimulated with anti-CD3 plus APCs in the presence of TGF-β. In these cultures, SN from cultured OX40Ltg memory T cells was added along with a panel of neutralizing mAbs against various cytokines, and the induction of Foxp3$^+$ T cells was determined 4 days later and compared with cultures without the addition of memory cell SN. As shown in Fig 4*A*, only the anti-IFN-γ neutralizing Ab abrogated the inhibition of Foxp3$^+$ Treg induction by the SN, whereas blocking other cytokines had no obvious effect at all, suggesting that IFN-γ is a key cytokine in the SN in suppressing the induction of Foxp3$^+$ Tregs.

To ascertain that the CD4$^+$ memory T cells in OX40Ltg mice are truly IFN-γ-producing cells, naive and memory CD4$^+$ T cells were sorted from Wt and OX40Ltg mice, the sorted cells were briefly stimulated with PMA and ionomycin in vitro, and the expression of IFN-γ was analyzed by intracellular staining. As shown in Fig. 4*B*, OX40Ltg memory T cells expressed copious amounts of IFN-γ while IFN-γ in naive CD4$^+$ T cells was virtually undetectable. Furthermore, stimulation of memory cell-rich CD4$^+$ T effector cells from OX40Ltg mice with anti-CD3/APCs plus TGF-β failed to induce Foxp3$^+$ T cells (Fig. 1), but the inclusion of a neutralizing anti-IFN-γ mAb resulted in the induction of Foxp3$^+$ T cells (Fig. 4*C*).

We performed two additional sets of experiments to further address the role of IFN-γ in our model. First, we FACS sorted memory CD4$^+$ T cells (CD44^{high}CD62L$^-$GFP$^-$) from Wt and IFN-γ-deficient mice and compared their effects on TGF-β-induced

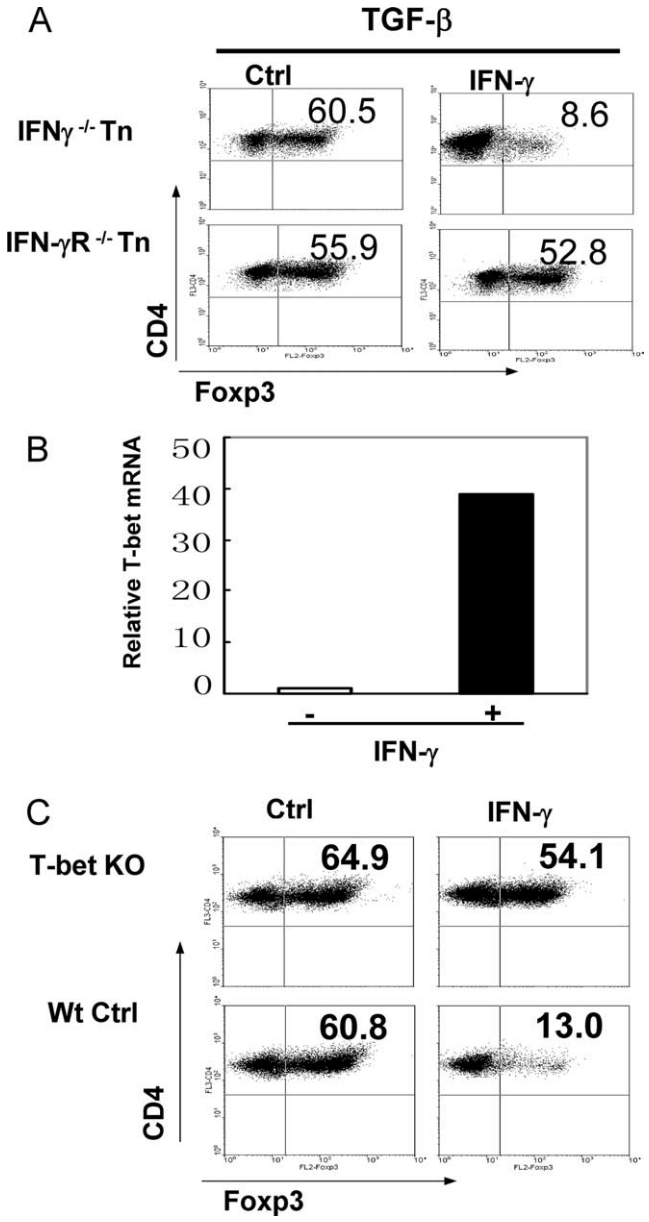

FIGURE 6. IFN-γR signaling and T-bet activation are required for the suppression of Foxp3$^+$ Treg induction. *A*, Naive CD4$^+$CD25$^-$ T effector cells (Tn) were sorted from IFN-γ KO and IFN-γR KO mice and stimulated in vitro with anti-CD3/APCs in the presence of TGF-β. In some cultures, exogenous IFN-γ (10 ng/ml) was added and the induction of Foxp3$^+$ T cells was determined 4 days later by intracellular staining for Foxp3. Data shown are from one of four individual experiments. Ctrl, Control. *B*, Wt naive CD4$^+$ T effector cells were stimulated with anti-CD3/APCs in the presence of TGF-β, and the expression of T-bet gene transcripts with or without the addition of IFN-γ in the cultures was determined four days later by real-time PCR. Data shown are from one of three experiments. *C*, Naive CD4$^+$ T effector cells were sorted from Wt BALB/c foxp3gfp mice and T-bet KO mice and stimulated in vitro with anti-CD3/APCs in the presence of TGF-β. In some cultures, exogenous IFN-γ (10 ng/ml) was added and the induction of Foxp3$^+$ T cells was determined 4 days later by intracellular staining for Foxp3. Data shown are from one of four individual experiments.

conversion of naive CD4$^+$ T cells to Foxp3$^+$ Tregs. As shown in Fig. 5*A*, memory CD4$^+$ T cells from Wt mice, but not from IFN-γ deficient mice, inhibited the conversion of naive CD4$^+$ T cells to Foxp3$^+$ Tregs. Second, we compared the conversion of Wt and IFN-γR KO naive CD4$^+$ T cells to Foxp3$^+$ Tregs in the presence of Wt memory T cells. As shown in Fig. 5*B*, memory CD4$^+$ T cells from congenic CD45.1 B6 mice inhibited the conversion of Wt CD4$^+$ T cells, but not that of the IFN-γR knockout CD4$^+$ T cells, to Foxp3$^+$ Tregs.

To further confirm the inhibitory role of IFN-γ in an Ag-specific setting, we stimulated FACS-sorted naive CD4$^+$GFP(Foxp3)$^-$ T effector cells from TEa and OT II mice with their cognate Ags in the presence of TGF-β with or without IFN-γ, and induction of Foxp3$^+$ T cells was determined 4 days later by FACS. As shown in Fig. 5*C*, IFN-γ strongly inhibited the induction of Foxp3$^+$ T cells from TEa and OT II cells, and the inhibitory effect of IFN-γ in this model is clearly dose dependent (Fig. 5*B*). Taken together, these data suggest strongly that memory T cells that are capable of producing IFN-γ can antagonize the induction of Foxp3$^+$ Tregs in the periphery.

Blocking IFN-γ signaling or absence of T-bet overcomes the inhibition of Foxp3$^+$ Tregs

As shown in Fig. 6*A*, naive CD4$^+$ T effector cells from both IFN-γ KO mice and IFN-γR KO mice are equally responsive to TGF-β-mediated induction of Foxp3$^+$ Tregs, and a significant fraction of naive CD4$^+$ T effector cells could be converted to Foxp3$^+$ Tregs (~60%). In these cultures, the addition of exogenous IFN-γ inhibited the conversion of IFN-γ KO cells, but not that of the IFN-γR KO cells, suggesting that IFN-γR signaling is required for the suppression of Foxp3 induction. Because T-bet, which is required for programming the development of Th1 cells (29), has been shown to be rapidly induced by IFN-γ in lymphocytes (30), we assessed the expression of T-bet transcripts in naive Wt CD4$^+$ T effector cells stimulated with anti-CD3/APCs plus TGF-β with or without the addition of IFN-γ. Similarly as in previous reports (30), T-bet gene transcripts were highly expressed in CD4$^+$ T effector cells stimulated in the presence of IFN-γ (Fig. 6*B*). To further determine the role of T-bet in IFN-γ-mediated suppression of Foxp3 induction, naive CD4$^+$GFP$^-$ (or CD25$^-$) T effector cells were sorted from Wt BALB/c foxp3gfp mice and T-bet KO mice, the sorted CD4$^+$ T effector cells were stimulated in vitro with anti-CD3/APCs plus TGF-β, and the induction of Foxp3$^+$ T cells was determined by flow cytometry. As shown in Fig. 6*C*, CD4$^+$ T effector cells from both Wt and T-bet KO mice could be readily converted to Foxp3$^+$ Tregs by TGF-β (~50% Foxp3$^+$ cells). However, the addition of exogenous IFN-γ strongly inhibited the conversion of Wt CD4$^+$ T effector cells but completely failed to suppress the conversion of T-bet KO cells, suggesting that that transcriptional activation of T-bet is associated with the suppressive effect of IFN-γ in the induction of Foxp3$^+$ Tregs.

Discussion

Recent studies have identified additional roles for OX40/OX40L costimulation in the T cell response. OX40 appears to have diametrically different effects on T effector cells and Foxp3$^+$ Tregs; it delivers a potent costimulatory signal to T effector/memory cells but inhibits the induction and suppressor functions of Foxp3$^+$ Tregs (31). Thus, understanding precisely the role of OX40 in regulating the different aspects of T cell responses and the development of effective means to modulate such responses are important and clinically relevant issues. In the present study, we addressed the role of OX40 in the induction of new inducible Foxp3$^+$ Tregs using OX40KO-foxp3gfp and OX40Ltg-foxp3gfp mice and demonstrated several new and interesting findings that might be therapeutically important.

Clearly, induction of Foxp3$^+$ Tregs in the periphery can be regulated by many different pathways and, in addition to the proinflammatory cytokines (e.g., IL-1, IL-6, and TNF-α) (32), OX40/

OX40L costimulation also plays an important role in suppressing the induction of Foxp3$^+$ Tregs. It is notable that OX40 appears to use different mechanisms to suppress the conversion of naive CD4$^+$ T effector cells to Foxp3$^+$ Tregs. Our data suggest that one of the mechanisms by which OX40 inhibits Foxp3 induction is by expanding IFN-γ-producing memory T cells. This conclusion is based on the following observations. First, CD4$^+$Foxp3$^-$ T effector cells from OX40Ltg mice completely failed to convert to Foxp3$^+$ Tregs, even under optimal conditions that could readily convert Wt and OX40KO T effectors to Foxp3$^+$ Tregs (Fig. 1). OX40Ltg CD4$^+$ T effector cells contain a surprisingly large population of memory T cells (i.e., CD44^{high}CD62L$^-$). In fact, >37% of the OX40Ltg CD4$^+$ T effector cells express such a CD44^{high}CD62L$^-$ memory phenotype but this phenotype is largely absent in OX40KO mice, confirming that OX40/OX40L costimulation in vivo is critical to the generation of such memory phenotype (18). Second, FACS-sorted naive CD4$^+$ T cells (CD44^{low}CD62L$^+$) from OX40Ltg mice that are devoid of memory CD4$^+$ T cells can be readily converted to Foxp3$^+$ Tregs, although at a lower rate than that of the Wt naive CD4$^+$ T cells (Fig. 1). Furthermore, memory CD4$^+$ T cells, regardless of their origins and Ag specificities, inhibit the conversion of naive CD4$^+$ T cells to Foxp3$^+$ Tregs in a cell contact-independent fashion. Third, OX40Ltg memory CD4$^+$ T cells readily produce copious amounts of IFN-γ, and neutralizing IFN-γ using specific mAb can promote the conversion of memory-rich OX40Ltg CD4$^+$ T effector cells, which are usually resistant to TGF-β-mediated conversion, into Foxp3$^+$ T cells (Fig. 4). Also, IFN-γ by itself is extremely potent in suppressing the conversion of naive CD4$^+$ T cells to Foxp3$^+$ Tregs, and this effect requires the presence of IFN-γR signaling and T-bet activation (Fig. 6). In fact, our data showed that T-bet is a critical transcription factor with which the induction of Foxp3$^+$ Tregs from T effector cells is inhibited by IFN-γ. Thus, IFN-γ-producing memory T cells not only resist TGF-β-mediated induction of Foxp3 but also actively suppress the conversion of naive CD4$^+$ T cells to Foxp3$^+$ Tregs. Clearly, OX40/OX40L interactions play an important role in expanding such memory T cells in vivo (18, 33).

Our study does not suggest that the generation of memory T cells is the only mechanism by which OX40 inhibits the induction of Foxp3$^+$ Tregs. In fact, direct OX40 signaling to activated CD4$^+$ T effector cells has also been shown to block the conversion of T effector cells to Foxp3$^+$ Tregs (22, 23). Using a TCR transgenic system, So and Croft showed that directly engaging OX40 on the surface of CD4$^+$ T effector cells using an agonist anti-OX40 mAb prevented the induction of Foxp3$^+$ Tregs by TGF-β (23). In our own studies, we found that the stimulation of CD4$^+$ T effector cells with anti-CD3 plus OX40Ltg APCs to directly engage OX40 on the T effector cells also blocked the induction of Foxp3$^+$ Tregs by TGF-β (22). Thus, OX40 costimulation also directly antagonizes the induction of Foxp3$^+$ T cells. In the present study, we also observed that purified naive OX40Ltg CD4$^+$ T effector cells are not as easily convertible as naive Wt CD4$^+$ T effector cells to Foxp3$^+$ Tregs (Fig. 1), suggesting that besides the presence of memory T cells, other mechanisms might also be involved in blocking Foxp3 induction. The precise molecular pathways triggered by OX40 engagement in blocking Foxp3 induction have yet to be resolved, but it is an area of considerable interests. In an in vitro model, stimulation of CD4$^+$ T effector cells with OX40Ltg APCs appears to inhibit Foxp3 gene expression (22), suggesting a possibility that OX40 signaling pathways and TGF-β-mediated Foxp3 induction may cross-regulate each other. However, the in vivo relevance of this finding remains unclear. Also, induction and maintenance of Foxp3 expression in activated T effector cells involve many other pathways, including TCR signaling, IL-2 signaling, CD28 signaling, and TGF-β signaling, and the role of OX40 costimulation in modulating such molecular pathways in the context of Foxp3$^+$ Treg induction is completely unknown. More studies are clearly required to further address this issue. Nonetheless, data from our studies and those of others suggest that OX40 signaling to T effector cells can directly suppress the induction of Foxp3$^+$ Tregs, or do so indirectly via the induction of memory T cells.

In our model system, IFN-γ appears to be a key cytokine from memory T cells that inhibit the induction of Foxp3$^+$ Tregs from CD4$^+$ T effector cells. Clearly, CD4$^+$ memory T cells from IFN-γ KO mice failed to inhibit the conversion of naive CD4$^+$ T effector cells to Foxp3$^+$ Tregs, and neutralizing IFN-γ in memory T cell-rich cultures that usually resist TGF-β-induced conversion can partially overcome such resistance (Figs. 4). Moreover, IFN-γ by itself strongly inhibits the conversion of Wt CD4$^+$ T effector cells, but not that of IFN-γR knockout T effector cells, to Foxp3$^+$ Tregs (Fig. 6). Additionally, the inhibitory effects of IFN-γ are also observed in cultures using TCR transgenic models in which CD4$^+$ T effector cells expressing a TCR transgene are stimulated with cognate Ags; in some cultures the transgenic CD4$^+$ T effector cells are selected based on whether they express Foxp3(GFP) and, therefore, concerns over the expansion of contaminating Foxp3$^+$ T cells in the cultures are completely excluded. Nonetheless, our findings appear to be in contrast with other reports showing that IFN-γ rather stimulates the induction or suppressor functions of Foxp3$^+$ Tregs (34, 35). The likely cause for such a discrepancy is unclear but may be related to the different culture systems used. In our studies we focused specifically on the conversion of naive CD4$^+$ T effector cells to Foxp3$^+$ Tregs, and the T effector cells were stringently selected based on whether they express Foxp3(GFP). In other studies, however, either unfractionated CD4$^+$ T cells are used or CD4$^+$ Tregs are selected based on CD25 expression (34). It has been well documented that IFN-γ can preferentially induce the apoptotic cell death of T effector cells, but not that of Tregs (36), and that the CD4$^+$Foxp3$^+$ Tregs can be CD25$^+$ or CD25$^-$ (37). Because only proportional changes of Foxp3$^+$ T cells were reported in those studies, the possibility that the increase in Foxp3$^+$ Tregs may only be relative to the selective death of T effector cells cannot be excluded. In fact, this possibility is supported by a recent report showing the antagonistic nature of Th1 and Th2 programs in opposing the induction of Foxp3$^+$ Tregs from naive T effector cells (38). In this study, IFN-γ is shown to be a key cytokine from Th1 cells that inhibits the induction of new Foxp3$^+$ Tregs from CD4$^+$ T effector cells (38), which is consistent with our current study.

Our finding may have far-reaching implications. First, CD4$^+$ T effector cells, once committed to a particular effector program (e.g., Th1, Th2, Th17, or T memory), will certainly counterbalance the induction of Foxp3$^+$ Tregs from T effector cells (38) and, besides cytokines, certain costimulatory pathways also regulate such effector programs. Clearly, OX40 plays a critical role in this regard by regulating Th1 and Th2 or the generation of memory cells (31), which hinders the induction of Tregs. Second, in addition to Th1 cells, the activation of innate NK cells and memory recall responses that result in the production of high levels of IFN-γ may also antagonize the induction of Ag-specific Tregs; this may have further impact on the induction of peripheral tolerance. Finally, induction of Treg-mediated tolerance in transplantation and autoimmunity may be difficult to achieve in memory-rich environments (e.g., primates, humans) (39), and strategies to target memory T cells may be required for the induction of Treg-mediated tolerance under such conditions.

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Disclosures

The authors have no financial conflict of interest.

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