The Journal of Immunology

TNFR2 Is Critical for the Stabilization of the CD4 $^{+}$Foxp3^{+} Regulatory T Cell Phenotype in the Inflammatory Environment

Xin Chen, Xueqiang Wu, Qiong Zhou, O. M. Zack Howard, Mihai G. Netea and Joost J. Oppenheim

J Immunol 2013; 190:1076-1084; Prepublished online 31 December 2012;
doi: 10.4049/jimmunol.1202659
http://www.jimmunol.org/content/190/3/1076

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

TNFR2 Is Critical for the Stabilization of the CD4$^+$Foxp3$^+$ Regulatory T Cell Phenotype in the Inflammatory Environment

Xin Chen,* Xueqiang Wu,† Qiong Zhou,† O. M. Zack Howard,† Mihai G. Netea,‡ and Joost J. Oppenheim†

Several lines of evidence indicate the instability of CD4$^+$Foxp3$^+$ regulatory T cells (Tregs). We have therefore investigated means of promoting the stability of Tregs. In this study, we found that the proportion of Tregs in mouse strains deficient in TNFR2 or its ligands was reduced in the thymus and peripheral lymphoid tissues, suggesting a potential role of TNFR2 in promoting the sustained expression of Foxp3. We observed that upon in vitro activation with plate-bound anti-CD3 Ab and soluble anti-CD28 Ab, Foxp3 expression by highly purified mouse Tregs was markedly downregulated. Importantly, TNF partially abrogated this effect of TCR stimulation and stabilized Foxp3 expression. This effect of TNF was blocked by anti-TNFR2 Ab, but not by anti-TNFR1 Ab. Furthermore, TNF was not able to maintain Foxp3 expression by TNFR2-deficient Tregs. In a mouse colitis model induced by transfer of naive CD4 cells into Rag1$^{-/-}$ mice, the disease could be inhibited by cotransfer of wild-type Tregs, but not by cotransfer of TNFR2-deficient Tregs. Furthermore, in the lamina propria of the colitis model, most wild-type Tregs maintained Foxp3 expression. In contrast, an increased number of TNFR2-deficient Tregs lost Foxp3 expression. Thus, our data clearly show that TNFR2 is critical for the phenotypic and functional stability of Tregs in the inflammatory environment. This effect of TNF should be taken into account when designing future therapy of autoimmunity and graft-versus-host disease by using TNF inhibitors. ***The Journal of Immunology*****, 2013, 190: 1076–1084.**

It is well established that CD4$^+$Foxp3$^+$ regulatory T cells (Tregs) play a nonredundant role in the maintenance of immunological homeostasis and in the prevention of autoimmune disorders, and they also represent a major cellular mechanism in immune evasion by tumors (1, 2). The transcription factor Foxp3 is a unique marker specific for the Treg lineage, which determines the phenotype and immunosuppressive function of Tregs (3). Abrogation of Foxp3 gene function leads to the development of lethal multiorgan autoimmune disorders in mice and humans (4, 5). In contrast, forced expression of Foxp3 in CD4$^+$CD25$^-$ effector T cells (Teffs) is sufficient to convert them into functional Tregs (6, 7).

Contrary to the previous notion that Tregs were stable and terminally differentiated cells, recent evidence revealed the phenotypic and functional plasticity of Tregs in response to inflammatory stimulation. For example, IL-1β and IL-6 each reprogrammed Treg cells and induced them to express IL-17 (8-10). However, there is compelling contrasting evidence that the number of highly suppressive Foxp3-expressing cells actually increased in various inflammatory sites (11). Therefore, the inflammatory environment seems to have the capacity to restrain the plasticity and promote the phenotypic and functional stability of Tregs. Clarification of the molecular basis of such an effect may be therapeutically beneficial and can further improve our understanding of Treg biology.

TNF is a pleiotropic cytokine and upregulation of TNF expression is a benchmark of inflammatory responses. The biological functions of TNF are mediated by two structurally related, but functionally distinct, receptors, TNFR1 (or p55) and TNFR2 (or p75) (12). With a death domain in its cytoplasmic tail, TNFR1 is the primary signaling receptor on most cell types and accounts for most of the proinflammatory, cytotoxic, and apoptotic effects classically attributed to TNF (13, 14). In contrast, TNFR2 lacks an intracellular death domain and predominantly mediates signals promoting lymphocyte activation and proliferation (15, 16). Although counterintuitive, the accumulated evidence indicates that TNF by signaling through TNFR2 promotes Treg activity. For example, we reported that TNF by activating TNFR2 is able to activate and expand Tregs (17), and TNFR2 expression identifies the maximal suppressive (18) and replicating Tregs (19) in mice and helps identify functional Tregs in human PBMCs (20). Furthermore, TNF preferentially upregulates TNFR2 expression on Tregs (21). In confirmation of our observation, Housley et al. (22) have shown that TNFR2 is critical for the in vivo immunosuppressive function of naturally occurring Tregs. Grinberg-Bleyer et al. (23) showed that pathogenic Teffs stimulated the activation

*Basic Science Program, Science Applications International Corporation–Frederick, National Cancer Institute–Frederick, Frederick, MD 21702; †Laboratory of Molecular Immunoregulation, Cancer Inflammation Program, Center for Cancer Research, National Cancer Institute–Frederick, Frederick, MD 21702; and ‡Department of Medicine, Radboud University Nijmegen Medical Center, 6500 HB Nijmegen, The Netherlands

Received for publication September 21, 2012. Accepted for publication December 3, 2012.

This work was supported by National Cancer Institute, National Institutes of Health Contract HHSN261200800001E. This work was also supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research. X.W. and Q.Z. were supported by the China Scholarship Council.

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organization imply endorsement by the U.S. Government.

Address correspondence and reprint requests to Dr. Xin Chen, Basic Science Program, Science Applications International Corporation–Frederick, Inc., Laboratory of Immunoregulation, Cancer Inflammation Program, National Cancer Institute–Frederick, P.O. Box B, Building 560, Room 31-19, Frederick, MD 21702-1201. E-mail address: chenxin@mail.nih.gov

Abbreviations used in this article: cLP, colon lamina propria; KI, knockin; LN, lymph node; LT, lymphotoxin; SP, single-positive; Teff, effector T cell; TβRI, TGF-β receptor I; Treg, regulatory T cell; WT, wild-type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1202659

of Tregs in vivo, at least partially through TNF/TNFR2 interaction. Mougiakakos et al. (24) found that TNF/TNFR2 interaction enhanced thioredoxin-1 expression on human Tregs, but not on Teffs, and therefore preferentially promoted Treg survival and activity within the inflammatory milieu. However, it remains elusive whether the replicating Tregs driven by TNF/TNFR2 interaction retain phenotypic and functional attributes of naturally occurring Tregs, especially in the chronic inflammatory condition. This question is clinically relevant now that TNF blockage therapy has become an important tool in the treatment of autoimmune disorders.

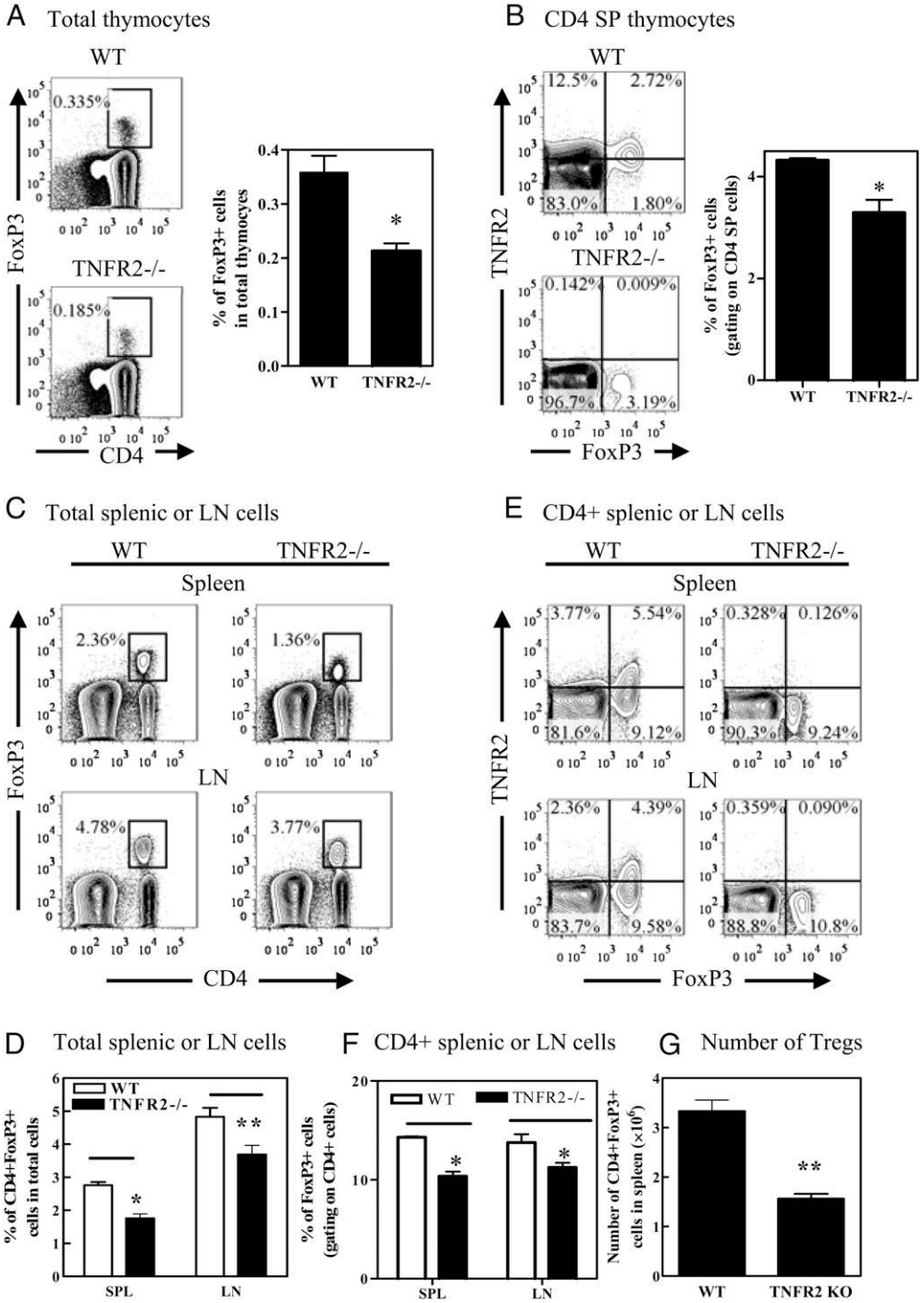

FIGURE 1. Reduced number of Tregs in TNFR2-deficient mice. Cells from thymus, spleen, and LNs in WT mice (C57BL/6) and TNFR2$^{-/-}$ mice were stained with CD3, CD4, CD8, TNFR2, and Foxp3. The expression of Foxp3 was analyzed by FACS, gating on CD3$^+$CD4$^+$ cells or CD3$^+$CD4$^+$CD8$^-$ cells (CD4 SP cells). (**A**) Proportion of CD4$^+$Foxp3$^+$ cells in the total thymocytes derived from WT or TNFR2$^{-/-}$ mice. (**B**) Expression of Foxp3 and TNFR2 on CD4 SP thymocytes from WT or TNFR2$^{-/-}$ mice. (**C** and **D**) Proportion of CD4$^+$Foxp3$^+$ cells in the total splenic and LN cells from WT or TNFR2$^{-/-}$ mice. In (A) and (B), *left panels* show the typical FACS plots, and *right panel* shows summary ($n = 3$). In (C) typical FACS plots are shown, and (D) shows the summary ($n = 3$). (**E** and **F**) Expression of Foxp3 and TNFR2 on CD4$^+$ T cells in the spleen and LNs from WT or TNFR2$^{-/-}$ mice. In (E) the typical FACS plots are shown, and (F) shows the summary ($n = 3$). (**G**) Number of CD4$^+$Foxp3$^+$ Tregs in the spleen from WT or TNFR2$^{-/-}$ mice. Number in the FACS plot shows the proportion of positive cells in the indicated gating or respective quadrants. Comparisons are between two indicated groups. Data shown are representatives of at least three separate experiments with the same results. *$p < 0.05$, **$p < 0.01$.

In this study, we observed that the frequency of Tregs in the thymus of mouse strains genetically deficient in TNFR2 or its ligands is reduced. Expression of Foxp3 is induced in developing thymocytes upon self-reactive TCR engagement (25). Thus, stimulation of TNFR2 may stabilize Foxp3 expression in response to activation of TCR of Tregs in immune or inflammatory responses, as shown by our in vitro and in vivo studies.

Materials and Methods

Mice

Wild-type (WT) C57BL/6 mice, congenic Ly5.2 C57BL/6 mice, Rag1$^{-/-}$ mice, TNFR2$^{-/-}$ mice, TNF$^{-/-}$ mice, and TNN/lymphotoxin (LT)α/LTβ$^{-/-}$ mice were provided by the Animal Production Area of the National Cancer Institute (Frederick, MD). Foxp3/GFP knockin (KI) mice were provided by Dr. Yasmine Belkaid at Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, and maintained in the Animal Production Area of the National Cancer Institute (Frederick, MD). National Cancer Institute-Frederick is accredited by American Association for the Accreditation of Laboratory Animal Care International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the *Guide for Care and Use of Laboratory Animals* (National Research Council, revised 1996). Anti-mouse Abs were purchased from BD Biosciences (San Diego, CA) and consisted of anti-mouse CD3 (145-2C11), CD4 (GK1.5), CD25 (PC61), TNFR2 (TR75-89), Ki-67 (B56), INF-γ (XMG1.2), and IL-17A (TC11-18H10). A leukocyte activation cocktail was also purchased from BD Biosciences. Functional grade purified anti-mouse CD3e (eBio500A2), CD28 (37.51), and IL-4 (11B11) Abs, a Foxp3 staining set (FJK-16s), and anti-mouse TCRβ (H57-597), CD45 (30-F11), and CD45RB (C363.16A) Abs were purchased from eBioscience (San Diego, CA). Functional-grade anti-mouse TNFR1 (55R-170), TNFR2 (TR75-32.4) Ab, and hamster IgG (HTK888) were purchased from BioLegend (San Diego, CA). Murine IL-6, IL-12, and TNF were purchased from PeproTech (Rocky Hill, NJ). Human rTGF-β1 was from R&D Systems (Minneapolis, MN).

T cell transfer model of colitis

Naive CD4$^+$CD25$^-$CD45RB^{hi} T cells were isolated from WT congenic B6 (CD45.1$^+$) mice and injected i.p. into Rag1$^{-/-}$ immunodeficient recipients (4×10^5 cells/mouse) alone, or cotransferred with WT or TNFR2$^{-/-}$ CD4$^+$CD25$^+$CD45RB^{lo} Treg cells (1.6×10^5/mouse, CD45.2$^+$). In Treg transfer experiments, CD4$^+$CD25$^+$CD45RB^{lo} Tregs flow-sorted from WT congenic B6 mice (CD45.1+) and TNFR2$^{-/-}$ mice (CD45.2$^+$) were mixed at 1:1 ratio and i.p. injected into Rag1$^{-/-}$ mice (1.2×10^5/mouse, each). Mice were

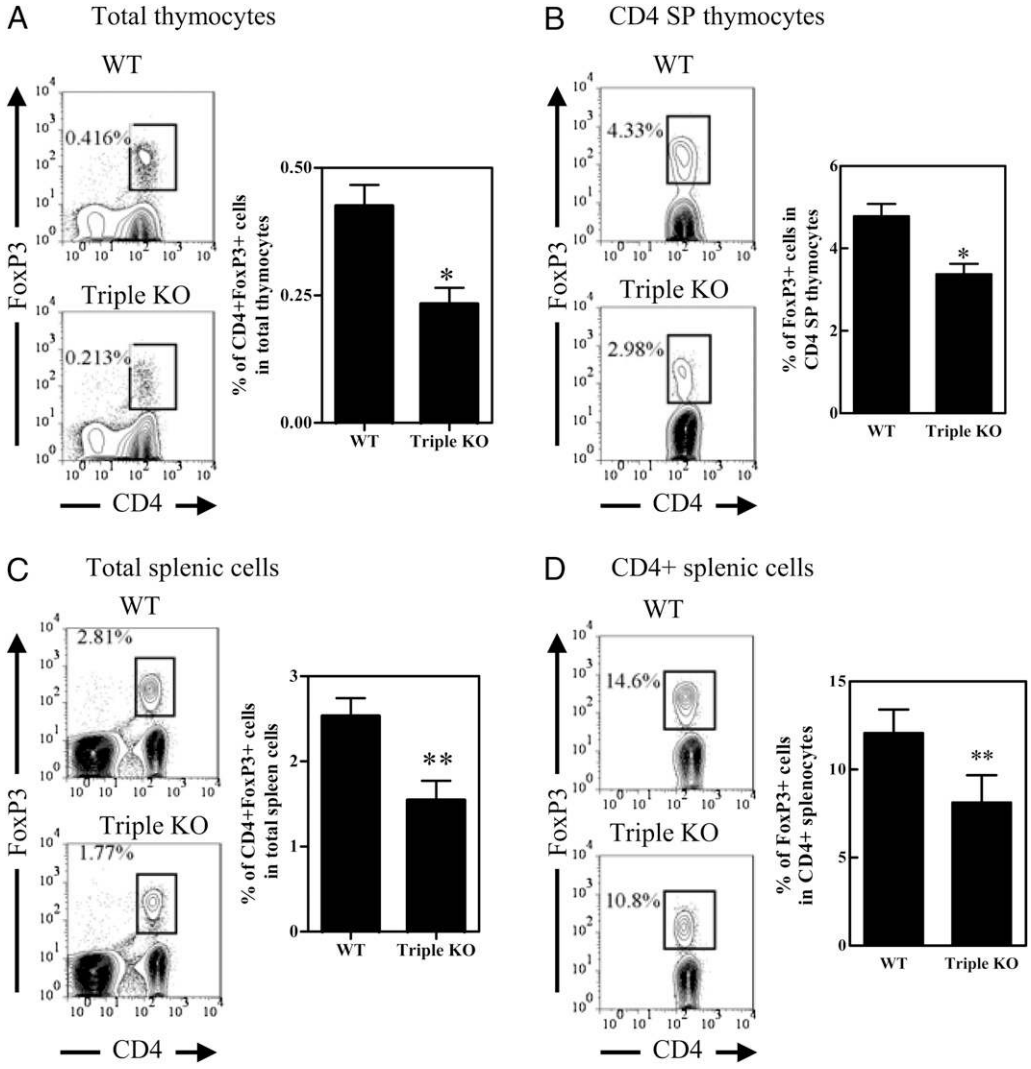

FIGURE 2. Reduced number of Tregs in TNFR2 ligand-deficient mice. Cells from thymus, spleen, and LNs in WT mice (C57BL/6) or TNF/LTα/LTβ$^{-/-}$ mice were stained with CD3, CD4, CD8, TNFR2, and Foxp3. The expression of Foxp3 was analyzed by FACS, gating on CD3$^+$CD4$^+$ cells or CD3$^+$CD4$^+$CD8$^-$ cells. (**A**) Proportion of CD4$^+$Foxp3$^+$ cells in total thymocytes from WT or TNF/LTα/LTβ$^{-/-}$ mice. (**B**) Proportion of Foxp3$^+$ cells in the CD4$^+$CD8$^-$ thymocytes from WT or TNF/LTα/LTβ$^{-/-}$ mice. In (A) and (B), *left panels* show the typical FACS plots, and *right panel* shows summary (n = 3–6). (**C**) Proportion of CD4$^+$Foxp3$^+$ cells in the spleen from WT or TNF/LTα/LTβ$^{-/-}$ mice. (**D**) Proportion of Foxp3$^+$ cells in CD4$^+$ splenic cells from WT or TNF/LTα/LTβ$^{-/-}$ mice. Number in the FACS data shows the proportion of cells in the indicated gating. Comparisons are between two indicated groups. Data shown are representative of at least three separate experiments with the same results. *$p < 0.05$, **$p < 0.01$. TNF/LTα/LTβ$^{-/-}$, Triple knockout (KO).

monitored weekly by animal facility staff for the clinical symptoms of colitis such as rectal bleeding, loose feces/diarrhea, rough/hunched posture, and decreased body weight. Any mice losing >20% of its starting body weight or showing severe signs of disease were euthanized.

Cell isolation

Single-cell suspensions from spleen and mesenteric lymph nodes (LNs) were prepared by filtration through a 70-μm cell strainer (BD Labware, San Jose, CA). Preparation of colon lamina propria (cLP) cells was as previously described (26). Briefly, colons were rinsed in PBS and cut into ~0.3-cm pieces. Intestinal epithelial cells were removed by incubation with Ca- and Mg-free PBS containing 10% FCS and 5 mM EDTA. Colon tissues then were incubated with RPMI 1640 containing 10% FCS and 1 mg/ml collagenase type 4 (Worthington Biochemical, Lakewood, NJ) for 30 min at 37°C.

In vitro T cell activation and differentiation

Flow-sorted $CD4^+Foxp3/GFP^+$ cells or $CD4^+Foxp3/GFP^-$ cells from Foxp3/GFP KI mice, or $CD4^+CD25^+$ cells from WT C57BL/6 mice or $TNFR2^{-/-}$ mice were seeded at 5×10^4 cells/well in a 96-well plate. The cells were stimulated with plate-bound anti-CD3e Ab (10 μg/ml) and soluble anti-CD28 Ab (2 μg/ml) for 5 d. In some experiments, the cells were activated in Th0, Th1, and Th17 polarizing conditions by adding medium alone, or IL-12 (10 ng/ml) plus anti–IL-4 Ab (10 μg/ml), or IL-6 (10 ng/ml), or IL-6 plus TGF-β (1 ng/ml), respectively. In some experiments, the cells were activated in the presence of TNF (10 ng/ml) and/or IL-6 (10 ng/ml) without or with 10 μg/ml hamster IgG, or anti-TNFR1 Ab or anti-TNFR2 Ab. IMDM (Sigma-Aldrich) was used in Th17 polarizing culture, and RPMI 1640 (Lonza BioWhittaker, Walkersville, MD) was used in all other cultures. The medium was supplemented with 10% FBS (HyClone, Logan, UT) containing 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 50 μM 2-ME.

Flow cytometry

After blocking FcR, cells were incubated with appropriately diluted Abs. Acquisition was performed using an SLRII (BD Biosciences, Mountain View, CA) and data analysis was conducted using FlowJo software (Tree Star, Ashland, OR). For intracellular cytokine staining, cells were restimulated with a leukocyte activation cocktail (BD Biosciences) for 4 h. FACS analysis was gated on the live cells only by using a Live/Dead fixable dead cell stain kit.

Statistical analysis

Cumulative incidence of colitis was graphed as survival plot and analyzed with a log-rank test, and comparison of other data were analyzed by a two-tailed Student *t* test using Graphpad Prism 4.0.

Results

Reduction of thymic and peripheral Tregs in mice deficient in TNFR2 or its ligands

In normal mice, most thymic Tregs express TNFR2 (18). All human thymic $CD4^+CD25^+$ Tregs constitutively express TNFR2, whereas thymic $CD4^+CD25^-$ cells do not express this receptor (27). TNF is expressed in the thymus of mice and humans, and it participates in the development of thymocytes (28). Thus, we investigated the possibility that TNF or LTα, the ligands for TNFR2, contribute to the thymic differentiation and generation of Tregs.

We first compared the Foxp3-expressing Tregs in $TNFR2^{-/-}$ and normal WT mice. In adult $TNFR2^{-/-}$ mouse thymus, the proportion of $CD4^+Foxp3^+$ Tregs in total thymocytes was reduced by 45% as compared with WT control B6 mice ($p < 0.05$, Fig. 1A). The proportion of $Foxp3^+$ cells in CD4 single-positive (SP) thymocytes was also reduced by ~30% ($p < 0.05$, Fig. 1B). It was reported that the cellularity of the thymus of $TNFR2^{-/-}$ mice was greater than that of WT mice; however, the most affected subset of thymocytes was naive triple-negative cells ($CD3^-CD4^-CD8^-$), whereas both CD4 and CD8 subsets were not altered (29). Because thymic Tregs were almost exclusively contained in the CD4 SP population, the absolute number of Tregs in the thymus of $TNFR2^{-/-}$ mice was reduced proportionally.

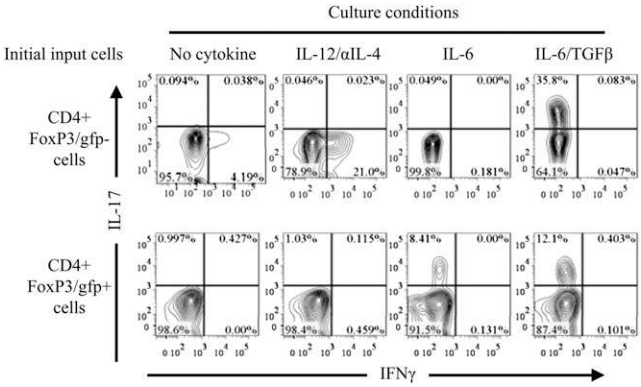

FIGURE 3. Plasticity of Tregs under proinflammatory stimulation in vitro. Splenic and LN cells from Foxp3/GFP KI mice were stained with CD4. $Foxp3/GFP^+$ or $Foxp3/GFP^-$ CD4 cells were flow sorted. The cells were activated in vitro with plate-bound anti-CD3 and soluble anti-CD28 Abs, in Th0 (no cytokine added) or in Th1 (IL-12 plus anti–IL-4 Ab) or Th17 (IL-6, or IL-6 plus TGF-β) polarizing culture condition. After 5 d, the cells were restimulated with a leukocyte activation cocktail and intracellular IFN-γ and IL-17A were analyzed by FACS. Numbers in FACS plots indicate the percentage of cells in the respective quadrant. Data shown are representative of at least three separate experiments with the same results.

In the periphery, the percentage of $CD4^+Foxp3^+$ cells in total splenic cells and LN cells was decreased by 42 and 21%, respectively ($p < 0.01$~0.05, Fig. 1C, 1D). The proportion of $Foxp3^+$ cells in the $CD4^+$ splenic cells and $CD4^+$ LN cells was decreased by 36 and 22%, respectively ($p < 0.05$, Fig. 1E, 1F). The absolute number of splenic Tregs in $TNFR2^{-/-}$ mice was reduced by ~50% ($p < 0.05$, Fig. 1G). These data suggest that TNFR2 may participate in the development of Tregs in the thymus. Although $TNFR2^{-/-}$ mice do not spontaneously develop apparent autoimmune disorders, this strain of mouse nevertheless shows more severe inflammation upon induction of autoimmune disease (30), presumably attributed by the reduced number of Tregs.

The development of $Foxp3^+$ Tregs in mice with depletion of TNFR2 ligands was also investigated. Both $TNF^{-/-}$ mice or $LT\alpha/\beta^{-/-}$ mice did not exhibit any deficiency in Tregs in the spleen (data not shown). However, the proportion of Tregs in the pe-

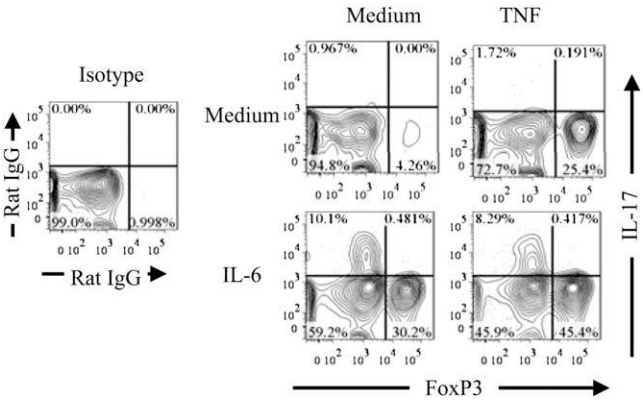

FIGURE 4. TNF stabilizes Foxp3 expression on Tregs in vitro. Flow-sorted $CD4^+Foxp3/GFP^+$ cells were stimulated as described in Fig. 3, with medium alone or with IL-6 in the presence of TNF or not. After reactivation, intracellular expression of Foxp3 and IL-17A was analyzed with FACS. Numbers indicate the percentage of cells in the respective quadrant. Data shown are representative of at least three separate experiments with the same results.

riphery and thymus of TNF/LTα/LTβ$^{-/-}$ mice was decreased. Despite a profound defect of peripheral lymphoid organs of TNF/LTα/LTβ$^{-/-}$ mice, this strain of mouse had no change in the major thymocyte populations and T/B cell ratio in the spleen (31). In the thymus, the proportion of CD4$^+$Foxp3$^+$ Tregs in total thymocytes was reduced by ~50% ($p < 0.05$, Fig. 2A). The proportion of Foxp3$^+$ Tregs in CD4 SP thymocytes was reduced by 31% ($p < 0.05$, Fig. 2B). In the spleen, the proportion of CD4$^+$Foxp3$^+$ Tregs in total splenic cells was reduced by 37% ($p < 0.01$, Fig. 2C). The proportion of Foxp3$^+$ Tregs in CD4$^+$ splenic cells was reduced by 26% ($p < 0.01$, Fig. 2D). TNF treatment in vitro largely restored the proportion of Foxp3$^+$ Tregs in CD4 cells derived from TNF/LTα/LTβ$^{-/-}$ mice (data not shown). Therefore, genetic ablation of TNFR2 or its ligands resulted in a reduction of Tregs in both thymus and peripheral lymphoid tissue.

TNF stabilizes Foxp3 expression by TCR-stimulated Tregs in vitro

Self-reactive TCR signaling plays a central role in the thymic generation of Tregs (25). Reduced thymic Tregs in mice deficient in TNFR2 and its ligands suggest that TNF/TNFR2 signaling may play a role in stabilizing Foxp3 expression by Tregs in response to TCR stimulation. We therefore examined the effect of exogenous TNF on Foxp3 expression by highly purified Tregs, which were stimulated in vitro with plate-bound anti-CD3 Ab and soluble anti-CD28 Ab. It was reported that, in the presence of proinflammatory cytokine, in vitro TCR stimulation could change the phenotype of Tregs (32). We confirmed this result and found that IL-6 plus TGF-β induced IL-17 expression by both Tregs and Teffs, IL-6 alone only induced IL-17 expression by TCR-stimulated Tregs, but not by Teffs. In contrast, Th1 polarizing culture condition only induced IFN-γ expression by TCR-stimulated Teffs, but not by Tregs (Fig. 3). Unexpectedly, TCR stimulation alone markedly downregulated Foxp3 expression by Tregs (Fig. 4). This was not caused by activation-induced cell death, because FACS analysis only gated on the live cells by using a Live/Dead fixable dead cell stain kit described in *Materials and Methods*. Although IL-6 induced IL-17 expression by a substantial proportion of initial Tregs, which all downregulated their Foxp3 expression, this proinflammatory cytokine paradoxically supported the persistent expression of Foxp3 by 30% of input cells (Fig. 4). In contrast, TNF by itself consistently maintained high levels of Foxp3 expression by 20–30% of initial input Tregs, and it can cooperate with IL-6 to further enhance the proportion of Foxp3-expressing cells without

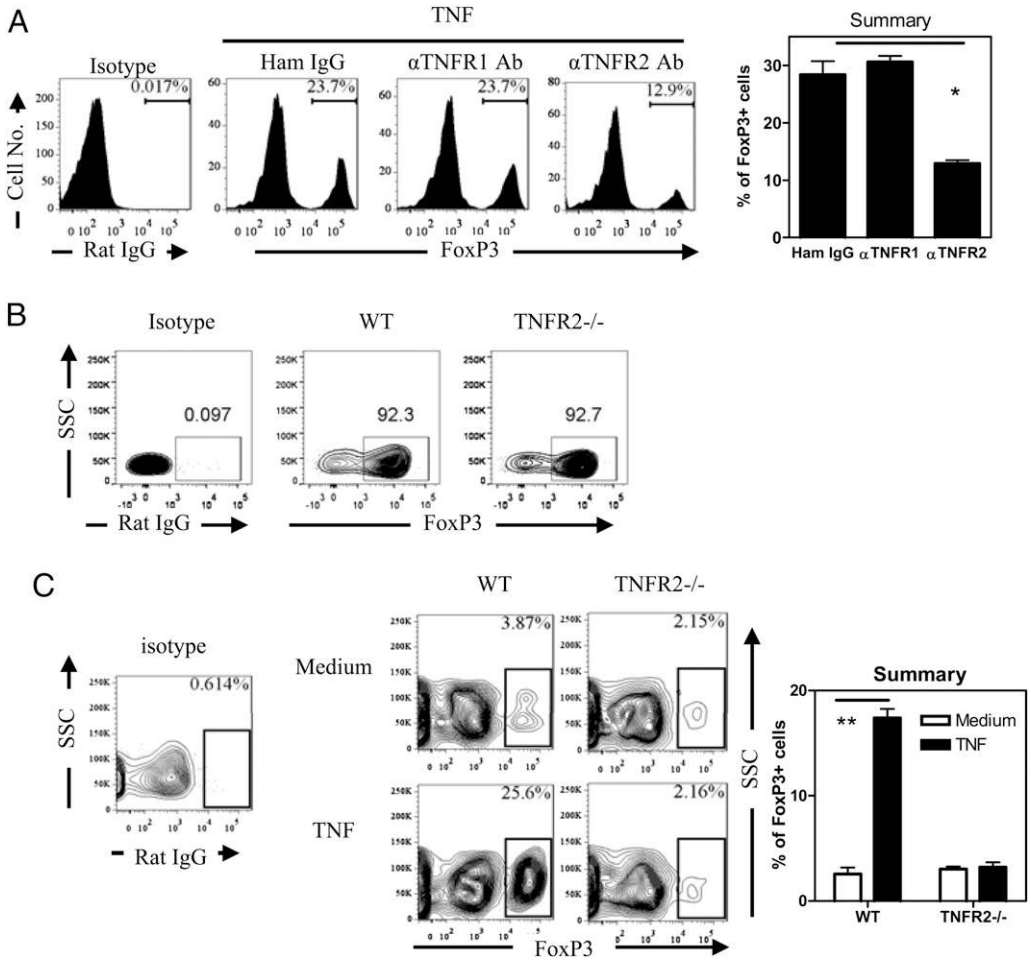

FIGURE 5. TNFR2 mediates the effect of TNF in maintaining Foxp3 expression in vitro. (**A**) Flow-sorted CD4$^+$Foxp3/GFP$^+$ cells were stimulated as in Fig. 3, in the presence of TNF (10 ng/ml) and 10 μg/ml isotype control hamster IgG or anti-TNFR1 Ab or anti-TNFR2 Ab. Foxp3 expression was analyzed by FACS. Typical histograms and summary ($n = 3$) are shown. (**B**) Tregs were flow sorted from WT and TNFR2$^{-/-}$ mice, based on surface expression of CD4$^+$CD25$^+$. The expression of Foxp3 was analyzed by FACS. (**C**) CD4$^+$CD25$^+$ cells from TNFR2 KO mice were activated as described in Fig. 3, in the presence of TNF (10 ng/ml) or not. The expression of Foxp3 was analyzed by FACS. Typical FACS plot and summary ($n = 3$) are shown. Numbers represent the percentage of cells within the indicated gate. Data shown are representative of at least three separate experiments with the same results. *$p < 0.05$, **$p < 0.01$ when compared with indicated group.

further increasing IL-17–producing cells (Fig. 4). Therefore, in addition to activating and expanding Tregs (17), TNF can also stabilize Foxp3 expression on Tregs.

TNFR2 mediates the effect of TNF in maintenance of Foxp3 expression in vitro

To further determine which TNF receptor was responsible for stabilizing Foxp3 expression, anti-TNFR1 or anti-TNFR2 neutralizing Abs were added to the TNF-treated Tregs. The anti-TNFR2 Ab, but not the anti-TNFR1 Ab, markedly reduced the proportion of Foxp3-expressing cells ($p < 0.05$, Fig. 5A). We further clarified this issue by comparing the response of Tregs from WT mice or TNFR2$^{-/-}$ mice. Although TNFR2$^{-/-}$ mice had reduced number of Tregs, the residual Tregs nevertheless expressed the same levels of Foxp3 as did WT Tregs (Fig. 5B). As shown in Fig. 5C, TCR stimulation reduced Foxp3 expression by TNFR2$^{-/-}$ Tregs and this was not reversed by TNF. In contrast, Foxp3 expression by TNFR2$^{-/-}$ Tregs was partially sustained by IL-6 (data not shown). Thus, our data clearly show that TNF activation of TNFR2 is able to stabilize Foxp3 expression by Tregs upon potent TCR stimulation.

TNFR2 is required for the in vivo immunosuppressive function of Tregs

The critical role of functional Tregs in immune homeostasis can be demonstrated in a mouse colitis model induced by transfer of naive CD4 T cells into Rag1$^{-/-}$ mice (26). In a preliminary experiment, we found that 8 wk after transfer, TNF can be expressed by both host cells and adoptively transferred cells in the cLP (data not shown), whereas TNFR2 expression was upregulated by both cotransferred initial naive CD4 cells and Tregs (data not shown). Thus, this model is appropriate to determine the role of interaction of TNF/TNFR2 on the function of Tregs. We confirmed that TNFR2 expression on Tregs was required for the suppression of colitis induced by transfer of naive CD4 cells (22) ($p < 0.05$, Fig. 6A). Furthermore, we compared the effect of WT or TNFR2-deficient Tregs on the development of Th1 or Th17 responses by cotransferred naive CD4 cells. As shown in Fig. 6B, naive CD4 cells transferred alone (without Tregs) into Rag1$^{-/-}$ mice could develop into both Th1 and Th17 cells in the colon, as indicated by their expression of IFN-γ and IL-17A. Consistent with a previous report (22), WT Tregs markedly inhibited a proportion of IFN-γ–producing cells ($p < 0.01$), whereas TNFR2$^{-/-}$ Tregs failed to do so. It has been shown in a mouse model where colitis was induced by transfer of naive CD4 cells that Th17 cells had paradoxically tissue protective and immunosuppressive effects and consequently suppressed colon inflammation by inhibiting pathogenic Th1 responses (33) or by enhancing barrier function of intestinal epithelial cells (34). In agreement with a number of recent studies (35, 36), we also found that transfer of WT Tregs resulted in a >2-fold increase in the proportion of IL-17A–producing cells developed from the initial naive CD4 cells, as compared with naive CD4 cells transfer alone ($p < 0.01$). In contrast, cotransfer of TNFR2-deficient Tregs failed to promote the generation of Th17 cells ($p > 0.05$, Fig. 6B). Therefore, TNFR2 is required for Tregs to suppress pathogenic Th1 response in this model.

TNFR2 is required for maintenance of Foxp3 expression on Tregs in vivo

The accumulation of Tregs and their Foxp3 expression were examined when full-fledged colitis was developed, which occurred typically 6–8 wk after cell transfer. As shown in Fig. 7A, the proportion of WT Tregs present in the total numbers of transferred

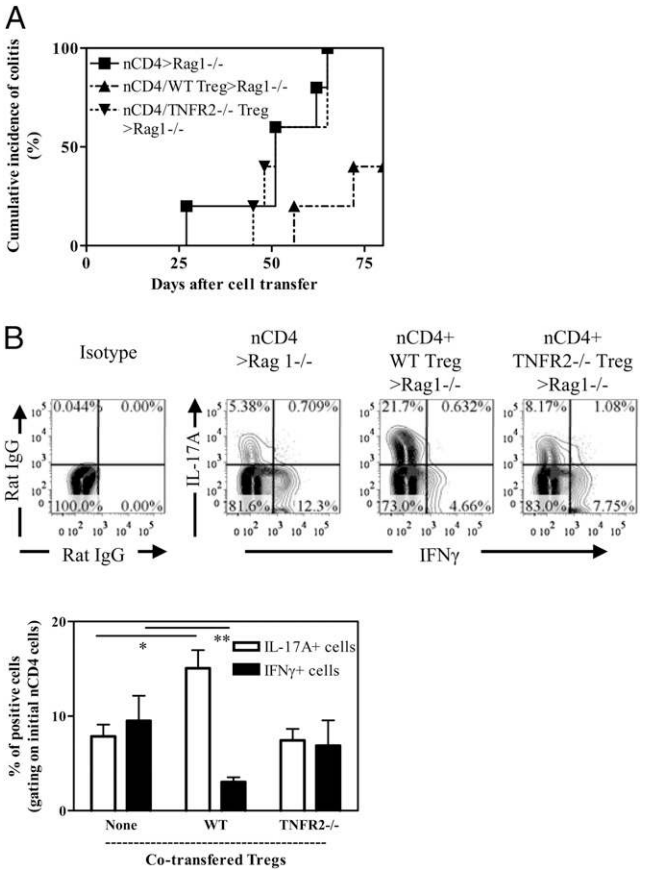

FIGURE 6. TNFR2 is required for the immunosuppressive function of Tregs in vivo. CD45.1$^+$CD4$^+$CD25$^-$CD45RB^{hi} naive T (nCD4) cells were transferred alone or cotransferred with CD45.2$^+$ WT or TNFR2$^{-/-}$ Treg cells into Rag1$^{-/-}$ mice. (**A**) Cumulative incidence of colitis. Incidence of colitis in mice cotransferred with naive CD4 cells and WT Tregs was markedly decreased by comparison with mice transferred with naive CD4 cells alone or cotransferred with naive CD4 cells and TNFR2$^{-/-}$ Tregs ($p = 0.0132$ and 0.0163, respectively). (**B**) After 8 wk, cLP cells were isolated. The intracellular expression of IFN-γ and IL-17A by initial transferred naive WT CD4 cells was analyzed by FACS, gating on CD45.1$^+$ cells. Typical FACS plot and summary ($n = 3$–5) are shown. Data shown are representative of three separate experiments with similar results. $^*p < 0.05$, $^{**}p < 0.01$.

cells was reduced from 28.6 to 12.5%, which may be based on the homeostatic restoration of Tregs to the normal 10–15% range in total CD4 pool. However, the proportion of Foxp3$^+$TNFR2$^{-/-}$ Tregs was reduced to 3.88%, which is markedly lower than 12.5% WT Tregs ($p < 0.05$).

Flow-sorted Tregs from WT and TNFR2$^{-/-}$ mice expressed the same high levels of Foxp3 (Fig. 7B). By 8 wk after transfer, most WT Tregs (> 80%) maintained their Foxp3 expression in cLP. However, Foxp3 expression by TNFR2-deficient Tregs was markedly reduced (~40%), as compared with WT Tregs ($p < 0.01$, Fig. 7B). The reduction of Foxp3 by TNFR2$^{-/-}$ Tregs was more profound in the cLP, whereas there was not a significant change in the spleen and mesenteric LNs (data not shown), suggesting a role of proinflammatory response in the colon. Therefore, TNFR2 expression is critical for the phenotypic stability of Tregs in the inflammatory environment and for the capacity of Tregs to compete efficiently with colitogenic T cells. The latter effect may be attributable to the lack of suppressive function of TNFR2-deficient Tregs in vivo found in our study and others (22, 37).

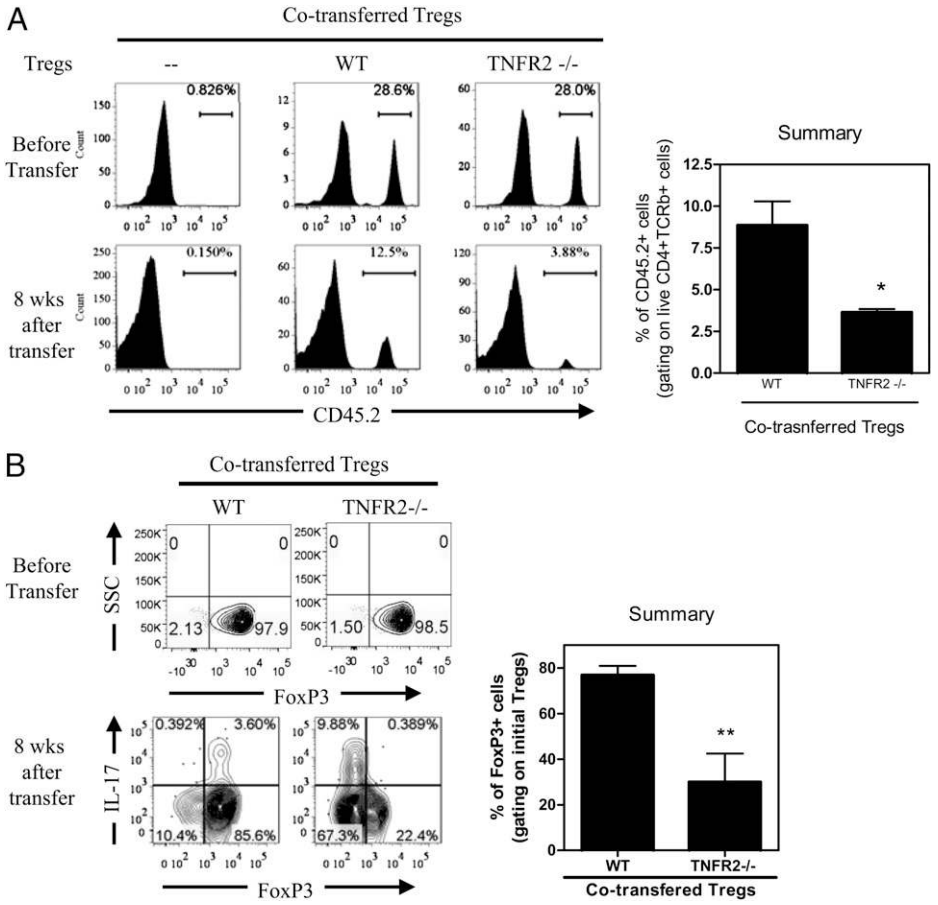

FIGURE 7. Critical role of TNFR2 in stabilizing Foxp3 expression in vivo. CD45.1$^+$ CD4$^+$CD25$^-$CD45RB^{hi} naive T cells were transferred alone or co-transferred with CD45.2$^+$ WT or TNFR2$^{-/-}$ Treg cells into Rag1$^{-/-}$ mice. After 8 wk, cLP cells were analyzed by FACS. (**A**) Reduced relative number of cotransferred TNFR2$^{-/-}$ Tregs. Proportion of CD45.2$^+$ initial Tregs in total transferred CD4 cells was determined. For comparison, pre-transferred cells are shown in the *upper panel*. (**B**) Reduced Foxp3 expression by initial Tregs from TNFR2$^{-/-}$ mice in cLP. Expression of IL-17A and Foxp3 was analyzed by FACS, by gating on CD45.2$^+$ initial Tregs. Numbers in the FACS plots represent the percentage of cells in the indicated gate or quadrant. Typical FACS plot and summary (n = 3–5) are shown. Data shown are representative of three separate experiments with similar results. *$p < 0.05$, ** $p < 0.01$.

TNFR2 expression is critical for the expansion of Tregs in competitive environment

The magnitude of inflammation was greater in Rag1$^{-/-}$ mice cotransferred with WT naive CD4 cells containing TNFR2$^{-/-}$ Tregs, which might be attributable to the profound loss of Foxp3 expression in TNFR2$^{-/-}$ Tregs. To clarify this issue, we injected a mixture of WT and TNFR2$^{-/-}$ Tregs into Rag1$^{-/-}$ mice to compare phenotypic stability of WT and TNFR2$^{-/-}$ Tregs in vivo in the identical environment. Tregs were flow sorted from CD45.1$^+$ WT and CD45.2$^+$ TNFR2$^{-/-}$ mice and transferred at a 1:1 ratio into Rag1$^{-/-}$ mice (Fig. 8A). Purified Tregs from these two strains of mice expressed the same high levels of Foxp3 (Fig. 8B). Ten weeks after transfer, the proportion of WT Tregs to TNFR2$^{-/-}$ Tregs was changed from 1:1 to roughly 4:1 ($p < 0.05$, Fig. 8C), indicating a greater capacity of WT Tregs than TNFR2$^{-/-}$ Tregs to reconstitute the lymphopenic environment (Fig. 8C). Most WT Tregs lost their Foxp3 expression and <40% expressed Foxp3 (Fig. 8D), which is much lower than Tregs cotransferred with naive CD4 cells (Fig. 7B). These data are consistent with previous studies showing that most transferred Foxp3/GFP$^+$ Tregs in cLP of recipient lymphopenic mice lose their Foxp3 expression, whereas cotransfer of Teffs resulted in maintaining Foxp3 expression by Tregs (38, 39), presumably by producing cytokines such as IL-2 and/or TNF. The reduction in Foxp3 expression by TNFR2-deficient Tregs was considerably greater than by WT Tregs, and >95% of initial Tregs derived from TNFR2$^{-/-}$ mice lost their Foxp3 expression (Fig. 8D). Consequently, <10% of Foxp3-expressing cells in cLP were derived from TNFR2$^{-/-}$ Tregs, which was markedly lower than those from WT mice ($p < 0.0005$, Fig. 8D). These data clearly show that TNFR2 per se is critical to sustain Foxp3 expression by Tregs in this model. In our experimental system, after transfer into Rag1$^{-/-}$ mice (CD45.2$^+$), TNFR2$^{-/-}$ Tregs isolated from CD45.2$^+$ mice were outnumbered by WT Tregs derived from either CD45.2$^+$ mice (Fig. 7) or CD45.1$^+$ mice (Fig. 8), mitigating the potential impact of different congenic marker expressed by WT Tregs and TNFR2$^{-/-}$ Tregs.

Discussion

Our data presented in this study clearly show that TNFR2 plays a critical role in sustaining Foxp3 expression and consequently maintaining the phenotypic and functional stability of Tregs. Therefore, at least in the inflammatory environment, the TNF/TNFR2 pathway is critical for the stabilization of Treg pool that is required to restrain the magnitude and length of an inflammatory immune response and to avoid harmful damage to self tissues.

A previous study reported that, in the same mouse colitis model, the accumulation and Foxp3 expression by TNFR2$^{-/-}$ Tregs in cLP were not changed when cells were harvested 2 wk after transfer (22). Because the development of colitis in this model usually starts 5 wk after transfer of naive cells, we examined accumulation of Tregs and their Foxp3 expression when full-fledged colitis was developed, which occurred typically 8 wk after cell transfer. In our transfer experiments, both WT and TNFR2$^{-/-}$ Tregs were flow sorted CD4$^+$CD25$^+$CD45RB^{lo} cells and contained similar high levels of Foxp3$^+$ cells (>95%). The expansion of contaminating Foxp3$^-$ Teffs present in the initial Treg population may partially account for the reduction in Foxp3-expressing cells after transfer into Rag1$^{-/-}$ mice. However, the impact of this contamination population is very limited since it has been shown that the transferred Foxp3$^+$ and Foxp3$^-$ T cells were replicating similarly in the lymphopenic mouse. There was no marked outgrowth of an intentionally added 3% Foxp3$^-$ Teffs in Tregs after

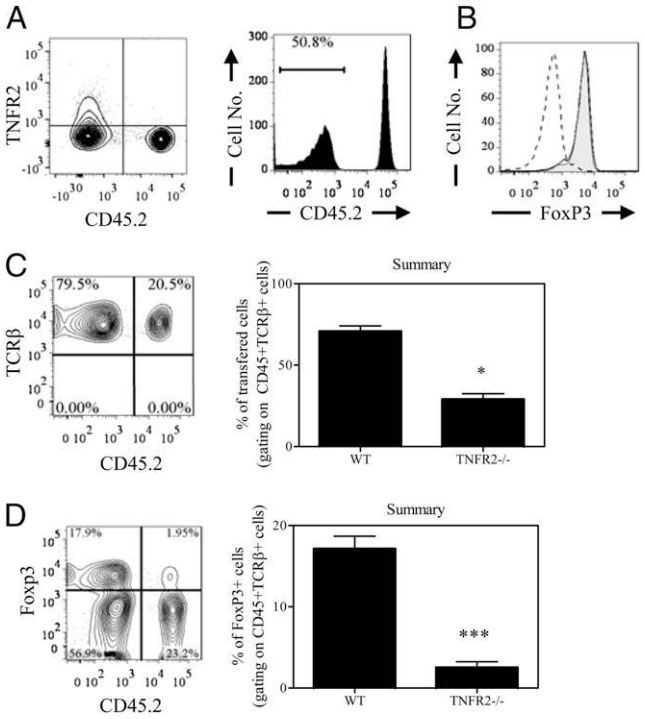

FIGURE 8. TNFR2 expression is critical for the expansion of Tregs in a competitive environment. CD45.1$^+$ Tregs and CD45.2$^+$ Tregs were flow-sorted from WT mice and TNFR2$^{-/-}$ mice, respectively, and cotransferred into Rag1$^{-/-}$ mice at a ratio of 1:1. (**A**) Profile of pretransferred Tregs. *Left panel* shows expression of TNFR2 and CD45.2 and *right panel* shows CD45.2 expression alone. (**B**) Flow-sorted Tregs from WT mice (solid line histogram) and TNFR2$^{-/-}$ mice (gray-filled histogram) express comparable levels of Foxp3. Dashed line indicates isotype control. (**C**) Ten weeks after transfer, Tregs present in the cLP were analyzed by FACS, gating on CD45$^+$TCRβ$^+$ cells. *Left panel*, Expression of TCRβ and CD45.2 on cLP Tregs; *right panel*, summary of proportion of WT and TNFR2$^{-/-}$ Tregs in total transferred cells present in cLP (n = 5). (**D**) Foxp3 expression on initial Tregs from WT or TNFR2$^{-/-}$ mice cotransferred into Rag1$^{-/-}$ mice (*left*) and summary of proportion of Foxp3-expressing cells in total transferred cells (*right*, n = 5). The number shown in the FACS plots represents the proportion of cells in the respective quadrant or gate. Data shown are representative of three separate experiments with similar results. *$p < 0.05$, ***$p < 0.001$.

transfer (38, 39). Furthermore, TNFR2 is well known for its costimulatory effect on Teffs (40), and thus it is likely that TNFR2$^{-/-}$ Teffs present in TNFR2$^{-/-}$ Tregs are less proliferative than WT Teffs.

IL-2 produced by Teffs has been proposed to be a paracrine factor to maintain Foxp3 expression and phenotypic as well as functional stability of Tregs (41). There are number of observations challenging the dispensable role of IL-2 on Tregs. First, only ~50% reduction of Foxp3$^+$ Tregs in CD4 cells was found in the thymus and peripheral lymphoid tissues of Il2$^{-/-}$ mice or Il2ra$^{-/-}$ mice, and residual Tregs had normal suppressive function (42). Second, a recent study found that the stability of transferred Tregs in the lymphopenic mouse was maintained by Teffs independent of IL-2, as the administration of IL-2 did not promote the maintenance of Foxp3 expression by Tregs (39). Third, the stimulatory effect of Teffs on Tregs in vivo was not abrogated by neutralization of IL-2, and IL-2–deficient Teffs still had the capacity to stimulate the activation of Tregs (23). TGF-β is able to induce Foxp3 expression (43) and thus may play a role in the persistent expression of Foxp3 on Tregs. Nevertheless, conditional deletion of TGF-β receptor I (TβRI) in T cells also only delayed the appearance of Foxp3$^+$ Tregs in neonatal mouse thymus; however, beginning 1 wk after birth, the same TβRI-mutant mice showed accelerated expansion of thymic Tregs (44). The proportion of Tregs in mice devoid of TNFR2 or its ligand was reduced by ~50% in both thymus and periphery. These data suggest that multiple factors, including TNF/TNFR2 signaling, contribute to the thymic generation and peripheral homeostasis of Tregs. Recently, Cuss and Green (45) reported that thymic Tregs actually were heterogeneic and contained resident Tregs and newly developed Tregs. They proposed that resident Tregs were IL-2 dependent for their homeostasis, whereas newly developed Tregs were not. Further study is warranted to determine which subset of thymic Tregs is defective in mouse strains deficient in TNFR2 or its ligands, as well as in Il2$^{-/-}$ or Il2ra$^{-/-}$ and TβRI$^{-/-}$ mice.

In conclusion, TNFR2 is a key factor in maintaining sustained Foxp3 expression and function of Tregs contributing to immune regulation in the inflammatory environment, which may explain why anti-TNF therapy fails or even at times exacerbates some autoimmune disorders (46). This should be taken into account when designing future therapy of autoimmunity by using TNF inhibitors.

Acknowledgments

We thank Drs. Giorgio Trinchieri and Scott K. Durum for discussion and critical review of the manuscript, Dr. Sergei A. Nedospasov for generating TNF/LTα/LTβ KO mice, and Drs. Yingzi Cong, Rosalba Salcedo, Ram Savan, and Miranda Hanson for help in the mouse colitis study. We thank the National Cancer Institute–Frederick Cancer Inflammation Program Fluorescence Cytometry Core for expert technical assistance with flow cytometry.

Disclosures

The authors have no financial conflicts of interest.

References

1. Wing, K., and S. Sakaguchi. 2010. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nat. Immunol.* 11: 7–13.
2. Shevach, E. M. 2009. Mechanisms of Foxp3$^+$ T regulatory cell-mediated suppression. *Immunity* 30: 636–645.
3. Rudensky, A. Y. 2011. Regulatory T cells and Foxp3. *Immunol. Rev.* 241: 260–268.
4. Brunkow, M. E., E. W. Jeffery, K. A. Hjerrild, B. Paeper, L. B. Clark, S. A. Yasayko, J. E. Wilkinson, D. Galas, S. F. Ziegler, and F. Ramsdell. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet.* 27: 68–73.
5. Bennett, C. L., J. Christie, F. Ramsdell, M. E. Brunkow, P. J. Ferguson, L. Whitesell, T. E. Kelly, F. T. Saulsbury, P. F. Chance, and H. D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* 27: 20–21.
6. Allan, S. E., A. N. Alstad, N. Merindol, N. K. Crellin, M. Amendola, R. Bacchetta, L. Naldini, M. G. Roncarolo, H. Soudeyns, and M. K. Levings. 2008. Generation of potent and stable human CD4$^+$ T regulatory cells by activation-independent expression of FOXP3. *Mol. Ther.* 16: 194–202.
7. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4$^+$CD25$^+$ regulatory T cells. *Nat. Immunol.* 4: 330–336.
8. Hori, S. 2010. Developmental plasticity of Foxp3$^+$ regulatory T cells. *Curr. Opin. Immunol.* 22: 575–582.
9. Beriou, G., C. M. Costantino, C. W. Ashley, L. Yang, V. K. Kuchroo, C. Baecher-Allan, and D. A. Hafler. 2009. IL-17-producing human peripheral regulatory T cells retain suppressive function. *Blood* 113: 4240–4249.
10. Yang, X. O., R. Nurieva, G. J. Martinez, H. S. Kang, Y. Chung, B. P. Pappu, B. Shah, S. H. Chang, K. S. Schluns, S. S. Watowich, et al. 2008. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity* 29: 44–56.
11. Chen, X., and J. J. Oppenheim. 2010. TNF-α: an activator of CD4$^+$FoxP3$^+$ TNFR2$^+$ regulatory T cells. *Curr. Dir. Autoimmun.* 11: 119–134.
12. Vandenabeele, P., W. Declercq, R. Beyaert, and W. Fiers. 1995. Two tumour necrosis factor receptors: structure and function. *Trends Cell Biol.* 5: 392–399.
13. Chen, G., and D. V. Goeddel. 2002. TNF-R1 signaling: a beautiful pathway. *Science* 296: 1634–1635.
14. Wajant, H., K. Pfizenmaier, and P. Scheurich. 2003. Tumor necrosis factor signaling. *Cell Death Differ.* 10: 45–65.

15. Grell, M., F. M. Becke, H. Wajant, D. N. Männel, and P. Scheurich. 1998. TNF receptor type 2 mediates thymocyte proliferation independently of TNF receptor type 1. *Eur. J. Immunol.* 28: 257–263.
16. Arnett, H. A., J. Mason, M. Marino, K. Suzuki, G. K. Matsushima, and J. P. Ting. 2001. TNFα promotes proliferation of oligodendrocyte progenitors and remyelination. *Nat. Neurosci.* 4: 1116–1122.
17. Chen, X., M. Bäumel, D. N. Männel, O. M. Howard, and J. J. Oppenheim. 2007. Interaction of TNF with TNF receptor type 2 promotes expansion and function of mouse $CD4^+CD25^+$ T regulatory cells. *J. Immunol.* 179: 154–161.
18. Chen, X., J. J. Subleski, H. Kopf, O. M. Howard, D. N. Männel, and J. J. Oppenheim. 2008. Cutting edge: expression of TNFR2 defines a maximally suppressive subset of mouse $CD4^+CD25^+FoxP3^+$ T regulatory cells: applicability to tumor-infiltrating T regulatory cells. *J. Immunol.* 180: 6467–6471.
19. Chen, X., R. Hamano, J. J. Subleski, A. A. Hurwitz, O. M. Howard, and J. J. Oppenheim. 2010. Expression of costimulatory TNFR2 induces resistance of $CD4^+FoxP3^-$ conventional T cells to suppression by $CD4^+FoxP3^+$ regulatory T cells. *J. Immunol.* 185: 174–182.
20. Chen, X., J. J. Subleski, R. Hamano, O. M. Howard, R. H. Wiltrout, and J. J. Oppenheim. 2010. Co-expression of TNFR2 and CD25 identifies more of the functional $CD4^+FOXP3^+$ regulatory T cells in human peripheral blood. *Eur. J. Immunol.* 40: 1099–1106.
21. Hamano, R., J. Huang, T. Yoshimura, J. J. Oppenheim, and X. Chen. 2011. TNF optimally activatives regulatory T cells by inducing TNF receptor superfamily members TNFR2, 4-1BB and OX40. *Eur. J. Immunol.* 41: 2010–2020.
22. Housley, W. J., C. O. Adams, F. C. Nichols, L. Puddington, E. G. Lingenheld, L. Zhu, T. V. Rajan, and R. B. Clark. 2011. Natural but not inducible regulatory T cells require TNF-α signaling for in vivo function. *J. Immunol.* 186: 6779–6787.
23. Grinberg-Bleyer, Y., D. Saadoun, A. Baeyens, F. Billiard, J. D. Goldstein, S. Grégoire, G. H. Martin, R. Elhage, N. Derian, W. Carpentier, et al. 2010. Pathogenic T cells have a paradoxical protective effect in murine autoimmune diabetes by boosting Tregs. *J. Clin. Invest.* 120: 4558–4568.
24. Mougiakakos, D., C. C. Johansson, R. Jitschin, M. Böttcher, and R. Kiessling. 2011. Increased thioredoxin-1 production in human naturally occurring regulatory T cells confers enhanced tolerance to oxidative stress. *Blood* 117: 857–861.
25. Hsieh, C. S., H. M. Lee, and C. W. Lio. 2012. Selection of regulatory T cells in the thymus. *Nat. Rev. Immunol.* 12: 157–167.
26. Griseri, T., M. Asquith, C. Thompson, and F. Powrie. 2010. OX40 is required for regulatory T cell-mediated control of colitis. *J. Exp. Med.* 207: 699–709.
27. Annunziato, F., L. Cosmi, F. Liotta, E. Lazzeri, R. Manetti, V. Vanini, P. Romagnani, E. Maggi, and S. Romagnani. 2002. Phenotype, localization, and mechanism of suppression of $CD4^+CD25^+$ human thymocytes. *J. Exp. Med.* 196: 379–387.
28. Chatzidakis, I., and C. Mamalaki. 2010. T cells as sources and targets of TNF: implications for immunity and autoimmunity. *Curr. Dir. Autoimmun.* 11: 105–118.
29. Baseta, J. G., and O. Stutman. 2000. TNF regulates thymocyte production by apoptosis and proliferation of the triple negative ($CD3^-CD4^-CD8^-$) subset. *J. Immunol.* 165: 5621–5630.
30. Eugster, H. P., K. Frei, R. Bachmann, H. Bluethmann, H. Lassmann, and A. Fontana. 1999. Severity of symptoms and demyelination in MOG-induced EAE depends on TNFR1. *Eur. J. Immunol.* 29: 626–632.
31. Kuprash, D. V., M. B. Alimzhanov, A. V. Tumanov, S. I. Grivennikov, A. N. Shakhov, L. N. Drutskaya, M. W. Marino, R. L. Turetskaya, A. O. Anderson, K. Rajewsky, et al. 2002. Redundancy in tumor necrosis factor (TNF) and lymphotoxin (LT) signaling in vivo: mice with inactivation of the entire TNF/LT locus versus single-knockout mice. *Mol. Cell. Biol.* 22: 8626–8634.
32. Xu, L., A. Kitani, I. Fuss, and W. Strober. 2007. Cutting edge: regulatory T cells induce $CD4^+CD25^-Foxp3^-$ T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-β. *J. Immunol.* 178: 6725–6729.
33. O'Connor, W., Jr., M. Kamanaka, C. J. Booth, T. Town, S. Nakae, Y. Iwakura, J. K. Kolls, and R. A. Flavell. 2009. A protective function for interleukin 17A in T cell-mediated intestinal inflammation. *Nat. Immunol.* 10: 603–609.
34. Yang, X. O., S. H. Chang, H. Park, R. Nurieva, B. Shah, L. Acero, Y. H. Wang, K. S. Schluns, R. R. Broaddus, Z. Zhu, and C. Dong. 2008. Regulation of inflammatory responses by IL-17F. *J. Exp. Med.* 205: 1063–1075.
35. Chen, Y., C. J. Haines, I. Gutcher, K. Hochweller, W. M. Blumenschein, T. McClanahan, G. Hämmerling, M. O. Li, D. J. Cua, and M. J. McGeachy. 2011. $Foxp3^+$ regulatory T cells promote T helper 17 cell development in vivo through regulation of interleukin-2. *Immunity* 34: 409–421.
36. Sujino, T., T. Kanai, Y. Ono, Y. Mikami, A. Hayashi, T. Doi, K. Matsuoka, T. Hisamatsu, H. Takaishi, H. Ogata, et al. 2011. Regulatory T cells suppress development of colitis, blocking differentiation of T-helper 17 into alternative T-helper 1 cells. *Gastroenterology* 141: 1014–1023.
37. van Mierlo, G. J., H. U. Scherer, M. Hameetman, M. E. Morgan, R. Flierman, T. W. Huizinga, and R. E. Toes. 2008. Cutting edge: TNFR-shedding by $CD4^+$ $CD25^+$ regulatory T cells inhibits the induction of inflammatory mediators. *J. Immunol.* 180: 2747–2751.
38. Duarte, J. H., S. Zelenay, M. L. Bergman, A. C. Martins, and J. Demengeot. 2009. Natural Treg cells spontaneously differentiate into pathogenic helper cells in lymphopenic conditions. *Eur. J. Immunol.* 39: 948–955.
39. Yurchenko, E., M. T. Shio, T. C. Huang, M. Da Silva Martins, M. Szyf, M. K. Levings, M. Olivier, and C. A. Piccirillo. 2012. Inflammation-driven reprogramming of $CD4^+$ $Foxp3^+$ regulatory T cells into pathogenic Th1/Th17 T effectors is abrogated by mTOR inhibition in vivo. *PLoS ONE* 7: e35572.
40. Kim, E. Y., J. J. Priatel, S. J. Teh, and H. S. Teh. 2006. TNF receptor type 2 (p75) functions as a costimulator for antigen-driven T cell responses in vivo. *J. Immunol.* 176: 1026–1035.
41. Malek, T. R., A. Yu, L. Zhu, T. Matsutani, D. Adeegbe, and A. L. Bayer. 2008. IL-2 family of cytokines in T regulatory cell development and homeostasis. *J. Clin. Immunol.* 28: 635–639.
42. Fontenot, J. D., J. P. Rasmussen, M. A. Gavin, and A. Y. Rudensky. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat. Immunol.* 6: 1142–1151.
43. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral $CD4^+CD25^-$ naive T cells to $CD4^+$ $CD25^+$ regulatory T cells by TGF-β induction of transcription factor Foxp3. *J. Exp. Med.* 198: 1875–1886.
44. Liu, Y., P. Zhang, J. Li, A. B. Kulkarni, S. Perruche, and W. Chen. 2008. A critical function for TGF-β signaling in the development of natural $CD4^+CD25^+$ $Foxp3^+$ regulatory T cells. *Nat. Immunol.* 9: 632–640.
45. Cuss, S. M., and E. A. Green. 2012. Abrogation of CD40-CD154 signaling impedes the homeostasis of thymic resident regulatory T cells by altering the levels of IL-2, but does not affect regulatory T cell development. *J. Immunol.* 189: 1717–1725.
46. Caminero, A., M. Comabella, and X. Montalban. 2011. Tumor necrosis factor α (TNF-α), anti-TNF-α and demyelination revisited: an ongoing story. *J. Neuroimmunol.* 234: 1–6.