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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...
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 blockade 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<sup>−/−</sup> mice, TNFR2<sup>−/−</sup> mice, TNF<sup>−/−</sup> mice, and TNN/lymphotoxin (LT)<sub>a</sub>/LT<sub>b</sub> 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 anti-mouse TCRβ (H57-597), CD45 (30-F11), and CD45RB (C363.16A) Abs were purchased from BioLegend (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**

Naïve CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>hi</sup> T cells were isolated from WT congenic B6 (CD45.1<sup>+</sup>) mice and injected i.p. into Rag1<sup>−/−</sup> immunodeficient recipients (4 × 10<sup>5</sup> cells/mouse) alone, or cotransferred with WT or TNFR2<sup>−/−</sup> CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>+</sup> Treg cells (1.6 × 10<sup>5</sup>/mouse, CD45.2<sup>+</sup>). In Treg transfer experiments, CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>+</sup> Tregs flow-sorted from WT congenic B6 mice (CD45.1<sup>+</sup>) and TNFR2<sup>−/−</sup> mice (CD45.2<sup>+</sup>) were mixed at 1:1 ratio and i.p. injected into Rag1<sup>−/−</sup> mice (1.2 × 10<sup>5</sup>/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/LT<sub>a</sub>/LT<sub>b</sub> mice were stained with CD3, CD4, CD8, TNFR2, and Foxp3. The expression of Foxp3 was analyzed by FACS, gating on CD3<sup>+</sup>CD4<sup>+</sup> or CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> cells. (A) Proportion of CD4<sup>+</sup>Foxp3<sup>+</sup> cells in total thymocytes from WT or TNF/LT<sub>a</sub>/LT<sub>b</sub> mice. (B) Proportion of Foxp3<sup>+</sup> cells in the CD4<sup>+</sup>CD8<sup>+</sup> thymocytes from WT or TNF/LT<sub>a</sub>/LT<sub>b</sub> mice. In (A) and (B), left panels show the typical FACS plots, and right panel shows summary (n = 3–6). (C) Proportion of CD4<sup>+</sup>Foxp3<sup>+</sup> cells in the spleen from WT or TNF/LT<sub>a</sub>/LT<sub>b</sub> mice. (D) Proportion of Foxp3<sup>+</sup> cells in CD4<sup>+</sup> splenic cells from WT or TNF/LT<sub>a</sub>/LT<sub>b</sub> 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/LT<sub>a</sub>/LT<sub>b</sub>, Triple knockout (KO).
monitored weekly by animal facility staff for the clinical symptoms of colitis such as rectal bleeding, loose feces/diarrhea, rough/bunched 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 (LN) 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 were then 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 × 10^6 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 polarization 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) with or without or with 10 µg/ml hamster IgG, or anti-TNFRI Ab or anti-TNFRII 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 re-stimulated 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.

In vitro T cell activation and differentiation

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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-TNFRI or anti-TNFR2 neutralizing Abs were added to the TNF-treated Tregs. The anti-TNFRI Ab, but not the anti-TNFRI 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 naïve 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 naïve 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 naïve 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 naïve CD4 cells. As shown in Fig. 6B, naïve 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 naïve CD4 cells that Th17 cells had paradoxically tissue protective and immunosuppressive effects and consequently suppressed colun 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 naïve CD4 cells, as compared with naïve 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 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).
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). 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. 8C), 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/TNF2 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 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\(^+\) 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\(^+\) Teffs were replicating similarly in the lymphopenic mouse. There was no marked outgrowth of an intentionally added 3% Foxp3\(^+\) Teffs in Tregs after
Thymus and peripheral lymphoid tissues of IL2 50% reduction of Foxp3+ Tregs in CD4 cells was found in the multiple factors, including TNF/TNFRII signaling, contribute to the thymic generation and peripheral homeostasis of Tregs. Recently, Cuss and Green (45) reported that thymic Tregs actually were heterogeneous 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 Ili2−/− or I2ra−/− 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.

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Disclosures
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