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Natural but Not Inducible Regulatory T Cells Require TNF-α Signaling for In Vivo Function

William J. Housley,* Catherine O. Adams,*† Frank C. Nichols,‡ Lynn Puddington,§ Elizabeth G. Lingenheld,§ Li Zhu,§ Thiruchandurai V. Rajan,* and Robert B. Clark*†

TNF-α has a multifunctional role in autoimmune diseases as reflected in the variable responses of different human diseases to anti–TNF-α therapy. Recent studies have suggested that TNF-α modulates autoimmunity partially via effects on regulatory T cells (Tregs) and that these effects are mediated through the type II TNFR (TNFR2). We have investigated the requirement for TNFR2-expression on murine natural Tregs (nTregs) and induced Tregs (iTregs) in mediating suppression of colitis. Surprisingly, we find that TNFR2-expression is required for both spleen- and thymus-derived nTreg-mediated suppression, but is not required for iTreg-mediated suppression. Abnormal TNFR2−/− nTreg function was not associated with an in vivo decrease in accumulation, stability, or expression of markers known to be relevant in Treg function. Because iTregs are generated in the presence of TGF-β, we investigated whether activation in the presence of TGF-β could overcome the functional defect in TNFR2−/− nTregs. Although preactivation alone did not restore suppressive function of nTregs, preactivation in the presence of TGF-β did. These results identify potentially critical differences in activation requirements for nTregs versus iTregs. Furthermore, our findings are consistent with reports suggesting that nTregs are activated in sites of inflammation while iTregs are activated in lymph nodes. Finally, by demonstrating that nTregs require TNF-α for optimal function whereas iTregs do not, our results suggest that the enigma of variable responses of different human diseases to anti–TNF-α therapy may relate to whether nTregs or iTregs have the predominant regulatory role in a given disease. The Journal of Immunology, 2011, 186: 6779–6787.
TNF-α (14). In contrast, it has been shown in humans that TNF-α decreases the in vitro suppressive function of both CD4+CD25+ peripheral blood cells and in vitro-generated iTregs (15–17). In addition, patients with rheumatoid arthritis, suggested to have Tregs with decreased suppressive function, have been reported to generate a population of CD62L+ iTregs with suppressive function after anti–TNF-α therapy (18). Although reports have shown opposite effects of TNF-α on mouse and human Tregs, both mouse and human studies have suggested that TNFR2 is the primary receptor required for these effects.

Recently, using murine models of diabetes, Grinberg-Bleyer et al. (19) demonstrated that the suppressive function of Tregs is optimized by the presence of Teffs and that TNF-α is one factor involved in this optimization (19, 20). In this study, we have investigated the role of TNFR2 on both murine nTregs and iTregs in the in vivo suppression of autoimmunity. Surprisingly, we find that TNFR2 expression is required for nTreg suppression but not for iTreg suppression. Furthermore, we find that preactivation in the presence of TGF-β can replace TNFR2 expression in optimizing nTreg function. These findings have significant implications for understanding differences in nTreg versus iTreg function, and they suggest a mechanism behind the dichotomous outcomes of anti–TNF-α therapy in human autoimmune diseases.

Materials and Methods

Mice

C57BL/6, TNFR2−/− (on a C57BL/6 background), and RAG-1−/− mice were purchased from Jackson Laboratories (Bar Harbor, ME). C57BL/6 Ly5.2+ mice were purchased from Charles River Laboratories. C57BL/6 mice expressing GFP under the control of the endogenous Foxp3 promoter (Foxp3.gfp.KI mice) were a kind gift from Vijay Kuchroo (Harvard University, Cambridge, MA). TNFR2−/− mice were crossed to the Foxp3.gfp.KI mice to produce TNFR2−/− Foxp3+ (CD45.2+) thymic WT or TNFR2−/− Foxp3−/− Foxp3+ (CD45.2+) thymic WT mice. For Foxp3 staining, cells were permeabilized with the BD Cell Permeabilization Kit (BD Biosciences). For Foxp3 staining, cells were permeabilized using the eBioscience Foxp3 staining kit (eBioscience). Analysis was performed on a BD FACSCalibur (BD Biosciences) or an LSRII (BD Biosciences).

Splenic cell purification and differentiation

Splenes were used as the source for all CD4+CD25− Teffs and CD4+CD25+ Foxp3+ Tregs except in the thymic Treg studies. Teff and Tregs were purified using a Milteny Treg purification kit (Milteny Biotech) except when, as noted, FACS-sorting was used for purification. iTregs were generated from T effs by culturing on plate-bound anti-CD3 Ab (pb-anti–CD3; 5 μg/ml; Biolegend) for 3 d with 100 U/ml IL-2 and 2 ng/ml TGF-β (R&D Biosystems). After in vitro differentiation, GFP+ iTregs from either Foxp3.gfp.KI or TNFR2−/− Foxp3.gfp.KI mice were FACS-sorted to ≥99% GFP+ prior to transfer into RAG-1−/− recipients. For Foxp3 stability studies, splenic CD4+CD25+ cells from Foxp3.gfp.KI or TNFR2−/− Foxp3.gfp.KI mice were purified using a Milteny Treg purification kit. The Tregs were then sorted into ≥99% GFP+ prior to transfer into RAG-1−/− mice. For nTreg activation in vitro, nTregs were cultured for 3 d on pb-anti–CD3 with 100 U/ml IL-2 in the presence or absence of 2 ng/ml TGF-β. In the presence of FACS-sorted CD4+CD25+Foxp3+ cells in the presence of TGF-β, splenic CD4+CD25+ cells were purified from Foxp3.gfp.KI or TNFR2−/− Foxp3.gfp.KI mice using a Milteny Treg purification kit and FACS-sorted to ≥99% GFP+.

Results

Colitis

Magnetic bead-purified, naïve CD4+CD25− WT Teffs (1 × 10⁶ cells) with or without nTregs, in vitro-differentiated iTregs, or in vitro-preactivated nTregs were transferred into RAG-1−/− mice by i.p. injection at varying ratios as noted. MLNs and colons were harvested 2 wk after transfer for further analysis and analysis of colitis. Colons were embedded in paraffin, sectioned, and stained with H&E. Colitis histology was graded blindly by a pathologist. Histologic evidence of colitis was scored as a sum of acute and chronic inflammation as follows. For acute inflammation: 1) neutrophil infiltration with rare or absent cryptitis; 2) cryptitis with rare or no crypt abscesses; 3) severe crypt abscesses; and 4) severe cryptitis with ulceration. For chronic inflammation: 1) mild inflammatory expansion of the lamina propria; 2) lamina propria fibrosis with minimal transmural inflammation; 3) lifting of crypts and goblet cell depletion; and 4) lymphoid follicles and intramural inflammation. The extent of inflammation was determined as a percent of the colon inflamed, and the total score was calculated by multiplying the extent by the score of acute and chronic inflammation. For experiments in which nTregs were adoptively transferred into RAG-1−/− mice without Teffs, splenic CD4+CD25− cells were purified using a Miltenyi Treg purification kit, and 0.5 × 10⁶ cells per mouse were injected i.p. Two weeks later, colons were taken for histologic analysis as described.

Colonic lamina propria purification

Colonic lamina propria cells were prepared by incubating colonic tissue sequentially with dithioerythritol (0.15 μg/ml; Calbiochem), EDTA (1.3 mM; Ambion), and a mixture of Collagenase D (0.5 mg/ml; Roche), DNase I (0.5 mg/ml; Sigma-Aldrich), Worthington, with disassociated. Tissue was crushed over a cell strainer, and lamina propria lymphocytes were purified by centrifugation on a 44%/67% Percoll gradient.

Statistics

The p values were determined with Student unpaired t tests of the Treg-suppressed samples compared with the Teff-alone samples. All graphs are shown as group means. Vertical bars are ±SEs.
Tregs that is maximally suppressive in vitro (11–13). To determine whether TNFR2-expression on nTregs delineates a unique subset or is a marker of activated Tregs, we FACs-purified CD4+CD25+ splenic nTregs into TNFR2low and TNFR2high populations and activated both populations on pb-anti-CD3 in vitro. We found that both the TNFR2low and TNFR2high populations upregulated TNFR2 after activation, suggesting that TNFR2 is an activation marker on all Tregs and not a unique subset (Fig. 1A, 1B).

To determine whether TNFR2 expression is required for nTreg suppressive function in vivo, we investigated the ability of TNFR2−/− nTregs to suppress an adoptive transfer model of Teff IFNγ production and colitis. Teffs were purified from C57BL/6 CD45.1+ mice (WT) and injected i.p. into RAG-1−/− mice alone or together with CD4+CD25+ nTregs from WT CD45.2+ or TNFR2−/− CD45.2+ mice. The number and frequency of Foxp3-expressing splenic cells and the level of Foxp3-expression after purification of CD4+CD25+ cells was similar between WT and TNFR2−/− nTreg populations prior to adoptive transfer (data not shown; Fig. 2A). Two weeks after transfer, the MLNs were harvested from the RAG-1−/− recipient mice, restimulated ex vivo, and IFN-γ production by the CD45.1+ Teffs was determined by flow cytometry. As expected, WT nTregs suppressed the absolute number of IFN-γ-producing Teffs in the MLNs by 60% (Fig. 2B).

In contrast, TNFR2−/− nTregs were unable to suppress the absolute number of IFN-γ-producing cells in the MLNs below that seen with Teffs alone (Fig. 2B).

It is postulated that peripherally derived CD4+CD25+Foxp3+ Tregs include a small percentage of iTregs. Although the exact proportion of nTregs to iTregs in the splenic Foxp3+ population is unknown, various approaches to differentiate the two populations have suggested that 10–30% of the splenic Foxp3+ population may be peripherally derived iTregs (21, 22). Given the possibility that our splenic magnetic bead-purified nTregs includes a sub-population of iTregs, we derived Foxp3+ nTregs from the thy-muses of Foxp3+gfp.KI (WT) and TNFR2−/− Foxp3+gfp.KI mice via FACs sorting (≥99% GFP+), yielding populations of nTregs theoretically devoid of contaminating iTregs. These thymus-derived nTregs (CD45.2+) were then tested for their ability to suppress WT Teffs (CD45.1+) after adoptive transfer into RAG-1−/− mice. Because the yields of thymus-derived nTregs were small, we studied in vivo function using fewer Teff and nTregs—though still using similar Teff:Treg ratios—and harvested the MLNs 1 wk later than in our other studies. As seen in Fig. 2C, whereas WT thymic nTregs significantly suppressed MLN Teff IFNγ production, TNFR2−/− thymic nTregs were unable to mediate significant suppression. These results are consistent with our findings with spleen-derived magnetic bead-purified nTregs, confirming that TNFR2−/− nTregs are defective in mediating suppression in vivo.

To determine whether TNFR2-expression was required for nTreg-mediated suppression of autoimmune disease, WT CD45.1+ Teffs were cotransferred into RAG-1−/− mice with or without spleen-derived magnetic bead-purified WT or TNFR2−/− nTregs (CD45.2+). Two weeks after cell transfer, the colons of the recipient RAG-1−/− mice were removed, fixed, embedded in paraffin, and stained with H&E, and the development of colitis was graded blindly by a pathologist. Teff transferred in the absence of nTregs resulted in severe colitis along the entire length of the colon (Fig. 2D, 2E). As expected, the cotransfer of WT nTregs with Teffs suppressed both the extent and severity of colonic inflammation. In contrast, and consistent with the lack of suppression of MLN IFNγ-producing cells, TNFR2−/− nTregs were unable to suppress colitis (Fig. 2D, 2E).

It has been shown that TNFR2−/− CD4+CD45RB+ T cells when transferred into RAG-1−/− mice can cause enhanced colitis (23). To ensure that a small population of contaminating TNFR2−/− Teffs was not responsible for causing disease in mice receiving TNFR2−/− nTregs, we adoptively transferred splenic magnetic bead-purified WT or TNFR2−/− nTregs alone (without Teff) into RAG-1−/− mice and assessed the colons of these recipient mice 2 wk later. We found that neither WT nor TNFR2−/− nTregs, when transferred in the absence of Teffs, were capable of inducing histologic evidence of colitis (data not shown). These results suggest that murine nTregs require TNFR2 expression to suppress both in vivo Teff cytokine production and autoimmunity.

TNFR2 is not required for iTreg suppressive function in vivo

TNFR2 expression and the relevance of this expression for suppressor function has not previously been characterized for murine

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**FIGURE 1.** TNFR2 is an activation marker on nTregs, Teffs, and iTregs. A. CD4+CD25+ nTregs were purified from WT Foxp3+gfp.KI or TNFR2−/− Foxp3+gfp.KI mice using magnetic beads and stained for CD4 and TNFR2 expression. Results prior to culture are shown gated on CD4+GFP+ cells. Dark gray, open histograms: TNFR2−/− GFP+ Teffs stained with anti-TNFR2 Ab; light gray, open histograms: WT GFP+ Teffs stained with anti-TNFR2 Ab and isotype control Ab; black, open histograms: WT GFP+ Teffs stained with anti-TNFR2 Ab. B. WT GFP+ Teffs were stained for TNFR2 and sorted into GFP+TNFR2high and GFP+TNFR2low populations. Cells were plated on 5 μg/ml pb-anti-CD3 with 100 U/ml IL-2 for 3 d. Before culture and after 3 d of culture, cells were stained for CD4 and TNFR2 expression. Results are gated on CD4+GFP+ cells. Light gray, filled histogram: GFP+TNFR2low preulture; dark gray, filled histogram: GFP+TNFR2high preulture; light gray, open histogram: GFP+TNFR2low after activation; black, open histogram: GFP+TNFR2high after activation. C. CD4+CD25+ Teffs were purified from Foxp3+gfp.KI or TNFR2−/− Foxp3+gfp.KI mice using magnetic beads and stained for CD4 and TNFR2. Results are gated on CD4+ T cells. Dark gray, open histograms: TNFR2−/− Teffs stained with anti-TNFR2 Ab; light gray, open histograms: WT Teff stained with anti-TNFR2 isotype control Ab; black, open histograms: WT Teff stained with anti-TNFR2 Ab. D. WT Teffs were plated on 5 μg/ml pb-anti-CD3 alone or with 100 U/ml IL-2 and TGF-β (2 ng/ml) to generate iTregs. Results for activated Teffs are gated on CD4+ T cells, and results for iTregs are gated on CD4+GFP+ T cells. Light gray, filled histogram: WT Teff preulture; dark gray, filled histogram: activated WT Teff; black, open histogram: iTregs. One representative experiment of three is depicted for A–D.
CD45.1+ Teff population by splenic magnetic bead-purified CD4+CD25+ suppress in vivo. WT Teff (CD45.1+), with or without magnetic-bead
pulsed to the total number of CD4+CD45.1+ IFN-γ* ratio = 2:1 to 2:1.5) into RAG-1
2 knockouts to TNFR2

Results are shown gated on CD4+ cells. One representative experiment of
twenty is shown.

A

CD4+CD25+ nTregs from TNFR2−/− mice are unable to suppress in vivo. WT Teff (CD45.1+), with or without magnetic-bead purified WT or TNFR2−/− CD45.2+ nTregs (nTregs) (Teff/nTreg ratio = 2:1), were transferred into RAG-1−/− mice. The recipient mice were analyzed 14 d later (B, D, E). For thymus-derived nTreg experiments (C), WT or TNFR2−/− thymic CD4+CD25+GFP+ cells (CD45.2+) were FACS-sorted to ≥99% GFP+ and cotransferred with CD45.1+ WT Teff (Teff/nTreg ratio = 2:1 to 2:1.5) into RAG-1−/− mice. The recipient mice were analyzed 21 d later. A. CD4+CD25+ nTregs were derived by magnetic bead purification from WT and TNFR2−/− spleens and stained for CD4 and Foxp3. Results are shown gated on CD4+ cells. One representative experiment of three is shown. B. Suppression of IFN-γ production from the MLN CD45.1+ Teff population by splenic magnetic bead-purified CD4+CD25+ nTregs. IFN-γ production by the MLN CD4+CD45.1+ T cells is normalized to the total number of CD4+CD45.1+ IFN-γ-producing cells in the Teff alone group (n = 7). C. Suppression of IFN-γ production from the MLN CD45.1+ Teff population by thymic CD4+CD25+GFP cells from WT Foxp3.gfp.KI or TNFR2−/−Foxp3.gfp.KI mice. IFN-γ production by the MLN CD4+CD45.1+ T cells is normalized to the total number of CD4+CD45.1+ IFN-γ-producing cells in the Teff-alone group (n = 3). D. Representative examples of colon histology from splenic magnetic bead-purified nTreg suppression experiments. E. Compilation of colon histologic scores from splenic magnetic bead-purified nTreg suppression experiments (n = 3).

*p < 0.05, ***p < 0.001, compared with Teff alone.

iTregs. We found that TNFR2 was expressed on ~30% of CD4+ CD25− Teffs in naive mice. However, after activation in vitro in the presence of exogenous TGF-β (to generate iTregs), TNFR2 was upregulated on both the activated Teff and iTreg populations, suggesting that TNFR2 is also an activation marker on Teff (Fig. 1C, 1D).

To determine whether TNFR2 expression is required for the suppressive function of murine iTregs in vivo, iTregs were generated in vitro from both Foxp3.gfp.KI Teff (WT) and TNFR2−/−Foxp3.gfp.KI Teffs using pb-anti–CD3, IL-2, and TGF-β. The percentage of iTreg differentiation was similar using WT and TNFR2−/− Teffs (Fig. 3A). After in vitro differentiation, CD45.2+ WT GFPl and CD45.2+ TNFR2−/− GFPl iTregs were FAC-sorted to ≥99% GFPl and tested for their ability to suppress colitis. In contrast to the lack of suppressive function seen using TNFR2−/− nTregs, TNFR2−/− iTregs suppressed MLN Teff IFN-γ production (Fig. 3B) and colitis development (Fig. 3C, 3D) equally to WT iTregs. These results demonstrate that although TNFR2 expression is required for nTreg suppression of autoimmunity, it is not required for iTreg suppression.

TNFR2−/− and WT nTregs express similar phenotypic markers of optimal suppressive function and activation

We next asked whether WT and TNFR2−/− nTregs differ in expression of molecules associated with optimal suppressive function (24–26). RAG-1−/− mice were adoptively transferred with WT Teffs (CD45.1+) and nTregs (CD45.2+) derived from either Foxp3.gfp.KI or TNFR2−/− Foxp3.gfp.KI mice. Two weeks later, cells were harvested from both the MLNs and colonic lamina propria (cLP) of recipient mice and the expression of markers on the CD4+CD45.2+GFPl population analyzed. Expression of the gut-homing integrin α4β7 and the homing receptor CCR6 were expressed equally on the WT and TNFR2−/− nTregs in the MLNs (Fig. 4A, 4B). CCR6 expression was also equal in the cLP, but α4β7 is downregulated in the cLP and little expression of α4β7 was found on either the WT or TNFR2−/− cLP iTregs. Similarly, we found that CTLA4 and OX40 were equally expressed on WT and TNFR2−/− nTregs in both the MLN and cLP (Fig. 4C, 4D).

IL-10 has been shown to be important in prevention of colitis, but it has been clearly demonstrated that IL-10 production by nTregs themselves is not required for suppression of this transfer model of colitis (26, 27). When cotransferred with Teffs, IL-10–deficient Tregs are fully capable of suppressing colitis in this model (26, 27). Rather, suppression of colitis in this model requires nTreg expression of the IL-10R and response to extrinsic IL-10 (26). We found IL-10 essentially undetectable in either WT or TNFR2−/− nTregs via intracellular staining 2 wk after transfer, but found that IL-10R was equally expressed on WT and TNFR2−/− nTregs in both the MLN and cLP (Fig. 4E). In addition, we examined the expression of the activation markers CD25, CD69, CD44, and CD62L as well as CD103 on the GFPl Treg population in the spleen and MLN on day 7 (before the onset of colitis). We found no difference in expression of activation markers between WT and TNFR2−/− nTregs at this early time point (Fig. 5). These results suggest that the difference in WT and TNFR2−/− nTreg function is not related to differential expression of molecules known to be required for optimal nTreg function or activation. Finally, we also measured the production of TGF-β by nTregs after in vitro stimulation and found that TGF-β production was similar between WT and TNFR2−/− nTregs (WT: 547 pg/ml ± 91 SEM; TNFR2−/−: 570 pg/ml ± 32 SEM).

TNFR2−/− nTreg accumulation and stability is normal in the MLN and in colonic lamina propria

To determine whether the abnormal TNFR2−/− nTreg-mediated suppression was due to defective accumulation of the nTregs in the spleen, MLN, or cLP, CD4+ CD25+ nTregs were purified from...
Foxp3.gfp.KI and TNFR2\(^{-/-}\) Foxp3.gfp.KI (CD45.2\(^{-}\)) mice were generated in vitro from CD4\(^{+}\)CD25\(^{-}\) Teffs derived from WT Foxp3.gfp.KI or TNFR2\(^{-/-}\) Foxp3.gfp.KI mice. After in vitro generation, GFP\(^{+}\) iTregs were FAC Sorted to \(\geq99\%\) purity and tested for the ability to suppress colitis. WT Teffs (CD45.1\(^{+}\)), with or without FAC Sorted WT or TNFR2\(^{-/-}\) iTregs (CD45.2\(^{+}\)) (Teff/iTreg ratio = 5:1 or 15:1), were transferred into RAG-1\(^{-/-}\) mice. The recipient mice were analyzed 14 d later. A. Percent GFP\(^{+}\) lymphocytes was assessed after 3 d of culture of CD4\(^{+}\)CD25\(^{-}\) Teffs from Foxp3.gfp.KI or TNFR2\(^{-/-}\) Foxp3.gfp.KI mice with 5 \(\mu\)g/ml pb-anti–CD3 in the presence of 100 U/ml IL-2 and 2 ng/ml TGF-\(\beta\) (\(n = 4\)). B. IFN-\(\gamma\) production by MLN CD4\(^{+}\)CD45.1\(^{+}\) T cells was assessed, and the data were normalized as in Fig. 2 (\(n = 5\) for 5:1; \(n = 4\) for 15:1). C. Representative examples of colon histology. D, Compilation of colon histologic scores (\(n = 5\) for 5:1; \(n = 4\) for 15:1), ***p < 0.001, **p < 0.01, *p < 0.05, compared with Teff alone.

\[\text{FIGURE 3.} \text{TNF-R2}^{-/-}\text{iTregs suppress colitis. iTregs (iT R) were generated in vitro from CD4}^{+}\text{CD25}^{-}\text{T effs derived from WT Foxp3.gfp.KI or TNF-R2}^{-/-}\text{Foxp3.gfp.KI mice. After in vitro generation, GFP}^{+}\text{iTregs were FAC Sorted to }\geq99\%\text{ purity and tested for the ability to suppress colitis. WT Teffs (CD45.1\(^{+}\)), with or without FAC Sorted WT or TNF-R2}^{-/-}\text{iTregs (CD45.2\(^{+}\)) (Teff/iTreg ratio = 5:1 or 15:1), were transferred into RAG-1}^{-/-}\text{ mice. The recipient mice were analyzed 14 d later. A. Percent GFP}^{+}\text{lymphocytes was assessed after 3 d of culture of CD4}^{+}\text{CD25}^{-}\text{T effs from Foxp3.gfp.KI or TNF-R2}^{-/-}\text{Foxp3.gfp.KI mice with 5 }\mu\text{g/ml pb-anti–CD3 in the presence of 100 U/ml IL-2 and 2 ng/ml TGF-}\beta\text{ (n = 4). B. IFN-}\gamma\text{ production by MLN CD4}^{+}\text{CD45.1}^{-}\text{T cells was assessed, and the data were normalized as in Fig. 2 (n = 5 for 5:1; n = 4 for 15:1). C. Representative examples of colon histology. D, Compilation of colon histologic scores (n = 5 for 5:1; n = 4 for 15:1), ***p < 0.001, **p < 0.01, *p < 0.05, compared with Teff alone.}
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FIGURE 4. WT and TNF-R2\(^{-/-}\) nTregs have similar phenotypes after transfer in vivo. RAG-1\(^{-/-}\) mice were adoptively transferred with WT Teffs (CD45.1\(^{+}\)) and magnetic bead-purified nTregs (CD45.2\(^{+}\)) derived from either Foxp3.gfp.KI or TNF-R2\(^{-/-}\) Foxp3.gfp.KI mice (Teff/nTreg ratio = 2:1). Two weeks later, cells were harvested from the MLN and cLP of recipient mice, and the CD4\(^{+}\)CD45.2\(^{+}\)GFP\(^{+}\) population was analyzed by FACS for expression of a4b7 (MLN only) (A), CCR6 (B), CTLA4 (C), OX40 (D), and IL-10R (E). Lines denote mean percent expression. Results are gated on CD4\(^{+}\)CD45.2\(^{+}\)CD45.1\(^{-}\) MHCII\(^{-}\) GFP\(^{+}\) cells.

\[\text{FIGURE 3.} \text{TNF-R2}^{-/-}\text{iTregs suppress colitis. iTregs (iT R) were generated in vitro from CD4}^{+}\text{CD25}^{-}\text{T effs derived from WT Foxp3.gfp.KI or TNF-R2}^{-/-}\text{Foxp3.gfp.KI mice. After in vitro generation, GFP}^{+}\text{iTregs were FAC Sorted to }\geq99\%\text{ purity and tested for the ability to suppress colitis. WT Teffs (CD45.1\(^{+}\)), with or without FAC Sorted WT or TNF-R2}^{-/-}\text{iTregs (CD45.2\(^{+}\)) (Teff/iTreg ratio = 5:1 or 15:1), were transferred into RAG-1}^{-/-}\text{ mice. The recipient mice were analyzed 14 d later. A. Percent GFP}^{+}\text{lymphocytes was assessed after 3 d of culture of CD4}^{+}\text{CD25}^{-}\text{T effs from Foxp3.gfp.KI or TNF-R2}^{-/-}\text{Foxp3.gfp.KI mice with 5 }\mu\text{g/ml pb-anti–CD3 in the presence of 100 U/ml IL-2 and 2 ng/ml TGF-}\beta\text{ (n = 4). B. IFN-}\gamma\text{ production by MLN CD4}^{+}\text{CD45.1}^{-}\text{T cells was assessed, and the data were normalized as in Fig. 2 (n = 5 for 5:1; n = 4 for 15:1). C. Representative examples of colon histology. D, Compilation of colon histologic scores (n = 5 for 5:1; n = 4 for 15:1), ***p < 0.001, **p < 0.01, *p < 0.05, compared with Teff alone.}
\]
inability of TNFR2
2
analyzed by FACS. Results are gated on CD4 +CD45.2+CD45.1
transferred with WT Teffs (CD45.1 +) and magnetic-bead purified CD4 +
B
or colitis (Fig. 7

restore their ability to suppress either MLN Teff IFN-
production
activation markers after transfer in vivo. RAG-1
KI (CD45.2 +) mice. Seven days later, cells were harvested from both the
Preactivation of TNFR2
2
not due to a defect in TCR mediated activation.

During in vitro derivation, iTregs are not only preactivated, but
preactivated in the presence of TGF-
preactivation in the presence of TGF-
b
CD25+ nTregs derived from either Foxp3.gfp.KI or TNFR2
in vitro for 3 d with pb-anti–CD3 and IL-2 and these preactivated
Foxp3+ WT or TNFR2
function. nTregs from WT or TNFR2
in vitro activation. To determine whether the abnormal function of
TNFR2
2
nTregs was a result of a defect in activation in vivo, we
next asked whether preactivation in vitro could restore in vivo function. nTregs from WT or TNFR2
2
mice were activated in vitro for 3 d with pb-anti–CD3 and IL-2 and these preactivated
Foxp3+ WT or TNFR2
2
Tregs were tested for their ability to suppress colitis. After 3 d of in vitro activation, Foxp3 expression by the WT and TNFR2
2
nTregs was similar (Fig. 7A). We found that adoptive transfer of preactivated TNFR2
2
nTregs did not restore their ability to suppress either MLN Teff IFN-γ production (Fig. 7B) or colitis (Fig. 7C, 7D). These results suggest that the inability of TNFR2
2
nTregs to mediate in vivo suppression is not due to a defect in TCR mediated activation.

Preactivation of TNFR2
2
nTregs in the presence of TGF-β leads to restoration of suppressive function

During in vitro derivation, nTregs are not only preactivated, but
preactivated in the presence of TGF-β. To determine whether activation in the presence of TGF-β might allow TNFR2
2
nTregs to function normally, splenic magnetic bead-purified nTregs from WT or TNFR2
2
mice were activated in vitro for 3 d with pb-anti–CD3, IL-2, and TGF-β. Using this in vitro protocol, Foxp3 expression by the WT and TNFR2
2
nTregs was again not significantly different on day 3 (Fig. 8A). We found no significant differences between WT and TNFR2
2
nTregs in the expression of CD62L, CD103, CD44, CD69, or CD25 after 3 d of in vitro activation with or without TGF-β (data not shown). We also found no difference in IL-10 production by WT and TNFR2
2
nTregs after in vitro stimulation with or without TGF-β (WT without TGF-β, 155 pg/ml ± 22 SEM, WT w/ TGF-β, 113 pg/ml ± 18 SEM; TNFR2
2
without TGF-β, 194 pg/ml ± 40 SEM, TNFR2
2
w/ TGF-β, 120 pg/ml ± 30 SEM). Equal numbers of preactivated, TGF-β–cultured Foxp3+
WT or TNFR2
2
nTregs were then tested for their ability to suppress in vivo. Surprisingly, preactivation of nTregs in the presence of TGF-β resulted in the restoration of the ability of TNFR2
2
nTregs to mediate suppression of MLN Teff IFN-γ production and colitis (Fig. 8B).

To rule out the possibility that preculturing in TGF-β resulted in iTreg generation from a small number of Teffs, potentially contaminating the magnetic bead-purified nTreg population, we repeated these studies using FACS-sorted splenic nTregs. FACS-
sorted nTregs (≥99% GFP+) from WT Foxp3.gfp.KI and TNFR2
2
Foxp3.gfp.KI mice were activated in vitro for 3 d with

FIGURE 5. TNFR2
2
and WT nTregs (nTR) express similar levels of activation markers after transfer in vivo. RAG-1
mice were adoptively transferred with WT Teffs (CD45.1 +) and magnetic-bead purified CD4+
CD25
nTregs derived from either Foxp3.gfp.KI or TNFR2
Foxp3.gfp.
KI (CD45.2 +) mice. Seven days later, cells were harvested from both the spleen (SPL) and MLN of recipient mice. SPL and MLN cells were stained for CD103 (A), CD25 (B), CD69 (C), CD44 (D), and CD62L (E) and analyzed by FACS. Results are gated on CD4+CD45.2+CD45.1
MHCII
GFP

cells.

FIGURE 6. TNFR2
2
nTregs accumulate normally in vivo and are equally stable in vivo compared with WT nTregs. A and B, RAG-1
mice were adoptively transferred with WT Teffs (CD45.1 +) and splenic nTregs (CD45.2 +) derived from either Foxp3.gfp.KI or TNFR2
Foxp3.
gfp.KI mice (Teff/nTreg ratio = 2:1). Tregs were magnetic bead-purified in A and B and purified by FACS sorting in C. A. Absolute number of CD4+
MHCII
CD45.2+GFP
cells in the MLN and spleen 7 d after transfer. B, Absolute number of CD4+MHCII
CD45.2+GFP
cells in the MLN and cLP 14 d after transfer. C, Foxp3.gfp.KI and TNFR2
Foxp3.gfp.KI (CD45.2 +) nTregs were FACS sorted into ≥99% GFP+ and transferred into RAG-1
mice with WT Teffs (CD45.1 +) (Teff/nTreg ratio = 2:1). Two weeks later, the percentage of GFP+ cells within the MLN and cLP CD4+
MHCII
CD45.2+ population was determined.
FIGURE 7. In vitro preactivation of TNFR2−/− nTregs does not restore in vivo suppressive function. Magnetic bead-purified nTregs from WT or TNFR2−/− mice were preactivated in vitro for 3 d. WT Teff (CD45.1+), with or without preactivated WT or TNFR2−/− CD45.2+ nTregs (Teff/nTreg ratio = 15:1), were transferred into RAG-1−/− mice. The recipient mice were analyzed 14 d later. A. Percent GFP+ lymphocytes was assessed after 3 d of culture of CD4+CD25+ nTregs from Foxp3.gfp.KI or TNFR2−/− Foxp3.gfp.KI mice with 5 μg/ml pb-anti–CD3 and 100 U/ml IL-2 (n = 4). B, IFN-γ production by MLN CD4+CD45.1+ T cells was assessed, and the data were normalized as in Fig. 2 (n = 3). C, Representative examples of colon histology. D, Compilation of colon histologic scores (n = 4). ***p < 0.001, **p < 0.01, compared with Teff alone.

FIGURE 8. Activation of TNFR2−/− nTregs in the presence of TGF-β restores suppressive function. Magnetic bead-purified splenic nTregs from WT or TNFR2−/− mice were preactivated in vitro in the presence of TGF-β for 3 d. WT Teffs (CD45.1+), with or without preactivated, TGF-β–cultured, WT or TNFR2−/− CD45.2+ nTregs (Teff/nTreg ratio = 30:1 or 40:1) were transferred into RAG-1−/− mice. The recipient mice were analyzed 14 d later. A. Percent GFP+ lymphocytes was assessed after 3 d of culture of magnetic bead-purified splenic CD4+CD25+ nTregs from Foxp3.gfp.KI or TNFR2−/− Foxp3.gfp.KI mice with 5 μg/ml pb-anti–CD3, 100 U/ml IL-2, and 2 ng/ml TGF-β (n = 4). B, IFN-γ production by MLN CD4+CD45.1+ T cells was assessed, and the data were normalized as in Fig. 2 (n = 3). C, Splenic CD4+CD25+ nTregs from Foxp3.gfp.KI or TNFR2−/− Foxp3.gfp.KI mice were FACs sorted to ≥99% GFP+ and preactivated in vitro in the presence of 100 U/ml IL-2 and 2 ng/ml TGF-β for 3 d. WT Teffs (CD45.1+), with or without FACs-sorted, preactivated, TGF-β–cultured, WT or TNFR2−/− CD45.2+ nTregs (Teff/nTreg ratio = 10:1 or 15:1) were transferred into RAG-1−/− mice. The recipient mice were analyzed 14 d later. IFN-γ production by MLN CD4+CD45.1+ T cells was assessed, and the data were normalized as in Fig. 2 (n = 3). D, Representative examples of colon histology from splenic magnetic bead-purified nTreg experiments. E, Compilation of colon histologic scores from splenic magnetic bead-purified nTreg suppression experiments (n = 3). ***p < 0.001, **p < 0.01, *p < 0.05, compared with Teff alone.
nTregs to suppress MLN Teff IFN-γ production, we found that TGF-β preculture also restored the capacity of TNFR2−/− nTregs to ameliorate the development of colitis (Fig. 8D, 8E). These results indicate that the inability of TNFR2−/− nTregs to mediate in vivo suppression can be overcome through activation of these Tregs in the context of TGF-β. Given that our investigations have revealed no identifiable phenotypic difference between WT and TNFR2−/− nTregs and that mechanisms of Treg suppression are still unclear, we are unable at this time to determine what abnormality in TNFR2−/− nTregs is corrected by activation in the presence of TGF-β. Nevertheless, whereas prior studies have indicated that TGF-β may be required for the peripheral maintenance of nTregs, a role for TGF-β in their functional activation has not been reported previously (29–31).

Discussion

Whereas Tregs have clearly been shown to be critical in immunoregulation and in preventing autoimmune, significant gaps remain in our understanding of Treg physiology. Recently, interest in the potential role of Tregs in immunotherapy has intersected with attempts to understand the enigmatic variable responses of human autoimmune diseases to anti-TNF-α therapy. These studies have resulted in a number of often conflicting results of the effects of TNF-α on Tregs (10, 15–19). Our results show that murine TNFR2−/− nTregs are deficient in suppressing autoimmunity and that, in contrast, murine TNFR2−/− iTregs are fully suppressive. These studies were performed using both spleen-derived CD4+CD25+ nTregs and thymus-derived FACS-sorted Foxp3+ nTregs (to eliminate the potential contamination with iTregs), and both approaches demonstrated a significant defect in TNFR2−/− nTreg function in vivo. A number of differences have been described for nTregs versus iTregs (21, 22, 32, 33), but our findings represent one of the few documentations of differences in vivo function. These results have a number of potentially significant implications regarding factors involved in vivo nTreg and iTreg activation. As a result, it should be noted that our studies address the functional relevance of Treg TNFR2 expression and not TNF-α effects directly. However, the other potential ligand for TNFR2, lymphotoxin-α homotrimer (LTα3), has been shown to signal primarily through TNFR1 during thymic development and inflammation in vivo (7, 8). Therefore, although it is unlikely that LTα3 is the primary factor relevant to our findings, we cannot rule out a possible role for LTα3 in the effects noted in this study.

In addition to identifying a novel difference in the in vivo function of murine natural versus induced Tregs, we have also demonstrated that preactivation in the presence of TGF-β can restore normal function in TNFR2−/− nTregs. We found this to be true using nTregs that were purified using either magnetic beads or FACS sorting, suggesting that the effects noted after preactivation in the presence of TGF-β are not a result of generating iTregs from a small contaminating population of Teffs. This apparent requirement for either TNF-α or TGF-β at the time of activation suggests that nTregs use TNF-α as a critical factor in their activation, whereas iTregs replace this requirement for TNF-α with TGF-β. These results are consistent with and expand on prior reports suggesting that nTregs initially require activation in tissue sites of inflammation, whereas iTregs are activated and generated in lymph nodes (34, 35). This paradigm, considered with our results, suggests that maximizing nTreg function involves interaction with the TNF-α in tissue sites of inflammation, whereas iTreg function is maximized by the TGF-β in lymph nodes that is required for their generation. Therefore, the functionally relevant activation of Tregs in vivo may depend on the availability of either TNF-α or TGF-β, and the requirement for TGF-β in iTreg generation may concomitantly lessen or remove the requirement for TNF-α to achieve maximal function of iTregs.

It has been reported previously that TNFR2−/− nTregs are able to suppress normally in vitro (14). In this study, we find no phenotypic differences between WT and TNFR2−/− nTregs that would account for the abnormal in vivo function of TNFR2−/− nTregs. The expression of activation markers and the homing receptors α4β7 and CCR6, as well as IL-10R, CTLA4, and OX40, are all expressed equally on WT and TNFR2−/− nTregs. In addition, we find no differences in TGF-β production, IL-10 production, or in vivo accumulation or stability between WT and TNFR2−/− nTregs. Given this phenotypic similarity and the fact that mechanisms of Treg suppression in vivo are still unclear, it is not yet possible to determine what abnormality in TNFR2−/− nTregs is corrected by activation in the presence of TGF-β. Nevertheless, our results suggest a previously unidentified role for TGF-β in the functional activation of Tregs.

TNFR2−/− mice do not develop a spontaneous autoimmune phenotype. This finding suggests that, under steady-state (i.e., noninflammatory) conditions, nTregs do not require TNF-α for the maintenance of immune homeostasis. Consistent with this concept is the recent report by Grinberg-Bleyer et al. (19) showing that TNF-α is necessary for optimizing, or “boosting,” Treg function in the context of autoimmunity, rather than being required for the baseline functioning of Tregs. Furthermore, TNFR2−/− mice demonstrate exacerbated disease when induced to develop experimental autoimmune encephalomyelitis, further suggesting that the anti-inflammatory effects of TNF-α are mediated through TNFR2 (36). Consistent with these findings, our results suggest that nTregs, but not iTregs, require TNF-α signaling through TNFR2 for optimal suppressive function under inflammatory conditions in vivo.

The use of anti–TNF-α therapy in treating human autoimmune diseases has been shown to be effective in some autoimmune diseases, but enigmatically can also exacerbate or even initiate some autoimmune conditions (1). Whereas the mechanisms underlying these dichotomous effects of anti–TNF-α therapy are unclear, our results suggest a new paradigm. First, anti–TNF-α therapy in human autoimmune diseases may differentially affect the function of nTregs versus iTregs. Second, and most interestingly, the effects of anti–TNF-α therapy in different human autoimmune diseases may depend on whether nTregs or iTregs have the predominant regulatory role in a given disease. Diseases in which iTregs are functionally predominant would demonstrate only the desired anti-inflammatory effects of anti–TNF-α therapy, with no concomitant deleterious effects on the iTregs. In contrast, diseases in which nTregs functionally predominate, in addition to having the anti-inflammatory effects of anti–TNF-α therapy, would also manifest a significant loss of Treg function, putatively counter-balancing or overcoming the anti-inflammatory effects. Thus, our findings may offer a novel mechanistic paradigm for understanding the puzzling dichotomous responses to anti–TNF-α therapy in different human autoimmune diseases.

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Disclosures

The authors have no financial conflicts of interest.