

TNF receptor 2 signaling prevents DNA methylation at the *Foxp3* promoter and prevents pathogenic conversion of regulatory T cells

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Regulatory T (Treg) cells expressing the transcription factor *Foxp3* play an important role in maintaining immune homeostasis. Chronic inflammation is associated with reduced *Foxp3* expression, function, and loss of phenotypic stability. Previous studies have established the importance of TNF receptor 2 (TNFR2) in the generation and/or activation of Treg cells. In this study, we assess the importance of TNFR2 in healthy mice and under inflammatory conditions. Our findings reveal that, in health, TNFR2 is important not only for the generation of Treg cells, but also for regulating their functional activity. We also show that TNFR2 maintains *Foxp3* expression in Treg cells by restricting DNA methylation at the *Foxp3* promoter. In inflammation, loss of TNFR2 results in increased severity and chronicity of experimental arthritis, reduced total numbers of Treg cells, reduced accumulation of Treg cells in inflamed joints, and loss of inhibitory activity. In addition, we demonstrate that, under inflammatory conditions, loss of TNFR2 causes Treg cells to adopt a proinflammatory Th17-like phenotype. It was concluded that TNFR2 signaling is required to enable Treg cells to promote resolution of inflammation and prevent them from undergoing dedifferentiation. Consequently, TNFR2-specific agonists or TNF1-specific antagonists may be useful in the treatment of autoimmune disease.

tumor necrosis factor receptor 2 | regulatory T cells | rheumatoid arthritis | *Foxp3* | DNA methylation

Regulatory T (Treg) cells are a subset of lymphocytes that play an indispensable role in maintaining self-tolerance in the periphery by regulating the activity of effector T (Teff) cells. The importance of Treg cells in homeostasis is underscored by the fact that loss-of-function mutations in the Treg cell signature transcription factor *Foxp3* result in catastrophic autoimmunity (1). In contrast, excessive Treg cell activity increases susceptibility to infection and is a hallmark of many cancers (2).

Treg cells receive cues from their local microenvironment that allow them to fine-tune their activity according to the degree of infectious or other risk. One factor in particular, TNF plays a key role in linking environmental cues to alterations in Treg cell activity, having either positive or negative effects on Treg cell activity (3, 4). One explanation for these differential effects is that TNF- α signals via 2 receptors, TNFR1 and TNFR2. TNFR1 contains an intracellular death domain and can activate either apoptotic or inflammatory pathways, whereas TNFR2 binds TNF receptor-associated factors and can activate the canonical and noncanonical NF- κ B pathway to control cell survival and proliferation (5). Inflammatory responses are mediated by TNFR1, whereas there is evidence of a role for TNFR2 in tissue regeneration and in the generation and activity of Treg cells. TNF- α interaction with TNFR2 was shown to promote Treg cell expansion and function in mice, and TNFR2 expression marks the maximally suppressive subset of Treg cells (4, 6, 7).

Following successful clinical trials of infliximab in rheumatoid arthritis (RA), TNF- α inhibitors have been shown to be effective

in controlling a number of diseases, including inflammatory bowel disease, ankylosing spondylitis, and psoriasis (8). However, there is increasing interest in the possibility of refining this approach through the use of selective TNFR1 antagonists (9) or TNFR2 agonists (10). In this study, we have performed a comprehensive analysis of the distinctive roles played by TNFR1 and TNFR2 in autoimmune arthritis with a particular emphasis on Treg cells. Our findings reveal that, under noninflammatory conditions, TNFR2 is important, not for generating Treg cells, but for maintaining them in a functionally active state. At a mechanistic level, we demonstrate that TNFR2 is critical for maintaining robust *Foxp3* expression by preventing aberrant methylation of CpG motifs at the *Foxp3* promoter and subsequent *Foxp3* gene silencing. Under inflammatory conditions, TNFR2-dependent signaling takes on greater significance by regulating numbers of Treg cells, particularly, at the site of disease activity as well as their functional activity and the intensity of the inflammatory response. Finally, we demonstrate that coculture of TNFR2-deficient Treg cells with Teff cells leads to up-regulation of Treg associated IL-17 production, suggesting that TNFR2 signaling is required to maintain Treg cells in an immunoregulatory (homeostatic) phenotype.

Significance

TNF signals via 2 receptors, TNFR1 and TNFR2. Anti-TNF biologics, which block signaling via both receptors, are now being used to treat millions of patients worldwide for immune-mediated inflammatory diseases. This study demonstrates that TNFR2 plays a major role in limiting the severity and duration of arthritis in animal models and that TNFR2 is important for maintaining the functional activity and phenotypic stability of Treg cells, which are major cellular mediators of immune homeostasis. Treg cells express the functionally important transcription factor *Foxp3*, and we show that TNFR2 is required for preventing DNA methylation at the *Foxp3* promoter thereby maintaining its transcriptional activity. This suggests that specific blockade of TNFR1 would be advantageous.

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Results

Absence of TNFR2 Does Not Affect Numbers of Treg Cells but Reduces Foxp3 Expression and Functional Activity. To assess the influence of TNFR1 and TNFR2 on numbers of Treg cells under resting (naive) conditions, the proportion of Foxp3⁺ Tregs was determined in wild-type (WT), TNFR1^{-/-}, and TNFR2^{-/-} mice. This work was approved by the University of Oxford Clinical Medicine Animal Welfare and Ethical Review Body and by the UK Home Office. No significant differences were observed between the numbers of CD4⁺CD25⁺Foxp3⁺ cells in the spleen, lymph node (LN), or thymus of the 3 strains (Fig. 1A). Similarly, the percentage of CD4⁺Foxp3⁺Helios⁺ Treg cells (a phenotype normally associated with thymus-derived Tregs) and CD4⁺Foxp3⁺Helios⁻ Treg cells (associated with induced Treg cells) were comparable among the 3 strains of naive mice in spleens, LNs, and thymuses (SI Appendix, Fig. S1A).

However, despite the similar levels of numbers of Foxp3⁺ T cells in WT, TNFR1^{-/-}, and TNFR2^{-/-} mice, there was a significant reduction of the level of Foxp3 mRNA expression in Treg cells of spleens and thymuses from TNFR2^{-/-} mice compared with WT mice (Fig. 1B). Similarly, a comparison of the level of Foxp3 protein expression among the 3 strains revealed that the median fluorescence intensity (MFI) and histograms of Foxp3 in CD4⁺CD25⁺ cells was significantly decreased in the spleens and thymuses of TNFR2^{-/-} mice compared to WT and TNFR1^{-/-} mice (Fig. 1B). The level of TGFβ1 mRNA expression was also reduced in Treg cells from TNFR2^{-/-} mice while proteins associated with Treg cells, cytotoxic T lymphocyte-associated protein 4 (CTLA-4), and glucocorticoid-induced TNFR, were unchanged (SI Appendix, Fig. S1B–D).

The reduced Foxp3 expression in TNFR2-deficient Treg cells led us to question whether their suppressor function was similarly compromised. We, therefore, used an assay in which TNFR1- or TNFR2-deficient or TNFR-sufficient Treg cells were cocultured with TNFR-sufficient Teff cells and APCs. Suppressor function was significantly decreased in TNFR2-deficient Treg cells and increased in TNFR1-deficient Treg cells (Fig. 1C). This confirms that TNFR2 is required for functionally active Treg cells and suggests that TNFR1 signaling compromises Treg cell activity.

TNFR2 Signaling Maintains Demethylation at the Foxp3 Promoter. To understand the mechanism of decreased expression of Foxp3 in TNFR2^{-/-} mice, we evaluated DNA methylation of Foxp3 cis-regulatory elements, and the methylation status was determined of the upstream enhancer, proximal promoter, and Treg-specific demethylated region (TSDR) of Treg cells from WT, TNFR1^{-/-}, and TNFR2^{-/-} mice. As Foxp3 is located on the X chromosome, male mice were used to avoid mosaicism in female mice. Treg cells were isolated from pooled spleen, and LN cells were isolated from WT, TNFR1^{-/-}, and TNFR2^{-/-} male mice by FACS. Genomic DNA was extracted from Tregs, followed by bisulfite conversion, purification, PCR amplification, and cloning. The degree of methylation of each regulatory element was determined by bisulfite sequencing. Substantially increased levels of CpG methylation were seen in the Foxp3 proximal promoter of TNFR2^{-/-} mice, compared with WT and TNFR1^{-/-} mice (Fig. 1D). In contrast, the methylation status of the upstream enhancer and TSDR did not differ significantly among the 3 strains.

TNFR2 Deficiency Results in Greater Severity and Chronicity of Arthritis with Reduced Numbers of Treg Cells. The effect of TNFR2 deficiency was assessed in collagen-induced arthritis (CIA), a widely used model of RA. WT, TNFR1^{-/-}, and TNFR2^{-/-} mice on a C57/BL6N.Q (H-2^q) background, were immunized with bovine type II collagen emulsified in complete Freund's adjuvant (CFA). As expected, TNFR1^{-/-} mice showed minimal disease activity throughout the 80-d observation period. WT and

TNFR2^{-/-} mice showed similar clinical scores in early arthritis (Fig. 2A). However, from day 75–80 after immunization, TNFR2^{-/-} mice had significantly higher clinical scores compared to WT mice. Histologically, while the paws of TNFR1^{-/-} mice were largely protected from arthritis, the joints of mice from TNFR2^{-/-} mice showed significantly increased inflammatory cell infiltration and bone erosion compared to WT mice (Fig. 2B).

Analysis of spleen and LNs of arthritic WT, TNFR1^{-/-}, and TNFR2^{-/-} mice revealed a significant decrease in CD4⁺Foxp3⁺ Treg cells in TNFR2^{-/-} mice, compared to WT mice. In contrast, there was a significant increase in CD4⁺Foxp3⁺ Treg cells in TNFR1^{-/-} mice (Fig. 2C). In addition, a significant decrease in Foxp3 expression (based on MFI) was observed in Treg cells from TNFR2^{-/-} mice with arthritis compared with WT mice (Fig. 2D). Phenotypic comparison of the Treg cell population in WT, TNFR1^{-/-}, and TNFR2^{-/-} mice revealed significant decreases in the percentages of Helios⁺ cells as well as cells positive for the checkpoint inhibitors PD-1 in spleens and LNs of TNFR2^{-/-} mice (Fig. 2E). Conversely, plasma levels of TNF-α were significantly increased (Fig. 2F), and analysis of gene expression revealed increased expression of a number of proinflammatory genes, including IL-6 and CD68 in the joints of arthritic TNFR2^{-/-} mice (Fig. 2G).

We then addressed the question of whether TNFR2 affects the chronicity of arthritis in antigen-induced arthritis (AIA), a model which normally resolves spontaneously by around day 7 post intra-articular injection. AIA was induced in TNFR1^{-/-}, TNFR2^{-/-}, and WT mice, and weight-bearing capacity was assessed beyond day 7 post intra-articular injection. As predicted, TNFR1^{-/-} mice were protected from inflammation, but notably TNFR2^{-/-} mice displayed no difference in weight bearing compared to WT mice up to day 3 post intra-articular injection (SI Appendix, Fig. S2A). However, analysis of the affected knees by qPCR did reveal a reduction in gene expression of Foxp3 in TNFR2^{-/-} mBSA injected knees at day 3 post intra-articular injection, indicative of an alteration in Treg phenotype. Proinflammatory genes including IL-6 and CXCL1 were unaffected (SI Appendix, Fig. S2B). This became evident at the functional level when AIA duration was extended beyond day 7 post intra-articular injection. The weight-bearing capacity of the injected knee in TNFR2^{-/-} mice was strikingly lower than that of WT mice on day 7, 10, and 14 post intra-articular injection (Fig. 3A). The increased severity of arthritis in TNFR2^{-/-} mice at this time was confirmed by histological examination of knee joints and micro-CT analysis of the epiphyseal plate on day 8 post intra-articular injection (Fig. 3B–D).

A comparison of the cellular composition of arthritic versus control knees on day 8 post intra-articular injection where the biggest differences in capacity for weight bearing were observed, revealed an accumulation of Treg cells in the arthritic joints of WT mice which failed to occur in TNFR2^{-/-} mice (Fig. 3E). TNFR2 deficiency also resulted in reduced numbers of CD4⁺Foxp3⁺ and CD4⁺IL-10⁺ cells in joints of TNFR2^{-/-} mice compared to WT mice (Fig. 3F and SI Appendix, Fig. S3) despite comparable numbers of total CD4⁺ cells.

We also compared the functional activity of Treg cells from arthritic TNFR2^{-/-} and WT mice in a suppression assay in which we titrated numbers of Treg cells against fixed numbers of Teff cells and APC to obtain different Treg:Teff ratios. The readouts for the assay were production of IFNγ, IL-17A, IL-10, and IL-2. WT Treg cells were able to suppress the production of IFNγ in a dose-dependent manner, and this ability was severely compromised with TNFR2-deficient Treg cells (Fig. 4A). Conversely, a dose-dependent increase in IL-10 production was observed following addition of WT Treg cells which was significantly decreased with TNFR2-deficient Treg cells. No effects of TNFR2 deletion were observed on IL-2 production. Remarkably, a dramatic increase in IL-17A production was observed when TNFR2-deficient Treg cells, but not WT Treg

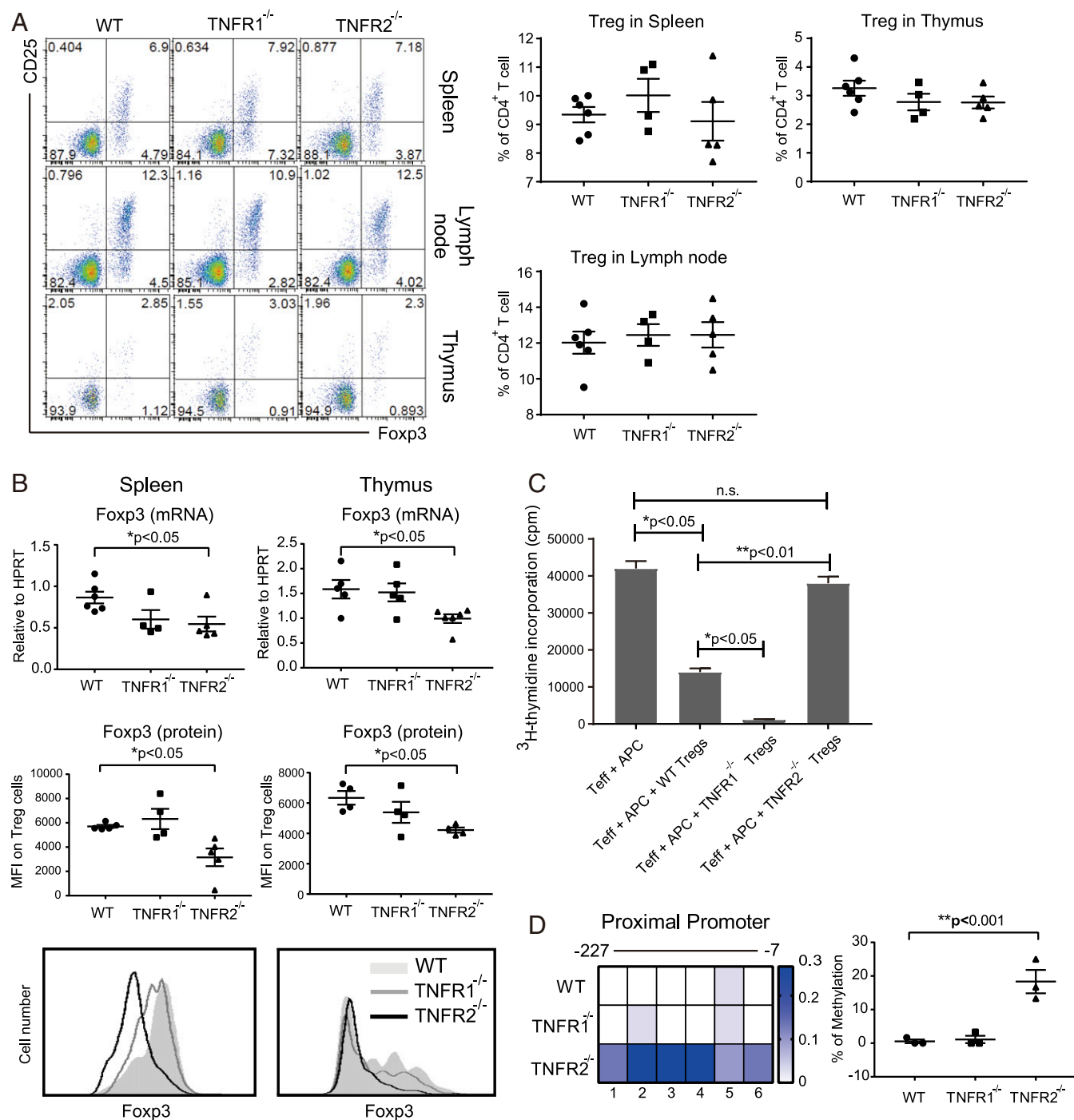


Fig. 1. TNFR2 is important for Foxp3 expression and functional activity of Treg cells. (A) The expression of CD25 and Foxp3 were analyzed by FACS, gating on CD4⁺ cells ($n = 4-6$). (B) RNA was isolated from spleen and thymuses, and expression of *Foxp3* was normalized to *Hprt* and calibrated relative to WT. Expression of Foxp3 protein in Treg cells was determined by FACS and expressed as MFI. Representative scatterplots are shown below. Values are the mean \pm SEM. * $P < 0.05$ for knockout versus WT by one-way ANOVA with Tukey's multiple comparison test ($n = 4-6$). (C) CD4⁺CD25⁺ Treg cells were isolated from spleens of TNFR1^{-/-}, TNFR2^{-/-}, and WT mice. The remaining CD4⁺ cells were treated with mitomycin C and used as antigen-presenting cells (APCs). Treg cells were cocultured with Teff cells and APCs (ratio 1:1:1) from WT mice in the presence of the anti-CD3 antibody and IL-2. (D) Genomic DNA was isolated from the FACS-sorted Treg cells, modified with sodium bisulfite, and the proximal promoter (-7 to -227) was then amplified using nested PCR, cloned into the pCR4-TOPO vector, and individual clones were sequenced. The methylation pattern of 6 CpG sites of the proximal promoter was presented by the percentage of methylation from dark blue (30%) to white (0%), which was determined by calculation of 30 clones (10 clones for each experiment). Total methylation degree of proximal promoter was calculated by 3 experiments. Values are the mean \pm SEM. ** $P < 0.01$ for knockout versus WT by 1-way ANOVA with Tukey's multiple comparison test.

cells, were cocultured with Teff cells at ratios of 1:1 or 1:2. The defects in suppressor function in TNFR2-deficient Treg cells were accompanied by phenotypic changes in the cells, including significantly reduced expression of latency-associated peptide

(LAP) and inducible costimulator (ICOS), 2 markers associated with functionally active Treg cells (Fig. 4B).

In order to establish whether TNFR2 signaling prevents Treg cells themselves from adopting a Th17-like phenotype, Treg cells

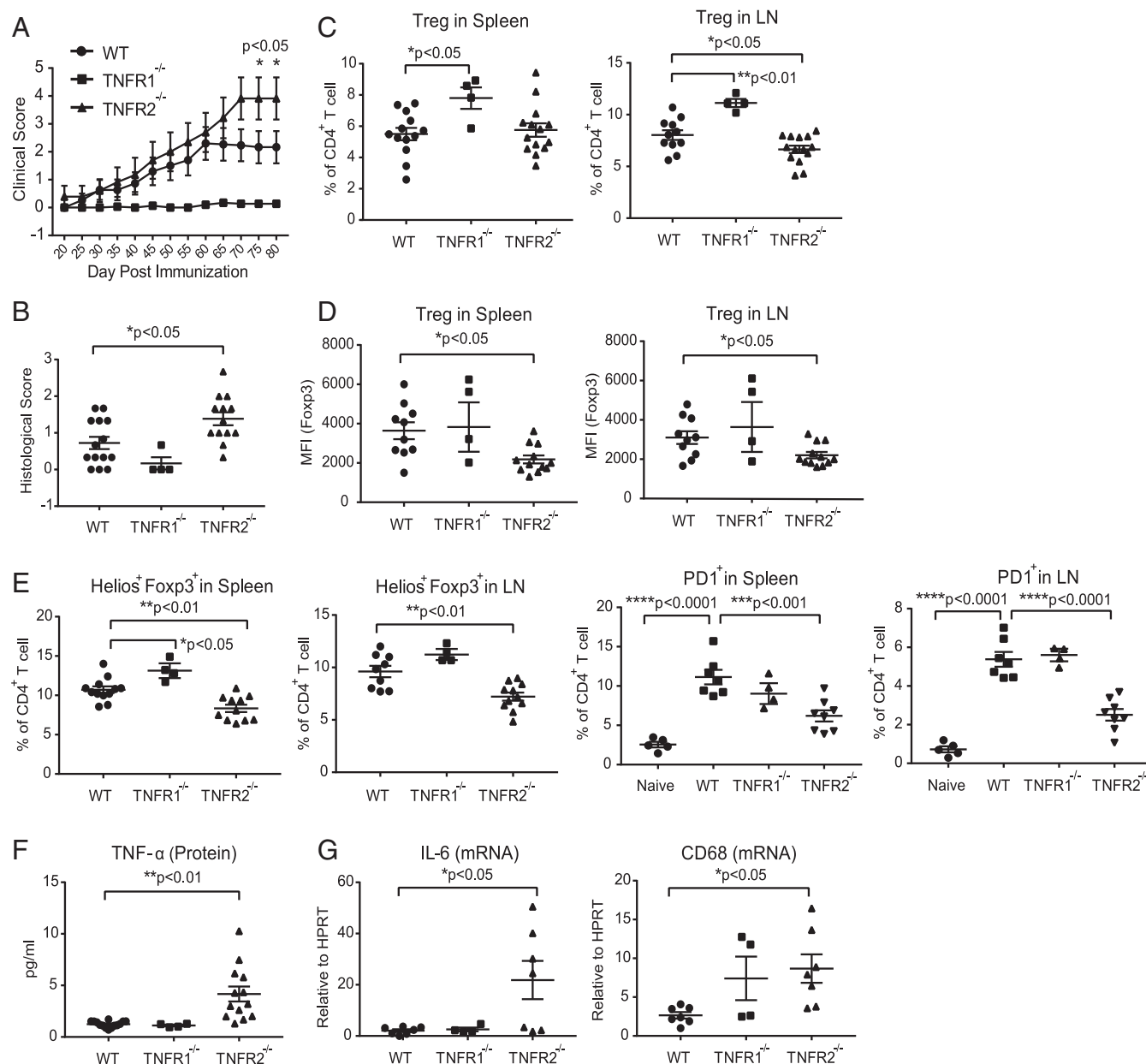


Fig. 2. TNFR2 is required for resolution of CIA and generation of Treg cells. (A) Clinical scores of WT (n = 30), TNFR1^{-/-} (n = 29), and TNFR2^{-/-} (n = 23) mice with CIA. (B) Histologic scores on day 10 of arthritis. (C) Percentages of CD4⁺ cells expressing Foxp3 and Helios or PD1. (D) MFI of Foxp3 expression in CD4⁺CD25⁺Foxp3⁺ cells of arthritic mice. (E) Percentages of CD4⁺ cells expressing Foxp3 and Helios or PD1. (F) Serum levels of TNF-α. (G) RNA was isolated from LNs harvested from arthritic mice on day 10 of arthritis and reverse transcribed to cDNA for RT-qPCR analysis using 384 well microfluidic card Taqman gene expression assays. Data were normalized to HPRT expression and were calibrated relative to WT mice (G). *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 for knockout versus WT. Groups are compared by 1-way analysis of variance with Dunnett's multiple comparison test.

were isolated from draining LNs of WT and TNFR2^{-/-} mice that had been previously immunized with type II collagen in CFA. There was a highly significant increase in the expression of RORγt at the gene and protein levels in Treg cells from TNFR2^{-/-} mice compared to WT mice as well as a significant increase in IL-17⁺, Foxp3⁺ double positive T cells (Fig. 5A–C). It was concluded that TNFR2 was responsible for preventing Treg cells from adopting a Th17-like phenotype under inflammatory conditions.

Discussion

Comparison of Treg cell numbers in healthy TNFR1^{-/-}, TNFR2^{-/-}, and WT mice failed to show any significant differences which was unexpected as it had previously been shown

that the interaction of TNFα with TNFR2 promotes the expansion of Treg cells under both resting and activated conditions (11). It was observed, however, that there was a significant reduction of the level of Foxp3 expression at the gene and protein levels in Treg cells from TNFR2^{-/-} mice compared with WT mice. Based on the reduced Foxp3 expression in TNFR2-deficient Treg cells, we hypothesized that the suppressor function of these cells would be compromised. Using an assay in which WT or knockout Treg cells were cocultured with WT Teff cells and APC plus anti-CD3 mAb, we confirmed that the suppressive activity was significantly decreased in TNFR2-deficient Treg cells and increased in TNFR1-deficient Treg cells. This supports previous findings that TNFR1 and TNFR2 play opposing roles in controlling Treg cell activity

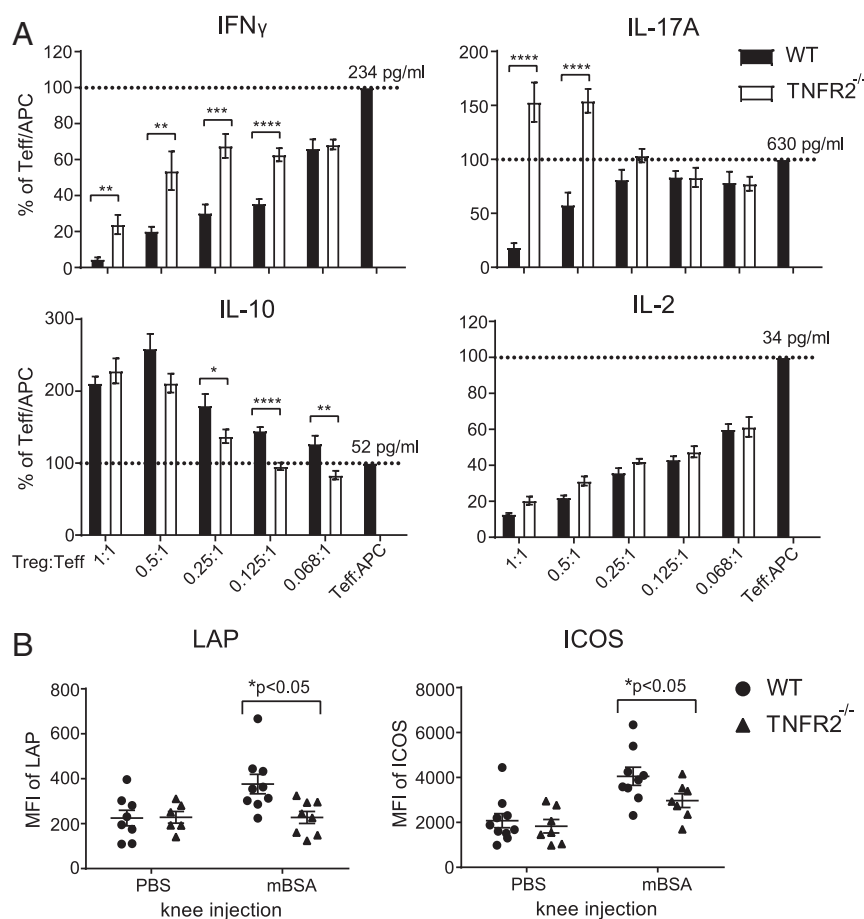


Fig. 4. TNFR2 is critical for Treg cell function in AIA. (A) Treg cells from spleens of WT and TNFR2^{-/-} mice of mice with AIA (day 8; $n = 9$) were isolated, and suppressive capacity was determined using specified ratios of 1:1 WT Teff and WT APC. Data were expressed as percentages of cytokine production by Teff and APC in the absence of Treg cells. (B) CD4⁺ and Foxp3⁺ Treg cells or bulk CD4⁺ T cells from knees of day 8 mice with AIA ($n = 8-9$) were analyzed for expression of a panel of function-associated proteins by FACS. Values in A and B are the mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ for versus WT by unpaired t test.

model, TNFR1^{-/-} mice showed minimal disease activity during the early phase of the disease and WT and TNFR2^{-/-} mice exhibited similar levels of disease activity. However, in the later stages of the disease, TNFR2^{-/-} mice had significantly more severe arthritis than WT mice with an increased level of joint damage. TNFR2^{-/-} mice also had reduced numbers of Treg cells which expressed reduced levels of Foxp3 and PD-1, and there was increased expression of proinflammatory cytokines, including TNF- α and IL-6. One caveat, however, is that we used global TNFR2^{-/-} mice, therefore, we cannot attribute the effects on arthritis solely on altered activity of Treg cells. Nevertheless, these results are consistent with the recently reported finding that intrinsic TNFR2 signaling in Treg cells is protective in experimental autoimmune encephalomyelitis (14).

Based on the above findings, it was hypothesized that TNFR2 deficiency would affect the chronicity of arthritis, and, to address this, we used the self-remitting AIA model. Arthritis was, indeed, found to be more chronic in TNFR2^{-/-} mice, a finding that was confirmed by micro-CT analysis and by the weight-bearing capacity of the affected knee. It was concluded that the absence of TNFR2 signaling resulted in a more sustained inflammatory response in this model and perhaps led to impaired capacity for resolution of local inflammation. Of further interest was the deficit in Treg cells in the arthritic joints of TNFR2^{-/-} mice despite similar numbers of total CD4⁺ T cells, indicating a specific failure in recruitment of Treg cells in the absence of TNFR2 signaling. The functional

activity of Treg cells from immunized TNFR2^{-/-} mice was also diminished, but it was of particular interest that coculture of TNFR2-deficient Treg cells with Teff cells led to a pronounced increase in IL-17A production, suggesting pathological conversion of Treg cells to Th17-like cells. TNFR2^{-/-} mice were, subsequently, found to have higher expression of ROR γ t in Treg cells and greater numbers of IL-17⁺ and Foxp3⁺ double positive T cells.

From these findings, it was concluded that TNFR2 deficiency not only impairs Treg suppressive function, but also leads to increased production of IL-17. Miller et al. (15) similarly reported increased IL-17 production in TNFR2-knockout T cells which could be abrogated by the addition of exogenous IL-2. These findings are consistent with a recent report that TNFR2 is important for maintaining the stability of the Treg cell phenotype in experimental colitis (16). Currently approved TNF- α inhibitors block soluble and membrane TNF- α and inhibit the activity of both TNFR1 and TNFR2. The findings from this study clearly suggest that a TNFR1-specific antagonist would inhibit the proinflammatory activity of TNF- α , while sparing the regulatory functions of TNFR2 (9). An alternative approach may be the use of a selective agonist of TNFR2 (17), alone or in combination with a TNF- α inhibitor.

To summarize, this study confirms the importance of TNFR2 signaling in the generation of robust Treg cell responses. The findings also demonstrate the physiological importance of the TNFR2 pathway in the context of joint inflammation. Importantly, we show that TNFR2 is involved in epigenetic modulation of the

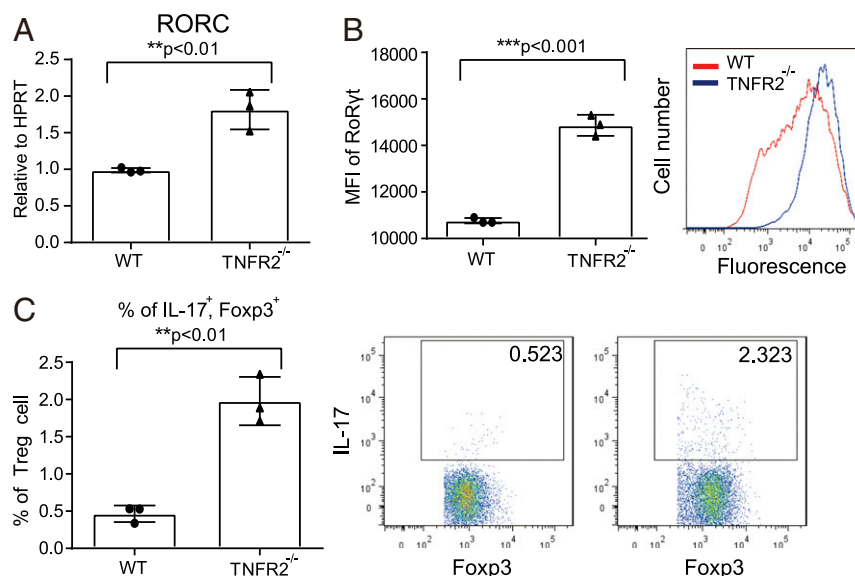


Fig. 5. TNFR2 prevents Treg cells from adopting a Th17-like phenotype under inflammatory conditions. WT and TNFR2^{-/-} mice were immunized with bovine collagen II ($n = 3$). After 10 d, Treg cells from spleens and LNs from WT and TNFR2^{-/-} mice were isolated. (A) RNA was isolated from Treg cells of WT and TNFR2^{-/-} mice. Gene expression of RORC was normalized to HPRT expression and calibrated relative to WT. (B) The expression of RORγt on Treg cells from WT and TNFR2^{-/-} mice was determined by FACS. (C) The percentage of CD4⁺, IL-17⁺, and Foxp3⁺ cells was analyzed by FACS. Values in a–c are the mean \pm SEM * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for knockout versus WT by unpaired t test.

Foxp3 gene by maintaining CpG demethylation. Activation of TNFR2 signaling has recently been shown to promote the generation and functional stability of human Treg cells (18, 19), and the therapeutic implications of these findings have not escaped us. While anti-TNF- α therapy has been successful in RA, Crohn's disease, ulcerative colitis, ankylosing spondylitis, psoriasis, psoriatic arthritis, and others, it is not a cure. Selective blockade of TNFR1, sparing TNFR2 and, hence, Treg cell function, might offer more durable therapeutic benefits.

Materials and Methods

Full details of materials and methods, including mice and arthritis models, flow cytometry, the Treg cell suppression assay, real time qPCR, DNA methylation analysis, cytokine measurement, and statistical analysis, are provided in the [SI Appendix](#).

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