

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

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Significance Statement

TNF signals via two receptors, TNFR1 and TNFR2. Anti-TNF biologics, which block signalling 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 regulatory T cells, which are major cellular mediators of immune homeostasis. Regulatory T 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.

Abstract

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 for the generation of Treg cells, but 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 pro-inflammatory, Th17-like phenotype. It was concluded that TNFR2 signalling 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.

Introduction

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 two 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 non-canonical 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 non-inflammatory conditions, TNFR2 is important, not for generating Treg cells, but for maintaining them in a functionally active state. At a mechanistic level, we demonstrate for the first time 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 signalling 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 co-culture of TNFR2-deficient Treg cells with Teff cells leads to up-regulation of Treg associated IL-17 production, suggesting that TNFR2 signalling is required to maintain Treg cells in an immunoregulatory (homeostatic) phenotype.

Results

1. 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 (naïve) conditions, the proportion of Foxp3⁺ Tregs was determined in wild-type, TNFR1^{-/-}, TNFR2^{-/-} mice. No significant differences were observed between the numbers of CD4⁺CD25⁺Foxp3⁺ cells in the spleen, lymph node or thymus of the three 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 between the three strains of naïve mice in spleens, lymph nodes and thymuses (*SI Appendix, Fig. S1a*).

However, despite the similar levels of numbers of Foxp3⁺ T cell in wild-type, TNFR1^{-/-}, 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 wild-type mice (Fig. 1b). Similarly, a comparison of the level of Foxp3 protein expression between the three 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 wild-type and TNFR1^{-/-} mice (Fig. 1b). The level of TGFβ1 mRNA expression was also reduced in Treg cells from TNFR2^{-/-} mice whilst proteins associated with Treg cells, CTLA-4 and GITR, 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 co-cultured with TNFR-sufficient Teff cells and APC. 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 signalling compromises Treg cell activity.

2. TNFR2 signalling 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, the methylation status was determined of the upstream enhancer, proximal promoter and TSDR of Treg cells from wild-

type, 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 from wild-type, 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 wild-type and TNFR1^{-/-} mice (Fig. 1d). In contrast, the methylation status of the upstream enhancer and TSDR did not differ significantly between the three strains.

3. TNFR2 deficiency results in greater severity and chronicity of arthritis, with reduced numbers of Treg cells

The effect of TNFR2 deficiency was assessed in CIA, a widely used model of RA. Wild-type, 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-day observation period. wild-type and TNFR2^{-/-} mice showed similar clinical scores in early arthritis (Fig. 2a). However, from day 75 to 80 after immunization, TNFR2^{-/-} mice had significantly higher clinical scores compared to wild-type 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 wild-type mice (Fig. 2b).

Analysis of spleen and lymph nodes of arthritic wild-type, TNFR1^{-/-} and TNFR2^{-/-} mice revealed a significant decrease in CD4⁺Foxp3⁺ Treg cells in TNFR2^{-/-} mice, compared to wild-type 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 wild-type mice (Fig. 2d). Phenotypic comparison of the Treg cell population in wild-type, 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 lymph nodes 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 pro-inflammatory 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 wild-type 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. Pro-inflammatory 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 wild-type 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 wild-type 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 wild-type 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 IFN γ , IL-17A, IL-10 and IL-2. Wild-type Treg cells were able to suppress the production of IFN γ 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 wild-type 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 wild-type Treg cells, were co-cultured 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 LAP and ICOS, two markers associated with functionally active Treg cells (Fig. 4b).

In order to establish whether TNFR2 signalling prevents Treg cells themselves from adopting a Th17-like phenotype, Treg cells were isolated from draining lymph nodes of wild-type and TNFR2^{-/-} mice that had been previously immunised with type II collagen in CFA. There was a highly significant increase in the expression of ROR γ t at the gene and protein level in Treg cells from TNFR2^{-/-} mice compared to wild-type mice as well as a significant increase in IL-17⁺, Foxp3⁺ double positive T cells (Fig. 5a-c). It was concluded that TNFR2 is 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 wild-type mice failed to show any significant differences which was unexpected as it had previously been shown that the interaction of TNF α 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 level, in Treg cells from TNFR2^{-/-} mice compared with wild-type mice. Based on the reduced Foxp3 expression in TNFR2-deficient Treg cells, we hypothesised that the suppressor function of these cells would be compromised. Using an assay in which wild-type or knockout Treg cells were co-cultured with wild-type 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 and that TNFR2 is required for functionally active Treg cells. These findings are also consistent with previous reports by ourselves and others showing an association between TNFR2 expression and correlated with suppressive function of Treg cells (9, 12). However, further studies are required to delineate the precise mechanisms by which TNFR2 regulates Treg cell function.

Previous studies have demonstrated an association between Treg cell functional activity and demethylation of CpG motifs at the *Foxp3* locus. There are several differentially methylated *cis*-regulatory elements that have been identified in the *Foxp3* locus. These are present upstream of the transcriptional start site (upstream enhancer and proximal promoter) and between non-coding exons (intronic enhancer; also known as the Treg specific demethylated region, TSDR). The methylation status of these elements negatively correlates with *Foxp3* gene expression and plays an indispensable role in controlling the differentiation and stabilization of Treg cells (4, 13). We therefore addressed the question of whether there was differential DNA methylation at the FoxP3 promoter in TNFR1/2-deficient *versus* TNFR-sufficient Treg cells. The results showed clearly increased levels of CpG methylation at the *Foxp3* proximal promoter of TNFR2^{-/-} mice, compared with wild-type and TNFR1^{-/-} mice whereas no differences were observed in the upstream enhancer and TSDR. These data are the first to show an association between TNFR2 signaling and maintenance of CpG demethylation at the proximal promoter at the *Foxp3* locus and provide a clear explanation for the importance of TNFR2 in maintaining optimal Foxp3 expression. The mechanism by

which TNFR2 regulates DNA methylation is unclear but may involve alterations in the activity of DNA methyltransferases and/or Ten-eleven translocation methylcytosine dioxygenases which cause demethylation of 5-methylcytosine. This question will be addressed in future studies.

Given the importance of TNFR2 in retaining the identity of Treg cells, we next assessed the impact of TNFR1 or TNFR2 deficiency on immune system driven inflammation. In the CIA model, TNFR1^{-/-} mice showed minimal disease activity during the early phase of the disease wild-type 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 wild-type 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 pro-inflammatory 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 signalling 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 signalling 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 signalling. The functional activity of Treg cells from immunised TNFR2^{-/-} mice was also diminished but it was of particular interest that co-culture 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⁺, 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* similarly reported increased IL-17 production in TNFR2-knockout T cells which could be abrogated by the addition of exogenous IL-2 (15). 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 pro-inflammatory activity of TNF- α , whilst 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.

In summary, this study confirms the importance of TNFR2 signaling in the generation of robust Treg cell responses. The findings also demonstrate the physiological importance of TNFR2 pathway in the context of joint inflammation. Importantly, we show for the first time that TNFR2 is involved in epigenetic modulation of the 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 SI Appendix.

Acknowledgments

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The authors declare no competing financial interests.

Author contributions: WYT, YSH, FILC, DPP, KM, JO and FM performed experiments and analyzed data. SFL and ROW helped in design, analysis and interpretation of the data. WYT, YSH, SFL, FM, MF and ROW helped in drafting the manuscript.

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Figure legends

Figure 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 wild-type. 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⁻ Teff cells and CD4⁺CD25⁺ Treg cells were isolated from spleens of TNFR1^{-/-}, TNFR2^{-/-} and wild-type mice. The remaining CD4⁻ cells were treated with mitomycin C and used as APCs. Treg cells were co-cultured with Teff cells and APCs (ratio 1:1:1) from wild-type mice in the presence of 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 three experiments. Values are the mean \pm SEM. **P<0.01 for knockout versus WT, by one-way ANOVA with Tukey's multiple comparison test.

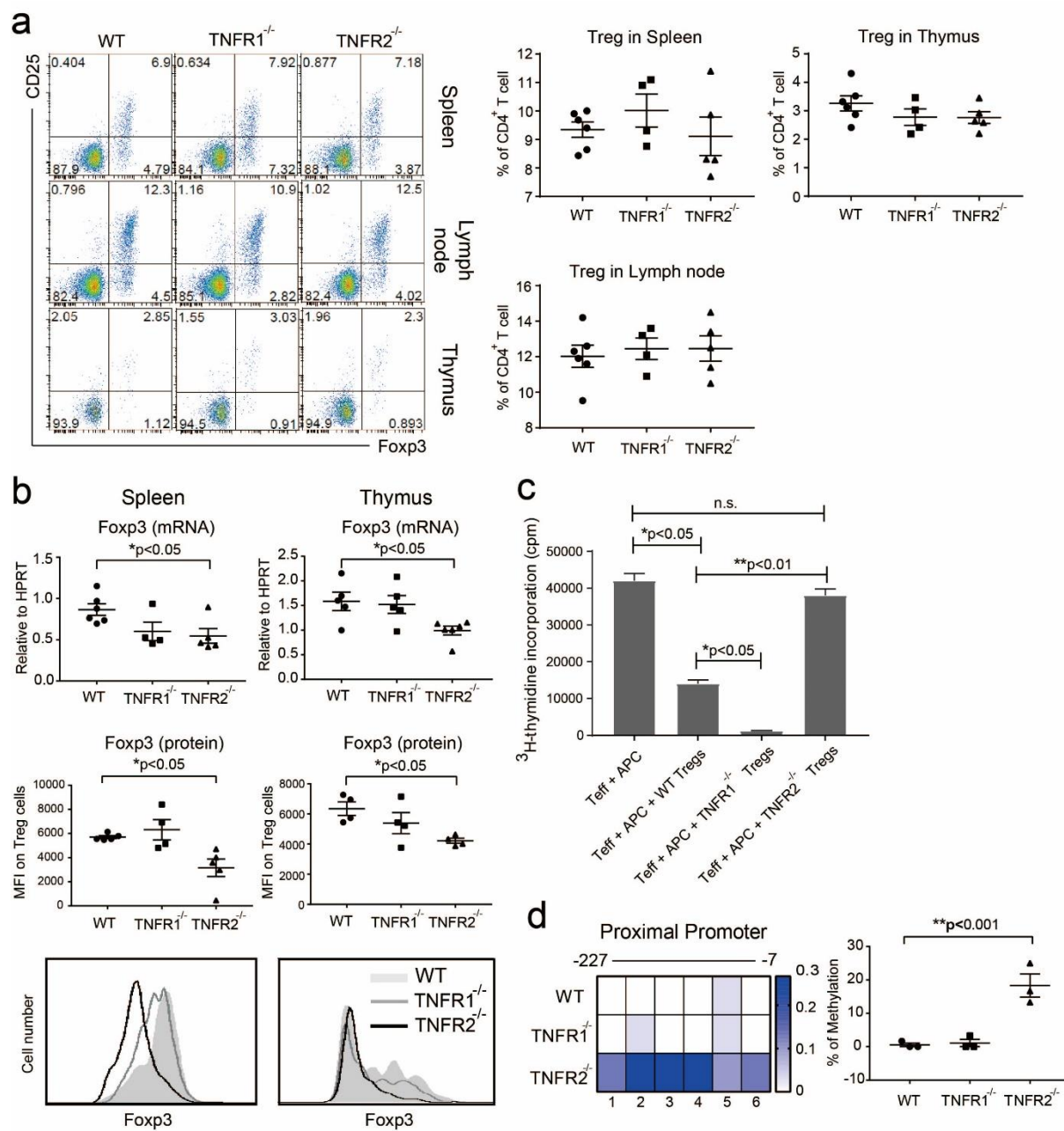
Figure 2. TNFR2 is required for resolution of CIA and generation of Treg cells. (a)

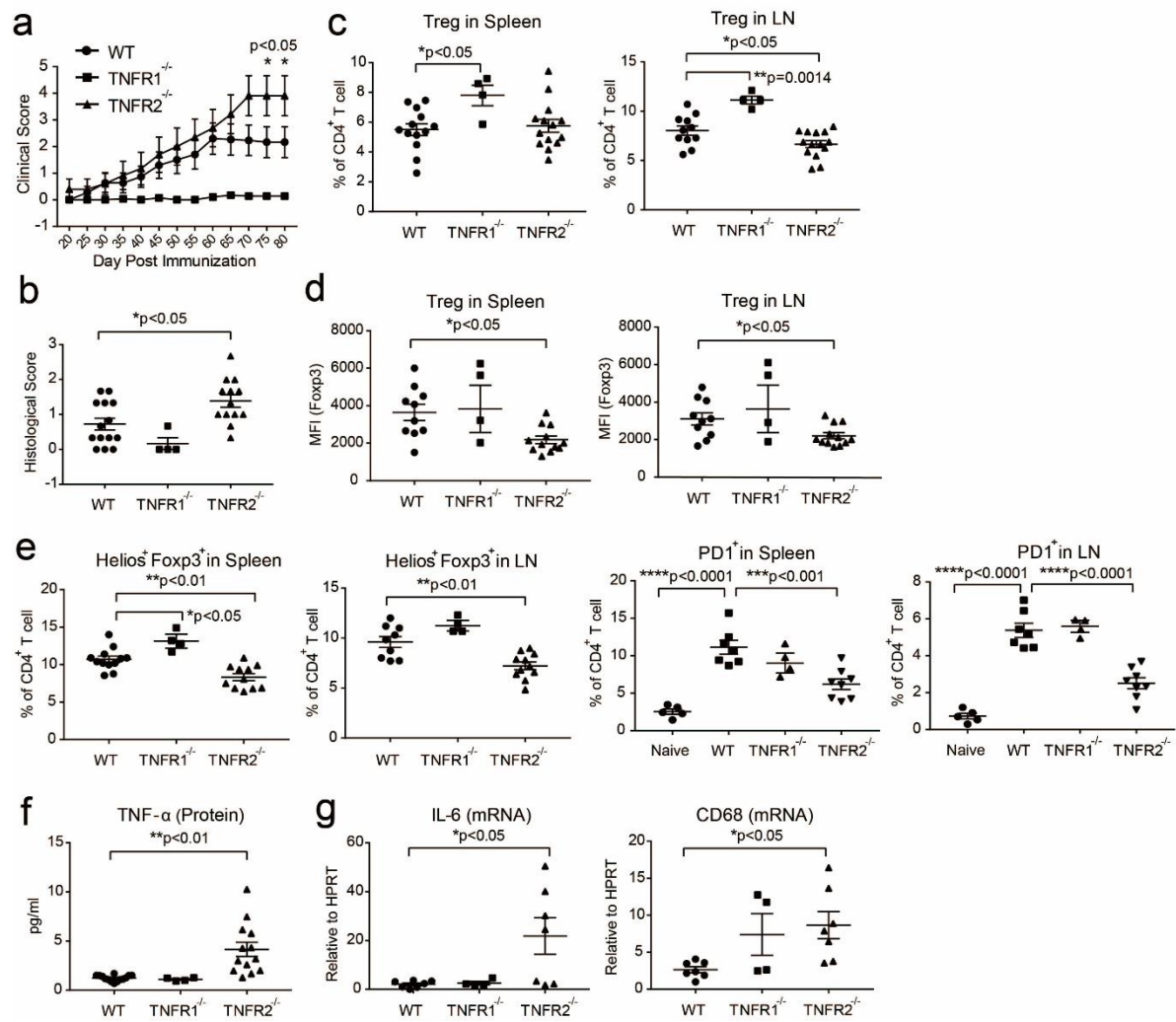
Clinical scores of WT (n=30), TNFR1^{-/-} (n=29) and TNFR2^{-/-} (n=23) mice with CIA. (b) Histologic scores on day 10 of arthritis. (c) Percentages of CD4⁺ cells expressing Foxp3 and CD25. (d) MFI of Foxp3 expression in CD4⁺CD25⁺Foxp3⁺ cells of arthritic mice. (e) Percentages of CD4⁺ cells expressing Foxp3 and Helios or PD1. (f) Serum levels of TNF- α . (g) RNA was isolated from lymph nodes 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 wild-type mice (g). *P<0.05; **P<0.01 ***P<0.001 ****P<0.0001 for knockout versus WT. Groups are compared by one-way analysis of variance with Dunnett's multiple comparison test.

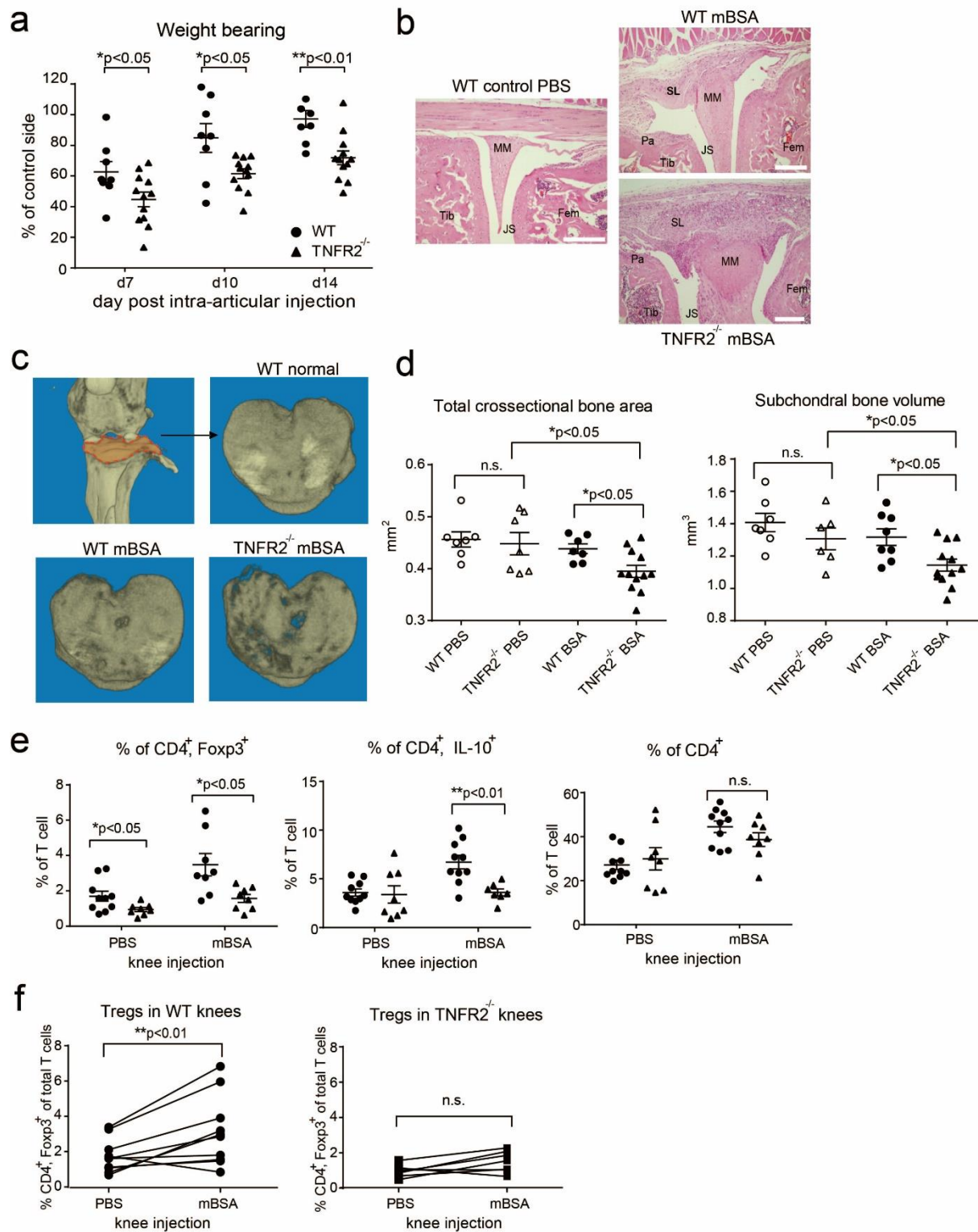
Figure 3. TNFR2 is important for resolution of AIA. Arthritis was induced in TNFR2^{-/-} and wild-type mice (n=6-12). **(a)** Weight bearing capacity was assessed from day 7 to day 14 post intra-articular injection. **(b, c, d)** In a subsequent experiment, histological examination of knee joints and micro-CT analysis of the epiphyseal plate of arthritic knees were carried out on day 8 post intra-articular injection. **(e, f)** On day 8, the knees were harvested and cellular composition was assessed by FACS. Proportions of T cell subsets are shown. Values are the mean \pm SEM. n.s.= not significant, *P<0.05, **P<0.01 for knockout versus WT, by unpaired t-test.

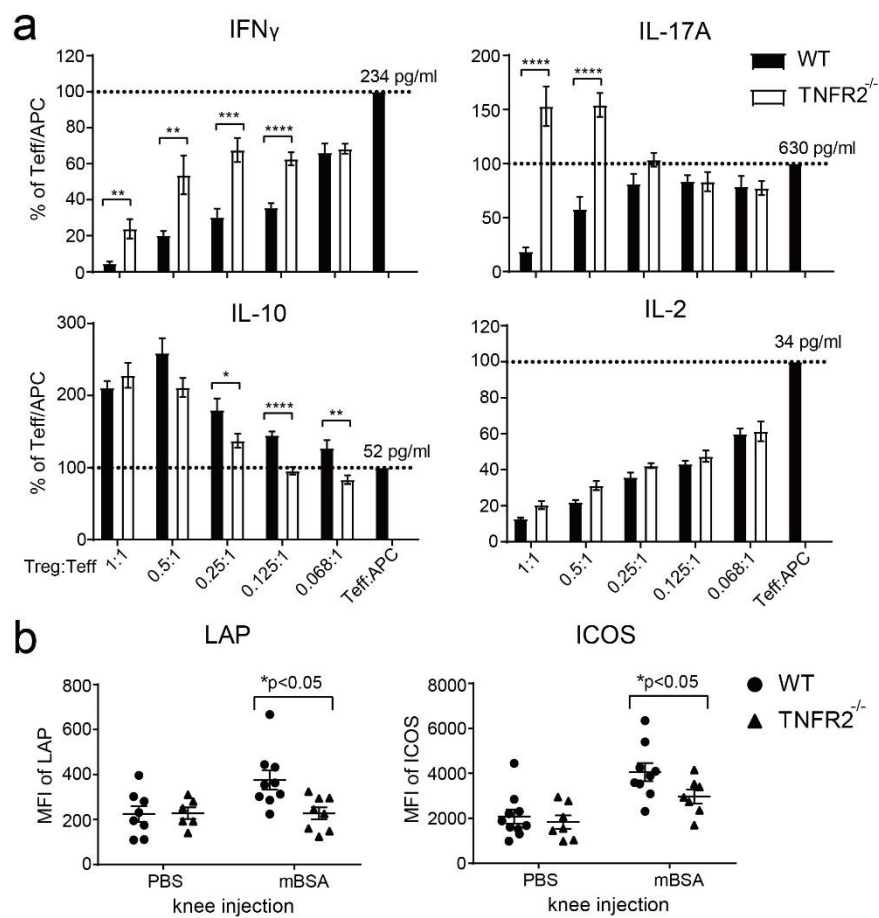
Figure 4. TNFR2 is critical for Treg cell function in AIA. **(a)** Treg cells from spleens of wild-type and TNFR2^{-/-} mice of mice with AIA (day 8; n=9) were isolated and suppressive capacity was determined using specified ratios of 1:1 wild-type Teff and wild-type APC. Data was expressed as % of cytokine 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 analysed 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, ****P<0.0001 for versus WT, by unpaired t-test.

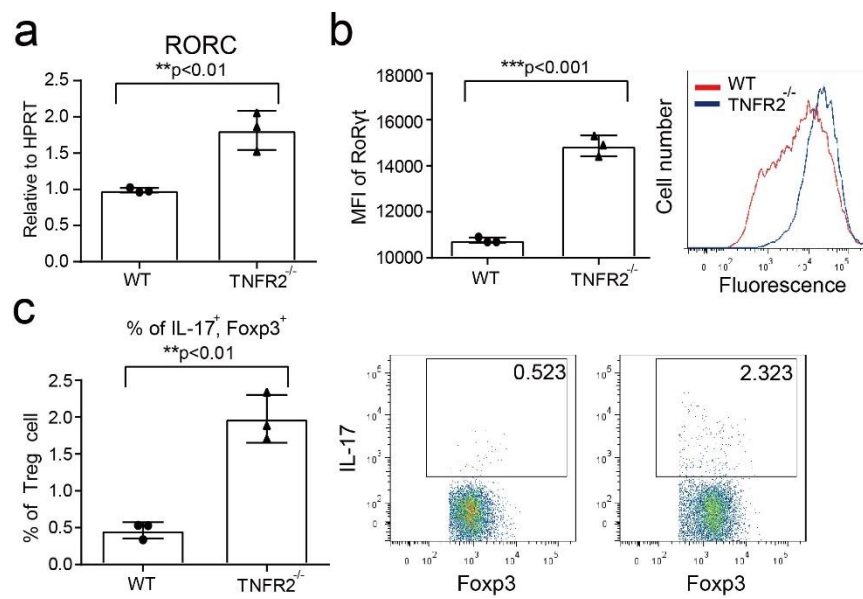
Figure 5. TNFR2 prevents Treg cells from adopting a Th17-like phenotype under inflammatory conditions. Wild-type and TNFR2^{-/-} mice were immunised with bovine collagen II (n=3). After 10 days, Treg cells from spleens and lymph nodes from WT & TNFR2^{-/-} mice were isolated. **(a)** RNA was isolated from Treg cells of WT and TNFR2^{-/-} mice. Gene expression of RORC was normalized to HPRT expression and calibrated relative to wild-type. **(b)** The expression of RoR γ t on Treg cells from wild-type and TNFR2^{-/-} mice was determined by FACS. **(c)** The percentage of CD4⁺, IL-17⁺ and Foxp3⁺ cells was analysed by FACS. Values in a, b and c are the mean \pm SEM. *P<0.05, **P<0.01, ***P<0.001 for knockout versus WT, by unpaired t-test.











Supplementary Information

Supplementary materials and methods

Mice. Adult male and female, wild type, TNFR1^{-/-}, and TNFR2^{-/-} mice on a C57BL/6N.Q (H-2^d) background were bred in-house. DBA/1 mice were purchased from Envigo (Huntingdon, UK). Mice were housed in ventilated cages, maintained at a 21°C ± 2°C and a 12-hour light/12-hour dark cycle, with food and water available *ad libitum*. All experimental procedures were approved by the Ethical Review Process Committee and the UK Home Office, in accordance with the 1986 Animals (Scientific Procedures) Act.

Animal models of arthritis. CIA was induced by immunization of collagen for induction of arthritis was carried out as previously described (1). In brief, a solution of bovine type II collagen (4 mg/ml) was emulsified with an equal volume of complete Freund's adjuvant (CFA; BD Biosciences, Oxfordshire, UK) on ice. C57BL/6 N.Q or DBA/1 mice were anaesthetized by using isoflurane and immunized by subcutaneous injection of 100 µl emulsion at two sites (one at the base of the tail and one on the flank). For the arthritis assessment of CIA, the clinical severity of arthritis was scored in each paw as follows: 0=normal, 1=slight swelling and/or erythema, 2=marked swelling, 3=ankylosis. Hindpaw thickness was measured using micro-calipers (Kroeplin, Schluchlem, Germany).

AIA was induced as previously described (2). C57BL/6 mice (10-12 weeks of age) were sedated using inhaled isoflurane anaesthesia and subsequently immunized with 100 µg of mBSA (Sigma-Aldrich) emulsified in 100 µL of complete Freund's adjuvant (BD Biosciences), administered subcutaneously at the base of the tail. At day 7, arthritis was induced by means of an intra-articular injection of mBSA (200 µg in 10 µL of sterile PBS) using a sterile 33-gauge micro-cannula into the knee joint. PBS was injected into the contralateral (control) knee. At defined time points post-immunisation the mice were culled and the knee joints, popliteal lymph nodes and spleens were excised. Throughout the experiment, knee joint swelling was measured with micro-calipers.

Linton incapacitance test for weight-bearing. Changes in weight distribution were measured blinded to the experimental groups with a Linton weight incapacitance tester (MJS Technology, Hertfordshire, UK), as described previously (1). Briefly, a single mouse was placed in the device and allowed to settle before a reading was taken. Each measurement was derived from the mean of 4 independent readings from a single mouse. Data are presented as weight bearing on antigen injected side as a percentage of the PBS injected side.

Micro-computed tomography (micro-CT). Knees from AIA mice were harvested at specified time points after intra-articular injection and fixed in 10% neutral buffered formalin prior to histological analysis, described above. Whole knees were imaged using a Skyscan 1174 (Bruker, Belgium)

compact micro CT scanner, at 50 kV, 800 μ A, 20.9 μ m isometric voxel resolution and 0.7 degree rotation step. The scans were reconstructed using NRecon software from Skyscan (3).

Histological assessment. For CIA, paws were fixed in 10% neutral buffered formalin followed by decalcification with 5.5% EDTA in buffered formalin. Fixed paws were embedded in paraffin, and sections were cut and stained with hematoxylin and eosin. Histopathologic changes in the joints were scored on a scale of 0–3, where 0=normal, 1=minimal synovitis without cartilage/bone erosion, 2=synovitis with some marginal erosion but joint architecture maintained and 3=severe synovitis and erosion with loss of normal joint architecture. For AIA, knees were removed and fixed in 10% neutral buffered formalin, then decalcified in 10% EDTA for 21 days before paraffin wax embedding. Coronal tissue sections (4 μ m) at seven depths throughout the joint, 80 μ m apart were stained with H&E or safranin-O to assess joint pathology. Histopathological changes were assessed using an adaptation of the parameters described (2). Inflammation, defined as the influx of inflammatory cells into the joint cavity was graded with an arbitrary scale from 0 (no inflammation) to 3 (severe inflammation). The medial capsule, including the diameter of the synovial membrane, but excluding overlying skeletal muscle layers and skin, was measured by drawing a line perpendicular to the medial meniscus, and the length calculated digitally with ImageJ software. All analyses were performed by a blinded investigator.

Single cell isolation. Spleens, inguinal lymph nodes and thymuses were harvested and disassociated by pushing through a Falcon 70 μ m strainer (BD Biosciences) atop a 50 ml Falcon tube (BD Biosciences). The spleen cells were incubated in 1 ml of red blood cell lysing buffer (Life Technologies, Paisley, U.K.) for 1 min, then diluted in 20 ml incomplete RPMI medium and centrifuged at 1500 rpm for 5 min. The cell pellets of spleen, lymph nodes or thymus were resuspended by complete RPMI media with 10% heat inactivated fetal bovine serum (FBS, Life technologies), 1% Penicillin/Streptomycin (Lonza, Basel, Switzerland) and 0.1 μ l β -Mercaptoethanol (Life technologies), and then counted with a hemocytometer. The cell suspension was diluted to 2 or 4 \times 10⁶ cells/ml for further analysis.

Flow cytometry and antibodies. Skin and surrounding muscle tissue was removed before knees were excised and trimmed, then placed in cold PBS. Knee joints were then cut into approximately 4 pieces and cells were liberated by enzymatic digestion in complete RPMI media containing 0.4 mg/ml Liberase TL and 1 mg/ml DNaseI (both Roche, Welwyn Garden City, UK) for 1.5 hr at 37°C. Digested tissues were to obtain the single cells as described above. The single cells of spleen, inguinal lymph node, thymus and knee joint were labelled with fluoro-chrome conjugated antibodies against cell surface markers and Zombie Fixable Viability dye. After washing by using FACS buffer (1X PBS contains 0.01% EDTA and 1% FBS), the cells were fixed and permeabilised using a mixture fixation/permeabilization solution (eBioscience, Hatfield, UK). After fixation, the cells were washed

and stained for intracellular markers. The labelled cells were washed in permeabilization buffer and resuspended in cell fix buffer (BD Biosciences) before running on the flow cytometer (BD Canto II or Fortessa X20). To determine cytokine expression, the single cells of spleen/lymph node were stimulated with 20 ng/ml of phorbol myristate acetate (PMA, Calbiochem, Darmstadt, Germany) and 2 μ M ionomycin (Calbiochem) in the presence of 12.5 μ g/ml Brefeldin A (Sigma-Aldrich, St. Louis, MO). After 3 hr, cells were then washed and stained with surface markers and intracellular cytokines as above.

Anti-mouse antibodies used were as follows: Fluorescence conjugated antibodies against CD3 (Alexa Fluor 700), CD4 (BV605 or PeCy7), Foxp3 (eFluor 450) were from ThermoFisher Scientific (Abingdon, UK). Anti-CD3 antibody and fluorescence conjugated antibodies against CD4 (PeCy7) and Foxp3 (PeCy7) were obtained from eBioscience. Zombie NIR™ Fixable Viability Kit and fluorescence conjugated antibodies against CD4 (BV510 or PerCP-Cy5.5), IL-17A (PE or APC), IFN γ (Alexa Fluor 647 or BV711), CD25 (PerCP-Cy5.5 or BV605), CD45 (PerCp-Cy5.5 or FITC), LAP (APC), ICOS (PerCP-Cy5.5), IL-10 (PE) and Helios (PB) were obtained from BioLegend (London, UK). Fluorescence conjugated antibodies against CTLA-4 (PE) was purchased from BD Biosciences.

Treg suppression assay. CD4⁺CD25⁻ Teff cells and CD4⁺CD25⁺ Treg cells were isolated from the spleens of TNFR1^{-/-}, TNFR2^{-/-} and wild-type littermate control mice with or without AIA by using the Regulatory T Cell Isolation Kit (Miltenyi Biotec, Bisley, UK) according to the manufacturer's instructions. Typically, 85-95% of the CD4⁺CD25⁺ cells expressed Foxp3, confirming Treg cell status. The remaining CD4⁺ cells were treated with mitomycin C (20 μ g/ml, Sigma-Aldrich) for 30 min and used as APCs. Graded numbers of Treg cells were co-cultured with 5×10^4 Teff cells and mitomycin C-pretreated APCs of wild-type mice in the presence of anti-CD3 antibody (1 μ g/ml) and IL-2 (10 ng/ml) (PeproTech, London, UK) for 72 hr. The ratio of Treg:Teff:APC ranged from 1:1:1 to 0.068:1:1. Culture supernatants were removed for analysis by cytokine bead array (BD Biosciences).

Real-Time Quantitative Reverse Transcription PCR. Surrounding skin and muscle were stripped from AIA knees before trimming to include only the knee joint before snap freezing in liquid nitrogen. Whole frozen knees were then pulverized on dry ice to a coarse powder before further homogenisation using tissue grinders in Trizol Reagent (Life Technologies). Cell samples were lysed using buffer RLT (Qiagen, Crawley, UK) with 1 in 100 dilution of 2-mercaptoethanol (Sigma-Aldrich). RNA was purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions and stored at -80°C until conversion to cDNA. For each cDNA reaction, RNA (25 ng/ μ l) was mixed with an equal volume of 2X Master Mix, containing 10X RT buffer, 25X dNTP mix, 10X random primers, reverse transcriptase (High Capacity cDNA Reverse Transcription Kit; Life Technologies) and nuclease-free water (Qiagen). The RNA was converted to cDNA under the cycling

condition: 25°C 10 min, 37°C 120 min, 85°C 5 min and 4°C ∞. cDNA was stored at -20°C until use in qPCR reaction.

For each 6 µl RT-qPCR reaction, 2.4 µl of template cDNA was placed into wells of MicroAmp® Optical 384-Well Reaction Plate with Barcode (Life Technologies) with 3 µl Takyon MatserMix (Eurogentee, Southampton Hampshire, UK), 0.3 µl TaqMan primer and 0.3 µl Nuclease-free water. Non-template controls were included on each plate. The plate was sealed with MicroAmp Optical Adhesive Film (Life Technologies) and loaded into the Applied Biosystems ViiATM 7 Real-Time PCR System. The target DNA was amplified under the cycling condition: 95°C 10 min (95°C 15 sec, 60°C 1min) × 45 cycles. Data were normalized to *Hprt* expression and calibrated relative to a control sample. The relative expression ratio was calculated using the "Delta Delta CT" ($\Delta\Delta CT$) method.

DNA methylation analysis. Treg cells were isolated by FACS (BD FACSAria™ III) using fluorochrome-conjugated anti-CD4 and anti-CD25 antibodies as mentioned above and then lysed by using buffer RLT plus (Qiagen) with 1% 2-ME for further genomic DNA and RNA extraction, according to the manufacturer instructions (ALL prep® DNA/RNA Mini Kit; Qiagen). Cytidine present in genomic DNA was converted to uracil using the EpiTect Fast Bisulfite Conversion Kit (Qiagen), according to the manufacturer instructions. The DNA fragments of the upstream enhancer, proximal promoter and Treg-specific demethylated region (TSDR) at the *Foxp3* locus were amplified using the bisulfite DNA as template and following primers listed in the Table 1, purified using the QIA quick PCR Purification kit (Qiagen) according to the manufacturer's instructions, and ligated into pCR4-TOPO vectors using the TOPO cloning kit (Invitrogen). Bisulfite genomic sequencing analysis was performed by Eurofins (Germany). The methylation rate was determined using the BiQ Analyzer software.

Cytokine analysis. Ten days after the onset of CIA, mice were sacrificed and bled by cardiac puncture. Blood was collected in 1.5 ml Eppendorf tubes containing heparin, and following centrifugation, plasma was collected and stored at -80°C prior to until cytokine analysis. The levels of cytokines were determined by Meso-Scale Discovery cytokine multiplex array.

Statistical Analysis. Data were analyzed by unpaired t-test, one-way ANOVA with Tukey's or Dunnett's multiple comparison test and two-way ANOVA with Tukey's multiple comparison test as appropriate. All calculations were made using GraphPad Prism 7 software. A P-value less than 0.05 was considered significant.

Supplementary figures and tables

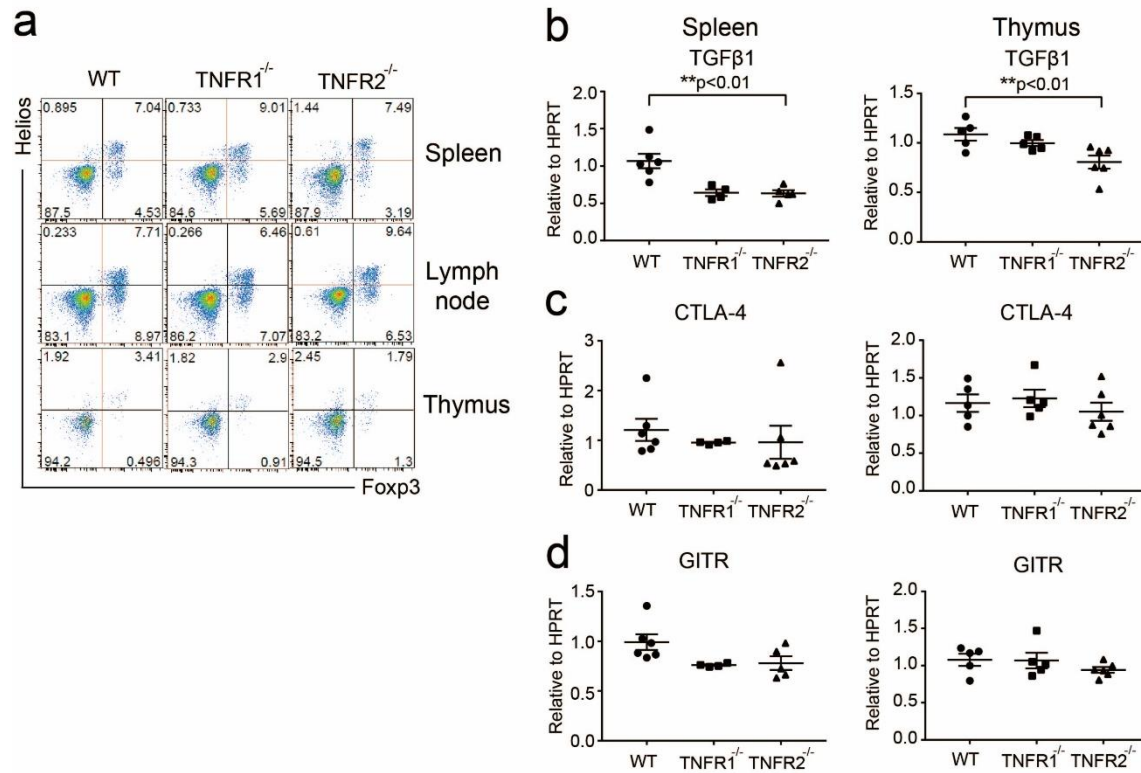


Figure S1. Absence of TNFR2 reduces Treg cell expression of TGFβ1 but not CTLA-4 and GITR. (a) The expression of helios and Foxp3 were analyzed by FACS, gating on CD4⁺ cells (n=4-6). The gene expression of TGFβ1 (b), CTLA-4 (c) and GITR (d) was normalized to HPRT and calibrated relative to wild-type

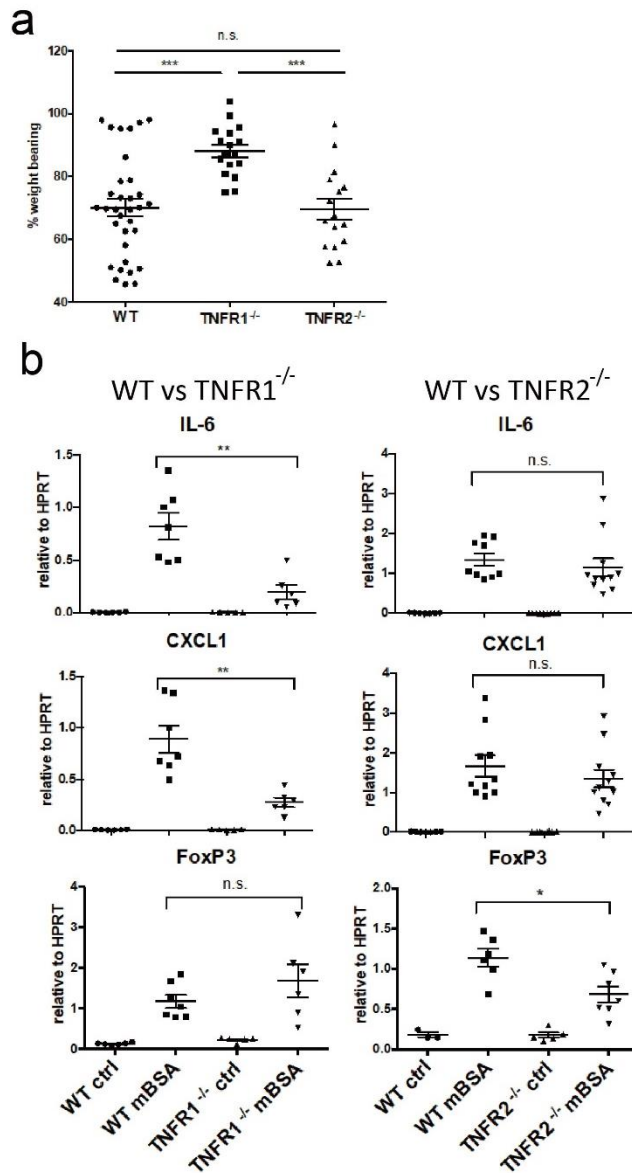
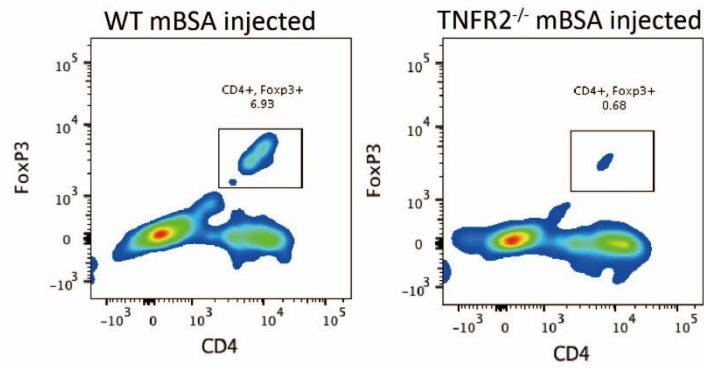


Figure S2. TNFR1 but not TNFR2 mediates inflammation and joint pathology in acute phase antigen induced arthritis. Arthritis was induced in TNFR1^{-/-}, TNFR2^{-/-} and wild-type mice (n=16-33). **(a)** Weight bearing capacity was assessed at day 2 post intra-articular injection. **(b)** In parallel, day 2 AIA knees were processed for IL-6, CXCL1 and Foxp3 gene expression. Values in a and b are the mean \pm SEM. n.s.= not significant, *P<0.05, **P<0.01, ***P<0.001 for knockout versus WT, by unpaired t-test.

a % of CD4⁺, Foxp3⁺



b % of CD4⁺, IL-10⁺

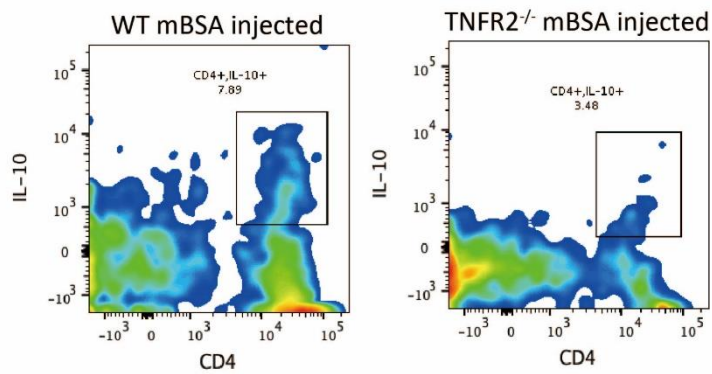


Figure S3. Two-parameter density plots showing reduced numbers of CD4⁺Foxp3⁺ and CD4⁺IL-10⁺ cells in joints of TNFR2^{-/-} mice with AIA. (a, b) On day 8 after intra-articular injection, mice were culled and the cellular composition of knee joints was assessed by FACS.

Primer	Sequence(5'-3')
UpEnhnc_F	AATGTGGGTATTAGGTAAAATTTTT
UpEnhnc_R	AAACCCTAAAACTACCTCTAAC
Promoter OF	TTTTGTGATTTGATTTATTTTTTTTT
Promoter OR	ATACTAATAAACTCCTAACACCCACC
Promoter IF	TATATTTTTAGATGATTTGTAAAGGGTAAA
Promoter IR	ATCAACCTAACTTATAAAAACTACCACAT
TSDR OF	TATTTTTTTGGGTTTTGGGATATTA
TSDR OR	AACCAACCAACTTCCTACACTATCTAT
TSDR IF	TTTGGGTTTTTTTTGGTATTTAAGA
TSDR IR	TTAACCAAATTTTCTACCATTAAC

Table S1. List of primers used in the methylation of Foxp3 study

UpEnhnc_F and _R: Forward and reverse primers for upper enhancer.

Promoter OF and OR, IF and IR: Outer forward and reverse primers for first amplification of promoter, inner forward and reverse primers for second amplification of promoter.

TSDR OF and OR, IF and IR: Outer forward and reverse primers for first amplification of TSDR, inner forward and reverse primers for second amplification of TSDR.

References

1. J. J. Inglis *et al.*, Regulation of pain sensitivity in experimental osteoarthritis by the endogenous peripheral opioid system. *Arthritis Rheum.* **58**, 3110-3119 (2008).
2. K. Midwood *et al.*, Tenascin-C is an endogenous activator of Toll-like receptor 4 that is essential for maintaining inflammation in arthritic joint disease. *Nat Med* **15**, 774-780 (2009).
3. Y. Vattakuzhi, S. M. Abraham, A. Freidin, A. R. Clark, N. J. Horwood, Dual-specificity phosphatase 1-null mice exhibit spontaneous osteolytic disease and enhanced inflammatory osteolysis in experimental arthritis. *Arthritis Rheum* **64**, 2201-2210 (2012).