Intrinsic TNFR2 signaling in T regulatory cells provides protection in CNS autoimmunity

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**Abstract**

TNF is a multifunctional cytokine involved in autoimmune disease pathogenesis that exerts its effects through two distinct TNF receptors, TNFR1 and TNFR2. While TNF- and TNFR1-deficient (but not TNFR2-deficient) mice show very similar phenotypes, the significance of TNFR2 signaling in health and disease remains incompletely understood. Recent studies implicated the importance of the TNF/TNFFR2 axis in T regulatory (Treg) cell functions. To definitively ascertain the significance of TNFR2 signaling, we generated and validated doubly humanized TNF/TNFR2 mice, with the option of conditional inactivation of TNFR2. These mice carry a functional human TNF-TNFR2 (hTNF-hTNFR2) signaling module and provide a useful tool for comparative evaluation of TNF-directed biologics. Conditional inactivation of TNFR2 in FoxP3+ cells in doubly humanized TNF/TNFFR2 mice down-regulated the expression of Treg signature molecules (such as FoxP3, CD25, CTLA-4, and GITR) and diminished Treg suppressive function in vitro. Consequently, Treg-restricted TNFR2 deficiency led to significant exacerbation of experimental autoimmune encephalomyelitis (EAE), accompanied by reduced capacity to control Th17-mediated immune responses. Our findings expose the intrinsic and beneficial effects of TNFR2 signaling in Treg cells that could translate into protective functions in vivo, including treatment of autoimmunity.

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**Significance**

In spite of TNF involvement in the pathogenesis of multiple sclerosis (MS), systemic TNF neutralization in MS patients was not successful. One of the possible reasons is that TNF possesses both pathogenic and protective features that may be related to TNFR1 versus TNFR2 receptor engagement. This study uncovers one of such protective functions of TNF mediated by intrinsic TNFR2 signaling in Treg cells. In mice bearing humanized TNF and TNFR2 genetic loci, TNFR2 ablation restricted to Treg cells led to reduced capacity to control Th17 cell responses, exacerbated experimental autoimmune encephalomyelitis (EAE) development, and affected the maintenance of Treg cells. These findings provide support for the emerging role of TNFR2 signaling in autoimmunity, as demonstrated here in mice with conditional inactivation of TNFR2.

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T cell is a cytokine with multiple functions in immune regulation, host defense, lymphoid tissue organogenesis, and control of inflammation. Dysregulation of TNF has been observed in numerous autoimmune diseases, including multiple sclerosis (MS). Strikingly, although anti-TNF therapy has shown remarkable efficacy in patients with rheumatoid arthritis (RA) (1) or inflammatory bowel disease (IBD) (2), this therapeutic approach was not successful in MS treatment (Lenercept study) (3). One possible reason is that TNF can acquire both pathogenic and protective features due to engagement with two distinct receptors, TNFR1 (CD120a or p55/p60) and TNFR2 (CD120b or p75/p80), respectively. TNFR1 is a death domain-containing receptor that can activate both cell death and expression of proinflammatory genes while TNFR2 is mostly linked to the NF-κB–mediated proliferative pathway (4). Many features in TNFR1-deficient mice mirror the major defects observed in TNF-deficient mice (5–7), suggesting that TNF signaling in vivo may be predominantly mediated by TNFR1, and not by TNFR2. However, recent studies revealed the involvement of TNFR2 in the maintenance and functions of various cell types, such as neurons, oligodendrocytes, myeloid-derived suppressors, and Treg cells (8–11). Moreover, loss-of-function studies in mice demonstrated that TNFR2 and the membrane-bound form of TNF, the preferred ligand for TNFR2, may have a protective role in experimental autoimmune encephalomyelitis (EAE), a mouse model of MS (12–14). In contrast, the soluble form of TNF through TNFR1 can trigger disease progression and demyelination (15, 16).

Emerging evidence indicates that TNFR2 is highly expressed on CD4+FoxP3+ Treg cells that are crucial for maintaining immune homeostasis and suppression of autoimmunity (17). Furthermore, TNFR2 is essential for the maintenance of FoxP3 expression by Treg cells and for their immunosuppressive functions in the colitis model (18). Nevertheless, the significance of intrinsic TNFR2 signaling in Treg cells in vivo remains incompletely defined due to the lack of mouse models with conditional TNFR2 ablation. In humans, TNFR2 acts on CD4+ T cells as the master control switch for Th17 expansion or Th17 contraction; therefore, TNFR2 agonists and antagonists may have therapeutic potential (19, 20). However, one of the limitations in the development of drugs targeting TNF/TNFFR2 signaling is the lack of preclinical animal models since many human TNF-directed biologics do not interact with murine TNF or TNFα receptors.
TNFR2. For this purpose, we developed and validated a doubly humanized TNF/TNF2 mouse line with the option of conditional Cre-mediated deletion of exons 2 to 6 of the TNFR2 gene. Such a mouse model allowed us to directly assess the consequences of $T_{reg}$-restricted TNFR2 ablation at steady state and during EAE. Furthermore, TNF/TNF2 doubly humanized mice have potential use for evaluation of agonistic and antagonistic antibodies against human TNF (hTNF) or human TNFR2 (hTNFR2) in various models of autoimmunity.

Results

Generation and Evaluation of Mice with Functional Signaling of Human TNF via both TNFR1 and TNFR2. To evaluate effects of clinically utilized and novel hTNF inhibitors in vivo, we previously generated a knock-in mouse (hTNFKI), in which the mouse Tnf gene was replaced by its human counterpart (21–23). Earlier biochemical studies suggested that human TNF can bind and engage murine TNFR1, but not TNFR2 (24). Therefore, in the current study, we aimed to generate mice with the additional humanization of the extracellular portion of TNFR2 to ensure functional TNF signaling through both receptors in vivo. In line with this, we generated a hTNFR2KI mouse (see SI Appendix, Fig. S1 for targeting strategy and technical details) and crossed these mice to hTNFKI mice to produce doubly humanized homozygous hTNFKI × hTNFR2KI mice (SI Appendix, Fig. S2). To directly address the role of TNFR2 signaling in distinct cell types, including $T_{reg}$ cells, we additionally inserted two LoxP sites within the human TNFR2 locus to allow for conditional Cre-mediated ablation of the extracellular portion of TNFR2.

Since TNFR2 signaling has been implicated in the proliferation of $T_{reg}$ cells (9), we first performed a comparative analysis of these cells in hTNFKI and hTNFKI × hTNFR2KI mice. We expected that hTNFKI mice might show defects in $T_{reg}$ cell maintenance due to the inability of hTNF to signal via murine TNFR2 while doubly humanized hTNFKI × hTNFR2KI mice would be comparable with WT C57BL/6 mice. Indeed, we noted diminished frequencies of CD4$^+$FoxP3$^+$ cells in the secondary lymphoid organs from hTNFKI mice, compared with WT controls, while additional humanization of TNFR2 resulted in normalized $T_{reg}$ frequencies (Fig. 1A), presumably due to restoration of hTNF signaling through hTNFR2. Additionally, frequencies of effecter CD44$^+$CD62L$^+$ $T_{reg}$ cells were increased in hTNFKI mice compared with WT and hTNFKI × hTNFR2KI mice (Fig. 1B). However, the total cell number and the expression levels of characteristic $T_{reg}$ markers, such as CD25, CTLA-4, and GITR, were not affected in either singly or doubly humanized mice (SI Appendix, Fig. S3).

To directly assess the functionality of TNFR2 signaling in $T_{reg}$ cells with humanized TNFR2, CD4$^+$CD25$^+$ $T_{reg}$ cells were sorted from spleens and lymph nodes of WT and hTNFKI × hTNFR2KI mice and stimulated in vitro with hTNF or mouse TNF (mTNF) in the presence of IL-2. In line with previous biochemical studies (24–26), $T_{reg}$ cells from hTNFKI × hTNFR2KI mice proliferated well in response to both mTNF and hTNF while proliferation of $T_{reg}$ cells isolated from WT mice was increased only in response to mTNF (Fig. 1C). These findings are consistent with the notion that hTNF cannot efficiently signal through murine TNFR2 (24) and suggest that this defect can be rescued by humanization of the extracellular part of TNFR2. To further validate our model and to test whether doubly humanized mice are suitable for evaluating TNFR2-specific biologies, we performed similar assays with recently characterized blocking or activating antibodies specific for hTNFR2 (27). We found that addition of antagonistic hTNFR2 antibody impaired hTNF-induced $T_{reg}$ cell proliferation while the agonistic hTNFR2 antibody alone induced a modest proliferative response (Fig. 1D and E).

In summary, we validated doubly humanized hTNFKI × hTNFR2KI mice and demonstrated “normalization” of $T_{reg}$ cell compartment, presumably due to functional TNF/TNF2 signaling.

hTNFKI Mice, but Not Doubly Humanized hTNFKI × hTNFR2KI Mice, Develop Exacerbated EAE. Since disrupted TNFR2 signaling in hTNFKI mice resulted in decreased $T_{reg}$ cell numbers at steadily
state, we next tested the development of EAE in WT, hTNFKI, and hTNFR2KI mice. We observed a much higher disease score and delayed EAE remission in hTNFKI mice (Fig. 2A), compared with WT mice and reminiscent of the phenotype of TNFR2-deficient mice in EAE (14). Importantly, humanization of TNFR2 in hTNFKI x hTNFR2KI mice rescued this phenotype and ameliorated disease to the level observed in WT mice (Fig. 2A), further supporting the notion that TNFR2 indeed provides a protective signal in EAE.

The T cell compartment was next analyzed in three groups of mice at the peak of disease. First, we found that total cellularity was diminished in the spleen while CNS infiltration was increased in hTNFKI mice, compared with WT and hTNFKI mice (Fig. 2B). Importantly, frequencies of Treg cells in spleen and CNS upon immunization were lower in hTNFKI mice, compared with both strains with functional TNF/TNFFR2 axis (Fig. 2C). Moreover, FoxP3 expression levels in Treg cells from hTNFKI mice, as judged by mean fluorescence intensity (MFI), were significantly reduced in spleen, but not in CNS, and were restored back to normal in mice with double humanization (Fig. 3A). Unexpectedly, the frequency of FoxP3+ cells among CD4+ T cells also appeared higher in hTNFKI x hTNFR2Areg transgenic mice (Fig. 3B), while the percentage of CD44hiCD62Llo Treg cells was not affected (SI Appendix, Fig. S5). Presumably, the shifted balance between Treg cells and effector T cells could be due to compromised Treg functions in the absence of TNFR2. Indeed, we found a significant reduction in the expression levels of Treg cell signature molecules, such as FoxP3, CD25, CTLA-4, and GITR, in Treg cells from hTNFKI x hTNFR2Areg mice (Fig. 3C). Interestingly, only the frequency of CD25+ cells among Treg cells lacking TNFR2 signaling was reduced whereas percentages of CTLA-4 and GITR positive cells among these Treg cells were not affected (in contrast to the expression levels) (SI Appendix, Fig. S5D). Additionally, hTNFKI x hTNFR2Areg mice showed diminished frequencies of CCR6+ Treg cells (SI Appendix, Fig. S5E).

To directly address a possible impact of TNFR2 deletion on Treg cell function, we evaluated suppressive capacity of Treg cells on T cell proliferation in vitro. To achieve this, CD4+CD25+ Treg cells were isolated from spleens and lymph nodes of hTNFKI x hTNFR2KI and hTNFKI x hTNFR2Areg mice and cocultured with responder T cells according to the standard protocol (30). We observed that TNFR2-deficient Treg cells showed reduced inhibitory capacity, compared with Treg cells with the functional TNFR2 (Fig. 3D). Altogether, these findings demonstrated the significance of intrinsic TNFR2 signaling for suppressive capacity and maintenance of Treg cells at steady state.

**TNFR2-Mediated Signaling in Treg Cells is Protective in EAE.** To assess the functional significance of TNFR2 expression by Treg cells in an autoimmunity model, we induced EAE in hTNFKI x hTNFR2Areg and control hTNFKI x hTNFR2 mice. Remarkably, hTNFKI x hTNFR2Areg mice showed exacerbated disease, compared with hTNFKI x hTNFR2KI mice (Fig. 4D), resembling our earlier observation for hTNFKI mice (Fig. 2A). The total number of cells and the frequency of CD4+ T cells in the spleen at the peak of disease were comparable in hTNFKI x hTNFR2KI and hTNFKI x hTNFR2Areg mice (SI Appendix, Figs. S6A and E), whereas frequencies of IFNγ-expressing T cells in the spleen were increased in hTNFKI mice (SI Appendix, Fig. S4D).

Altogether, we concluded that TNFR2-mediated signaling is required to sustain Treg cell functionality, which, in turn, controls pathogenic T cells during EAE.

**Intrinsic TNFR2-Mediated Signaling Is Essential for the Suppressive Capacity and Maintenance of Treg Cells.** To demonstrate the intrinsic role of TNFR2 for Treg cell development and function, we next generated mice with Treg cell-restricted deficiency in TNFR2 by conditional deletion of exons 2 to 6 of the human TNFR2 gene in doubly humanized mice after crossing hTNFKI x hTNFR2KI mice to FoxP3-Cre transgenic mouse (29) (SI Appendix, Fig. S2). The resultant mice were designated as hTNFKI x hTNFR2Areg.

Evaluation of these mice did not reveal any obvious abnormalities in the cellularity of secondary lymphoid organs or frequencies of CD4+ T cells (SI Appendix, Figs. S5 and B). However, further evaluation showed increased frequency of CD44hiCD62Llo effector T cells in the secondary lymphoid organs of hTNFKI x hTNFR2Areg mice, compared with mice with sufficient TNFR2 signaling in Treg cells (Fig. 3A). Unexpectedly, the frequency of FoxP3+ cells among CD4+ T cells also appeared higher in hTNFKI x hTNFR2Areg mice (Fig. 3B), while the percentage of CD44hiCD62Llo Treg cells was not affected (SI Appendix, Fig. S5C). Presumably, the shifted balance between Treg cells and effector T cells could be due to compromised Treg functions in the absence of TNFR2. Indeed, we found a significant reduction in the expression levels of Treg cell signature molecules, such as FoxP3, CD25, CTLA-4, and GITR, in Treg cells from hTNFKI x hTNFR2Areg mice (Fig. 3C). Interestingly, only the frequency of CD25+ cells among Treg cells lacking TNFR2 signaling was reduced whereas percentages of CTLA-4 and GITR positive cells among these Treg cells were not affected (in contrast to the expression levels) (SI Appendix, Fig. S5D). Additionally, hTNFKI x hTNFR2Areg mice showed diminished frequencies of CCR6+ Treg cells (SI Appendix, Fig. S5E).

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Altogether, we concluded that TNFR2-mediated signaling is required to sustain Treg cell functionality, which, in turn, controls pathogenic T cells during EAE.
Moreover, percentages of CD45RO+CD62Llow cells among CD4+TCRb+ cells in peripheral lymph nodes (LN) and spleen (Spl). (B) Representative frequencies of FoxP3+ cells among CD4+TCRb+ cells. (C) Representative FACS histograms and summary of FoxP3, CD25, CTLA-4, and GITR mean fluorescence intensity in FoxP3+ cells gated on CD4+TCRb+ live cells. FACS histograms are shown for spleen-derived Treg cells, both in CNS and peripheral immune response 2 wk postimmunization. (D) Total cell numbers in spleen (Spl) and CNS. (E) Frequencies of CCR6+ Treg cells in the presence of Treg cells isolated from hTNFKI×hTNFR2KI (gray) and hTNFKI×hTNFR2ΔTregs (red) mice at ratio 1:1, without T cell activation (dark blue). The graph represents summary of responder T cell proliferation in the presence of Treg cells isolated from hTNFKI×hTNFR2KI (black) or hTNFKI×hTNFR2ΔTregs (red) mice, n = 6. Paired one-tailed t test revealed: ***P < 0.001.

Fig. 3. TNFR2-mediated signaling is essential for the maintenance of Treg cells suppressive function. Analysis of hTNFKI×hTNFR2KI (gray) and hTNFKI×hTNFR2ΔTregs (red) mice at steady state. (A) Representative frequencies of CD45RO+CD62Llow cells among CD4+TCRb+ cells in peripheral lymph nodes (LN) and spleen (Spl). (B) Representative frequencies of FoxP3+ cells among CD4+TCRb+ cells. (C) Representative FACS histograms and summary of FoxP3, CD25, CTLA-4, and GITR mean fluorescence intensity in FoxP3+ cells gated on CD4+TCRb+ live cells. FACS histograms are shown for spleen-derived Treg cells. Data are representative of three independent experiments with four mice per group in each experiment. Each point in a diagram represents a single mouse; mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001 (two-tailed unpaired Student’s t test). (D) T cell suppression assay with TNFR2-sufficient and TNFR2-deficient Treg cells. Representative FACS histograms with Cell Trace Violet (CTV) dilution by viable TCRb+ cells. (E) Frequencies of CCR6+ Treg cells in the presence of Treg cells isolated from hTNFKI×hTNFR2KI (gray) and hTNFKI×hTNFR2ΔTregs (red) mice at ratio 1:1, without T cell activation (dark blue). The graph represents summary of responder T cell proliferation in the presence of Treg cells isolated from hTNFKI×hTNFR2KI (black) or hTNFKI×hTNFR2ΔTregs (red) mice, n = 6. Paired one-tailed t test revealed: ***P < 0.001.

Discussion

TNF is a pleiotropic cytokine and a critical target for therapeutic neutralization in several autoimmune diseases. Nevertheless, due to the complexity of TNF signaling through two different receptors, it appears important to define cellular sources of pathogenic and protective TNF, as well as the involvement of a particular TNF receptor expressed by distinct cell type in a given disease setting. Cell type-restricted targeting in autoimmune diseases may prevent side effects and increase efficiency of the treatment. However, the progress in the development of new biologics targeting hTNF is largely hampered by lack of adequate in vivo model systems.

In the present study, we describe doubly humanized mice with the functional human TNF-TNFR2 signaling axis and with the additional option for conditional TNFR2 inactivation in desired cell types.

Previous studies employing cell type-specific gene targeting suggested that myeloid-derived TNF is required for EAE initiation while TNF produced by T cells protected animals from exacerbation of EAE.

Thus, TNFR2 expression and its intrinsic signaling in Treg cells are essential for the functional status of Treg cells and provide an overall protective signal in EAE via control of autoreactive Th17 cells.

In summary, in this study, we developed and validated a humanized mouse with the conditional deletion of TNFR2 in Treg cells that allowed us to directly demonstrate the critical protective role of intrinsic TNFR2 signaling in EAE, a murine model of MS.

mice was characterized by increased levels of hTNF in CNS (SI Appendix, Fig. S6D) and severe demyelination, as well as elevated axonal damage (SI Appendix, Fig. S7).

CTLA-4, and GITR, in spleen while, in the CNS, only CD25 expression was compromised (Fig. 4 D–F), suggesting functional insufficiency of Treg cells lacking TNFR2. Moreover, percentages of CD25 and GITR positive Treg cells were diminished in the absence of TNFR2 on Treg cells in the spleen (SI Appendix, Fig. S6B). Additionally, hTNFKI×hTNFR2ΔTregs mice demonstrated lower percentage of CCR6+ Treg cells, both in CNS and in the spleen (Fig. 4G), and increased frequency of MOG-specific Th17 cells (Fig. 4H), but not of GM-CSF–producing T cells or Th1 cells (SI Appendix, Fig. S6C). Impaired CCR6 expression on Treg cells can affect their recruitment to the CNS during inflammation (31, 32), where Treg cells suppress inflammation. Exacerbated EAE in hTNFKI×hTNFR2ΔTregs mice is associated with increased levels of hTNF in CNS (SI Appendix, Fig. S6D) and severe demyelination, as well as increased axonal damage (SI Appendix, Fig. S7).
pathogenic T cell responses and subsequent CNS demyelination (33). Interestingly, TNFR2 signaling on myeloid cells may play contrasting roles, since TNFR2-expressing microglial cells appeared to be protective while TNFR2+ macrophages contribute to the severity of EAE (10). However, the impact of TNFR2 signaling in T cells, especially in Treg cells, to neuroinflammation remains incompletely understood. Despite the evidence that TNFR2 is highly expressed on Treg cells, the role of TNFR2 in differentiation and maintenance of Treg cells in vivo remains controversial since most reported studies were based on the adoptive transfer of Treg cells. Some studies indicated that TNF has a beneficial effect for Treg cells through increased proliferation and maintenance of suppressive phenotype (9, 27, 34) while others reported the opposite role or no role for TNFR2 (14, 35, 36).

Using humanized mice with the Cre-mediated TNFR2 deletion specifically in FoxP3+ cells, we directly demonstrated the loss of suppressive functions of Treg cells and increased EAE severity in the absence of TNFR2. Selective ablation of TNFR2 on Treg cells resulted in increased pathogenic T cell responses during EAE. Our data indicate that TNF-TNFR2 signaling in Treg cells is not essential for disease initiation but is critical for the control of autoreactive Th17 responses and disease severity. The dominant role of TNFR2 in Treg cells is mediated by both canonical and noncanonical NF-kB pathways (37). Through distinct combinations of NF-kB subunits, TNFR2 may regulate distinct Treg features, including the initial burst in FoxP3 expression and its stabilization during Treg cell proliferation and differentiation (18, 38). Indeed, we found that TNFR2 deficiency in Treg cells leads to reduced expression of its master regulator FoxP3 and Treg-associated molecules, such as CD25, CTLA-4, and GITR, that are known to be critical for dampening the inflammatory responses (39, 40). Interestingly, TNFR2 is highly expressed on most suppressive Treg cells (17, 41), and one possible role of this receptor could be its competition with other cells for TNF. Since TNFR2 has higher affinity for transmembrane TNF than TNFR1, Treg cells may bind this form of TNF more efficiently and, thus, acquire a proliferative or survival advantage. Another possible implication of TNFR2 signaling for Treg cell functions is the prevention of caspase-8–mediated degradation of FoxP3 during inflammation (35). Additionally, in the absence of TNFR2 signaling, expansion of CCR6+ Treg cells is reduced. This subpopulation can migrate following CCL20 chemokine gradient and regulate Th17 immune responses in target organs, including the CNS (32). It would be beneficial to conduct further experiments using inducible Treg-specific ablation of TNFR2 to shed light on the role of TNFR2 in the priming phase of T cell response and in EAE progression.

Dysfunctional Treg cells are a characteristic trait of autoimmunity. In some human autoimmune diseases, the total numbers of Treg cells are not altered, but the potent TNF2+ Treg cell subpopulation is underrepresented (42). Growing evidence suggests that TNFR2 may define a major central switch for Treg cell expansion or Treg cell contraction (19, 43). Recently, a TNFR2 agonist was used to expand a stable population of human Treg cells ex vivo that highly expressed FoxP3 and Helios, which was later confirmed by studies in mice (19, 44). The same TNFR2 agonist showed therapeutic potential in murine models of acute graft-versus-host disease (GVHD) (43), and in collagen-induced arthritis (45), although the exact cellular targets were not identified. Selective inhibition of TNFR2 and inhibition of TNFR1 by receptor-specific antibodies was beneficial in NMDA-induced acute neurodegeneration (46) while employing TNFR2 antagonist inhibited proliferation of tumor-associated Treg cells, suggesting TNFR2 as an important target also in cancer treatment (27). However, similarly to the checkpoint therapy paradigm, blockade of suppressive mechanisms may, on one hand, promote antitumor immunity but, at the same time, may lead to exacerbation of autoimmunity, due to compromised control of pathogenic T cells by Treg cells.

In summary, we have generated and validated a humanized mouse model with conditional ablation of TNFR2 in Treg cells that allowed us to unambiguously demonstrate the critical protective role of TNFR2 intrinsic signaling in EAE, a murine model of MS. Additionally, this mouse system can be used for evaluation of biologics that target hTNF or hTNFR2 signaling pathways.

Materials and Methods
Mice. hTNFKI mice and Foxp3-Cre mice have been described elsewhere (22, 23, 29). Generation of hTNFKI × hTNFR2KI and hTNFKI × hTNFR2Astro mice is described in SI Appendix, Figs. S1 and S2. All mice were bred and housed under specific pathogen-free (SPF) conditions at the Institute for Molecular Medicine (Mainz), at the German Rheumatism Research Center (DRFZ) (Berlin), and at the Pushchino Animal Breeding Facility (Branch of the Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences). Cohoused, age, and gender-matched animals (7 to 12 wk old) of different genotypes were utilized throughout the study. Experiments were performed in accordance with the local guidelines of the corresponding animal facility and the institution (Translational Animal Research Center (TARC), University of Mainz, Landesamt für Gesundheit und Soziales (LaGeSo), German Rheumatism Research Center and Scientific Council, Engelhardt Institute of Molecular Biology).

Organ Preparation and Cell Isolation. Single cell suspensions were prepared by mechanical dissection of spleen and lymph nodes in PBS supplemented with 2% FCS. To obtain CNS infiltrating lymphocytes, brain and spinal cord were isolated from transcardially perfused mice, pooled, enzymatically digested, and centrifuged in a 30/37/70 Percoll gradient (47).

In Vitro Treg Cultures. Treg cells were sorted from spleen and lymph nodes of C57Bl6 and hTNFKI × hTNFR2KI mice. Treg cells were labeled with CellTrace Violet (CTV) (ThermoFisher) at a concentration of 2.5 μM according to the manufacturer’s instructions. Then, 50,000 CD4+CD25− cells were cultured together with 200,000 irradiated antigen-presenting cells (APCs) in complete RPMI 1640 in the presence of either plate-bound (10 ng/mL) or soluble (0.5 μg/mL) anti-CD3 (145-2C11; in-house/DRFZ) and 10 to 100 ng/mL IL-2 (R&D systems) in a humidified atmosphere for 72 h at 37 °C and 5% CO2. Where indicated, murine TNF (ThermoFisher) or human TNF (Dr. Madaus GmbH) were added at 10 ng/mL. Anti-human TNFR2 blocking antibody (TY877), or anti-human TNFR2 activating antibody (TY101), or the respective isotype controls, rat IgG2b (RTK4530; Biolegend) or rat IgG1 (19E1; in-house/DRFZ), were added at 12.5 μg/mL (27).

In Vitro Treg Suppression Assay. Treg cell suppression assay was performed as previously described (30). Briefly, naive CD4+ T cells from spleen and lymph nodes of C57Bl6 mice were magnetically sorted (Milteny) and labeled with CellTrace Violet (CTV) (CTV) at a concentration of 5 μM according to the manufacturer’s instructions. CD4+CD25+ Treg cells were isolated from spleens and lymph nodes of hTNFKI × hTNFR2KI and hTNFKI × hTNFR2Astro mice using a magnetic bead separation kit (Milteny). CTV-labeled T cells were cocultured with Treg cells in complete RPMI 1640 medium in the presence of 1 μg/mL anti-CD3 (145-2C11; in-house/DRFZ) and 6 ng/mL of anti-CD28 (37.51; in-house/DRFZ) in a humidified atmosphere for 80 h at 37 °C and 5% CO2. T cell proliferation was analyzed by flow cytometry by measuring the CTV label.

MOG-Induced EAE. Mice were s.c. immunized with 50 μg of MOG35-55 peptide (Gene Script) emulsified in complete Freund’s adjuvant (CFA) supplemented with 5 mg/mL Mycobacterium tuberculosis (Bacillus Calmette-Guérin) (BCG), followed by 150 ng of Pertussis toxin (List Biological Laboratories) administration on day 0 and 2. Mice were scored daily, and clinical signs were assessed according to standard protocol. Briefly, the following scores were used: 0, no disease; 0.5, partial tail paralysis; 1, complete tail paralysis; 1.5, partially impaired righting reflex; 2, impaired righting reflex; 2.5, impaired gait with limping; 3, hind limbs paresis; 3.5, complete paralysis of hind limbs; 4, forelimbs paresis; 4.5, complete paralysis of forelimbs; 5, inability to move; 5.5, moribund.

ELISA Analysis. For hTNF measurement, brain and spinal cord homogenates were incubated in complete radioimmunoprecipitation assay (RIPA) buffer (Sigma Aldrich) with Protease Inhibitor Mixture (Roche) and centrifuged at
20,000 \times g for 30 min at 4 °C. Total protein concentration was measured with a Bradford Protein Assay (Bio-Rad). hTNF concentration in supernatants was measured using ELISA Ready-Set-Go kits (eBioscience) and normalized to total protein level.

**Histology.** A detailed procedure of histology analysis is provided in SI Appendix, Supplementary Materials. Briefly, spinal cords were isolated on day 17 after EAE immunization and fixed in 4% buffered paraformaldehyde (PFA). Specimens were dehydrated and embedded in paraffin and cut into 5-μm thick sections. Labeled primary antibodies for double-staining with LFB-PAS were used to assess the degree of demyelination and with antibody to amyloid precursor protein (APP) to determine axonal damage. Histological images were taken with a BX-9000 Microscope (BioRevo; Keyence) and quantified as described (48).

**Flow Cytometry.** Single cell suspensions prepared from lymph nodes, spleen, and central nervous system were stained with fluorescently labeled antibodies purchased from BD Biosciences, BioLegend, or eBioscience (SI Appendix, Table S2) and acquired with BD FACSCanto II or LSR II flow cytometers. Flow cytometry data were analyzed using FlowJo software. For intracellular staining of cytokines, cells were stimulated with MOG-peptide (20 μM) for 6 h in the presence of monensin or brefeldin A, harvested, washed, and stained with an eBioscience FoxP3 Fixation Permeabilization kit. Gating strategies are provided in SI Appendix, Figs. S8 and S9.

**Statistical Analyses.** All statistical analyses were done using Prism software (GraphPad). Unpaired or paired t tests and one-way or two-way ANOVA tests were used. Differences were considered significant when P values were <0.05.

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