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

Kamar-Sulu N. Atretkhany[a,b,c,1], Ilgiz A. Mufazalov[a,c,1], Josefine Dunst[d], Anna Kuchmiy[a,d], Violetta S. Gogoleva[a,b], David Andruszewski[c], Marina S. Drutskaya[a,b], Denise L. Faustman[e], Marius Schwabenland[f], Marco Prinz[f,g,h], Andrey A. Kruglov[b,d,i], Ari Waisman[c], and Sergei A. Nedospasov[a,b,d,i,2]

[a]Laboratory of Molecular Mechanisms of Immunity, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 119991 Moscow, Russia; [b]Department of Immunology, Faculty of Biology, Lomonosov Moscow State University, 119991 Moscow, Russia; [c]Institute for Molecular Medicine, University Medical Center of the Johannes Guttenberg University of Mainz, 55131 Mainz, Germany; [d]Research Group Inflammation Biology, German Rheumatism Research Center, Leibniz Institute, 10117 Berlin, Germany; [e]Department of Immunobiology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02129; [f]Institute of Neuropathology, Faculty of Medicine, University of Freiburg, D-79106 Freiburg, Germany; [g]BIOSS-Centre for Biological Signalling Studies, University of Freiburg, D-79104 Freiburg, Germany; [h]CIBSS-Centre for Integrative Biological Signalling Studies, University of Freiburg, D-79104 Freiburg, Germany; and [i]Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119991 Moscow, Russia

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/TNFR2 axis in T regulatory (T_{reg}) 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/TNFR2 mice down-regulated the expression of T_{reg} signature molecules (such as FoxP3, CD25, CTLA-4, and GITR) and diminished T_{reg} suppressive function in vitro. Consequently, T_{reg}-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 T_{reg} cells that could translate into protective functions in vivo, including treatment of autoimmunity.

neuroinflammation | EAE | TNF/TNFR2 | T regulatory cells | humanized mice

TNF 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 T_{reg} 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+ T_{reg} cells that are crucial for maintaining immune homeostasis and suppression of autoimmunity (17). Furthermore, TNFR2 is essential for the maintenance of FoxP3 expression by T_{reg} cells and for their immunosuppressive functions in the colitis model (18). Nevertheless, the significance of intrinsic TNFR2 signaling in T_{reg} 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 T_{reg} expansion or T_{reg} contraction; therefore, TNFR2 agonists and antagonists may have therapeutic potential (19, 20). However, one of the limitations in the development of drugs targeting TNF/TNFR2 signaling is the lack of preclinical animal models since many human TNF-directed biologics do not interact with murine TNF or

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 T_{reg} cells. In mice bearing humanized TNF and TNFR2 genetic loci, TNFR2 ablation restricted to T_{reg} cells led to reduced capacity to control Th17 cell responses, exacerbated experimental autoimmune encephalomyelitis (EAE) development, and affected the maintenance of T_{reg} cells. These findings provide support for the emerging role of TNFR2 signaling in autoimmunity, as demonstrated here in mice with conditional inactivation of TNFR2.

IMMUNOLOGY AND INFLAMMATION

TNFR2. For this purpose, we developed and validated a doubly humanized TNF/TNFR2 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/TNFR2 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. 1*A*), presumably due to restoration of hTNF signaling through hTNFR2. Additionally, frequencies of effector $CD44^{hi}CD62L^{lo}$ T_{reg} cells were increased in hTNFKI mice compared with WT and hTNFKI × hTNFR2KI mice (Fig. 1*B*). 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. 1*C*). 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 biologics, 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. 1 *D* and *E*).
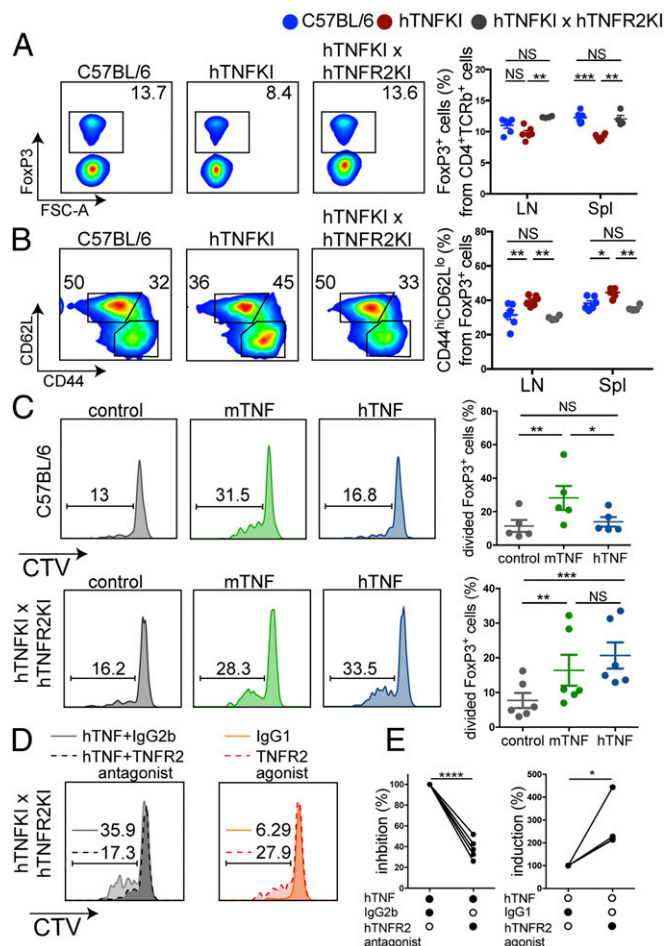

Fig. 1. TNFR2 humanization in hTNFKI mice restores frequency of T_{reg} cells at steady state. (*A* and *B*) Analysis of T_{reg} cells isolated from C57BL/6 mice (blue), hTNFKI (dark red), and hTNFKI × hTNFR2KI (gray) mice at steady state. (*A*) Representative FACS plots and frequencies of $FoxP3^+$ cells among $CD4^+TCRb^+$ cells gated on live cells. (*B*) Representative FACS plots and frequencies of $CD44^{hi}CD62L^{lo}$ cells among $FoxP3^+$ cells gated on $CD4^+TCRb^+$ live cells. Data are representative of two independent experiments with three or more mice per group in each experiment. Each point in a diagram represents a single mouse; mean ± SEM, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (one-way ANOVA test); NS, nonsignificant. (*C–E*) Humanization of TNFR2 in hTNFKI mice restores T_{reg} cell proliferation in vitro. (*C*) Representative FACS histograms with Cell Trace Violet (CTV) dilution and summarized graphs of viable $TCRb^+CD4^+FoxP3^+$ cells isolated from WT (C57BL/6, n = 5) and hTNFKI × hTNFR2KI mice (n = 6) and cultured under indicated conditions in the presence of aCD3, irradiated APC, and IL-2; repeated measures ANOVA with Bonferroni correction revealed: NS, nonsignificant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (*D*) Representative FACS histograms with CTV dilution of viable $TCRb^+CD4^+FoxP3^+$ cells from hTNFKI × hTNFR2KI mice cultured (*Left*) with hTNF in the presence of IgG2b or anti-hTNFR2 blocking antibody or (*Right*) with IgG1 or anti-hTNFR2 activating antibody. (*E*) Summary of anti-hTNFR2 blocking antibody-mediated inhibition of hTNF-induced proliferation of regulatory T cells from hTNFKI × hTNFR2KI mice for n = 5 experiments (*Left*). Summary of anti-hTNFR2 activating antibody-mediated induction of regulatory T cell proliferation for n = 4 experiments (*Right*). Paired one-tailed t test revealed: *$P < 0.05$; ****$P < 0.0001$. FSC-A, forward-scatter area; LN, lymph nodes; Spl, spleen.

In summary, we validated doubly humanized hTNFKI × hTNFR2KI mice and demonstrated "normalization" of T_{reg} cell compartment, presumably due to functional TNF/TNFR2 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 steady
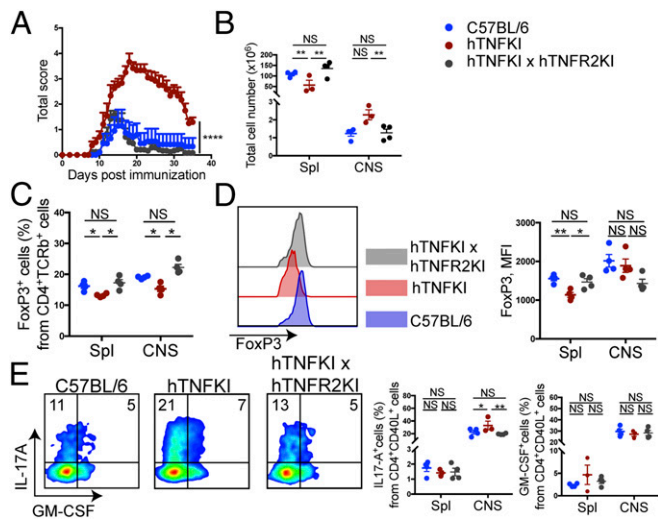
Fig. 2. hTNFKI mice, but not doubly humanized hTNFKI × hTNFR2KI mice, develop exacerbated EAE. EAE analysis of C57BL/6 mice (blue), hTNFKI (dark red), and hTNFKI × hTNFR2KI (gray) mice. (*A*) Representative EAE clinical scores. (*B–E*) Analysis of immune response 2 wk postimmunization. (*B*) Total cell numbers of splenocytes and CNS-infiltrates. (*C*) Frequencies of FoxP3⁺ cells among CD4⁺TCRb⁺ cells gated on live cells. (*D*) Representative FACS histograms and mean fluorescence intensity (MFI) of FoxP3 in FoxP3⁺ cells gated on CD4⁺TCRb⁺ live cells. (*E*) Representative FACS plots and frequencies of IL-17A⁺ and GM-CSF⁺ cells gated on CD40L⁺ cells among CD4⁺TCRb⁺ live cells. Data are representative of three independent experiments with three or more mice per group in each experiment. Each point in a diagram represents a single mouse; mean ± SEM. *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$; NS, nonsignificant. Two-way ANOVA (*A*) or one-way ANOVA (*B–E*) tests were used.

state, we next tested the development of EAE in WT, hTNFKI, and hTNFKI × hTNFR2KI mice. We observed a much higher disease score and delayed EAE remission in hTNFKI mice (Fig. 2*A*), compared with WT mice and reminiscent of the phenotype of TNFR2-deficient mice in EAE (14). Importantly, humanization of TNFR2 in hTNFKI × hTNFR2KI mice rescued this phenotype and ameliorated disease to the level observed in WT mice (Fig. 2*A*), 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 × hTNFR2KI mice (Fig. 2*B*). Importantly, frequencies of T$_{reg}$ cells in spleen and CNS upon immunization were lower in hTNFKI mice, compared with both strains with functional TNF/TNFR2 axis (Fig. 2*C*). Moreover, FoxP3 expression levels in T$_{reg}$ 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. 2*D*). Expression levels of CD25, CTLA-4, and GITR, as well as the frequencies of CD25⁺, CTLA-4⁺, GITR⁺, and CCR6⁺ T$_{reg}$ cells, were not affected in the spleen of hTNFKI mice (*SI Appendix*, Fig. S4 *A–C*). In contrast, CD25 and CCR6 expression by T$_{reg}$ cells was compromised in the draining lymph nodes of EAE-diseased hTNFKI mice, but restored in hTNFKI × hTNFR2KI mice (*SI Appendix*, Fig. S4 *A* and *C*). IL-17A–producing T cells represent the main pathogenic subset in CNS-directed autoimmunity (28). Analysis during EAE revealed significantly higher frequencies of MOG-specific Th17 cells in the CNS, but not in the spleen, of hTNFKI mice among all groups tested (Fig. 2*E*). Frequencies of GM-CSF and IFNγ-expressing T cells, as well as hTNF levels in CNS, remained unaffected, regardless of TNF/TNFR2 humanization (Fig. 2*E*

and *SI Appendix*, Fig. S4 *D* and *E*), whereas frequencies of IFNγ-expressing T cells in the spleen were increased in hTNFKI mice (*SI Appendix*, Fig. S4*D*).

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

Intrinsic TNFR2-Mediated Signaling Is Essential for the Suppressive Capacity and Maintenance of T$_{reg}$ Cells. To demonstrate the intrinsic role of TNFR2 for T$_{reg}$ cell development and function, we next generated mice with T$_{reg}$ 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 × hTNFR2KI mice to FoxP3-Cre transgenic mouse (29) (*SI Appendix*, Fig. S2). The resultant mice were designated as hTNFKI × hTNFR2^{ΔTregs}.

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*, Fig. S5 *A* and *B*). However, further evaluation showed increased frequency of CD44^{hi}CD62L^{lo} effector T cells in the secondary lymphoid organs of hTNFKI × hTNFR2^{ΔTregs} mice, compared with mice with sufficient TNFR2 signaling in T$_{reg}$ cells (Fig. 3*A*). Unexpectedly, the frequency of FoxP3⁺ cells among CD4⁺ T cells also appeared higher in hTNFKI × hTNFR2^{ΔTregs} mice (Fig. 3*B*) while the percentage of CD44^{hi}CD62L^{lo} T$_{reg}$ cells was not affected (*SI Appendix*, Fig. S5*C*). Presumably, the shifted balance between T$_{reg}$ cells and effector T cells could be due to compromised T$_{reg}$ functions in the absence of TNFR2. Indeed, we found a significant reduction in the expression levels of T$_{reg}$ cell signature molecules, such as FoxP3, CD25, CTLA-4, and GITR in T$_{reg}$ cells from hTNFKI × hTNFR2^{ΔTregs} mice (Fig. 3*C*). Interestingly, only the frequency of CD25⁺ cells among T$_{reg}$ cells lacking TNFR2 signaling was reduced whereas percentages of CTLA-4 and GITR positive cells among these T$_{reg}$ cells were not affected (in contrast to the expression levels) (*SI Appendix*, Fig. S5*D*). Additionally, hTNFKI × hTNFR2^{ΔTregs} mice showed diminished frequencies of CCR6⁺ T$_{reg}$ cells (*SI Appendix*, Fig. S5*E*).

To directly address a possible impact of TNFR2 deletion on T$_{reg}$ cell function, we evaluated suppressive capacity of T$_{reg}$ cells on T cell proliferation in vitro. To achieve this, CD4⁺CD25⁺ T$_{reg}$ cells were isolated from spleens and lymph nodes of hTNFKI × hTNFR2KI and hTNFKI × hTNFR2^{ΔTregs} mice and cocultured with responder T cells according to the standard protocol (30). We observed that TNFR2-deficient T$_{reg}$ cells showed reduced inhibitory capacity, compared with T$_{reg}$ cells with the functional TNFR2 (Fig. 3*D*). Altogether, these findings demonstrated the significance of intrinsic TNFR2 signaling for suppressive capacity and maintenance of T$_{reg}$ cells at steady state.

TNFR2-Mediated Signaling in T$_{reg}$ Cells Is Protective in EAE. To assess the functional significance of TNFR2 expression by T$_{reg}$ cells in an autoimmunity model, we induced EAE in hTNFKI × hTNFR2^{ΔTregs} and control hTNFKI × hTNFR2 mice. Remarkably, hTNFKI × hTNFR2^{ΔTregs} mice showed exacerbated disease, compared with hTNFKI × hTNFR2KI mice (Fig. 4*A*), resembling our earlier observation for hTNFKI mice (Fig. 2*A*). The total number of cells and the frequency of CD4⁺ T cells in the spleen at the peak of disease were comparable in hTNFKI × hTNFR2KI and hTNFKI × hTNFR2^{ΔTregs} mice (*SI Appendix*, Fig. S6*A*) whereas CNS infiltration was significantly increased in hTNFKI × hTNFR2^{ΔTregs} mice (Fig. 4*B*). Although frequencies of FoxP3⁺ cells among CD4⁺ T cells were comparable in both groups, the expression level of FoxP3 was significantly diminished in T$_{reg}$ cells from hTNFKI × hTNFR2^{ΔTregs} mice (Fig. 4*C*). We also observed a significant reduction in the expression levels of several signature T$_{reg}$ cell markers, such as CD25,
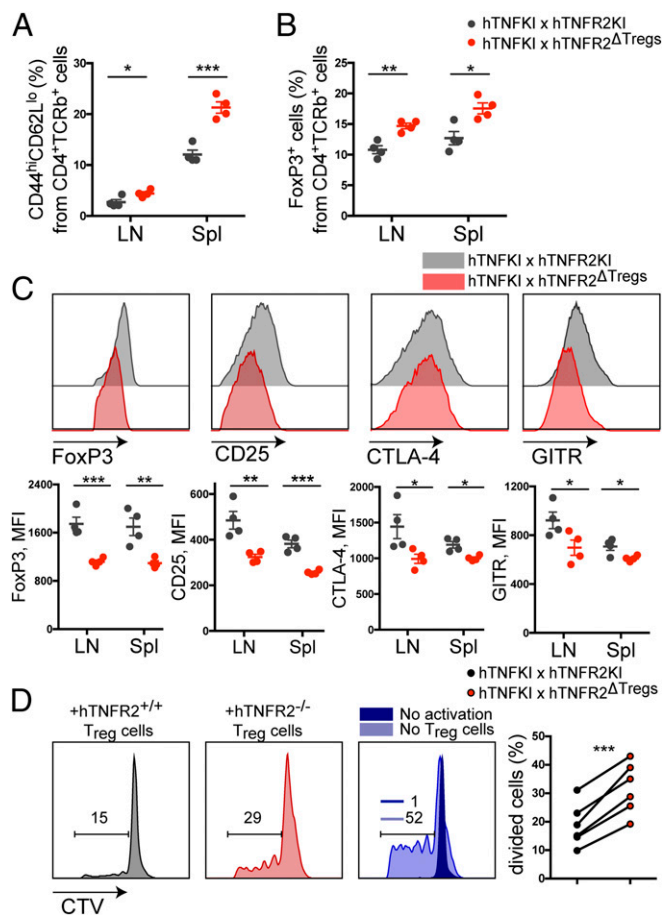
IMMUNOLOGY AND INFLAMMATION

Fig. 3. TNFR2-mediated signaling is essential for the maintenance of T_reg cells suppressive function. Analysis of hTNFKI x hTNFR2KI (gray) and hTNFKI x hTNFR2^ΔTregs (red) mice at steady state. (*A*) Representative frequencies of CD44^hiCD62L^low 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 T_reg 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 T_reg cells. Representative FACS histograms with Cell Trace Violet (CTV) dilution by viable TCRb^+CD4^+ responder T cells in the presence of T_reg cells isolated from hTNFKI × hTNFR2KI (gray) and hTNFKI × hTNFR2^ΔTregs (red) mice at ratio 1:1, without T_reg cells (blue) or without activation (dark blue). The graph represents summary of responder T cell proliferation in the presence of T_reg cells isolated from hTNFKI × hTNFR2KI (black) or hTNFKI × hTNFR2^ΔTregs (red) mice, *n* = 6. Paired one-tailed *t* test revealed: ***$P < 0.001$.

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).

Thus, TNFR2 expression and its intrinsic signaling in T_reg cells are essential for the functional status of T_reg 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 T_reg cells that allowed us to directly demonstrate the critical protective role of intrinsic TNFR2 signaling in EAE, a murine model of MS.

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
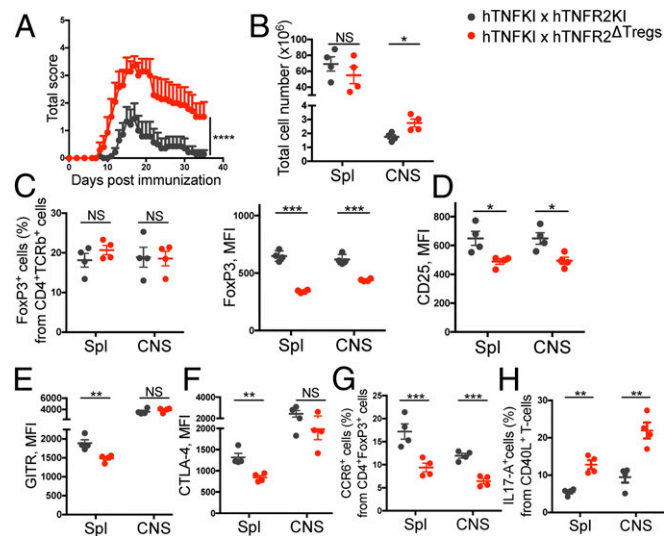
CTLA-4, and GITR, in spleen while, in the CNS, only CD25 expression was compromised (Fig. 4 *D–F*), suggesting functional insufficiency of T_reg cells lacking TNFR2. Moreover, percentages of CD25 and GITR positive T_reg cells were diminished in the absence of TNFR2 on T_reg cells in the spleen (*SI Appendix*, Fig. S6B). Additionally, hTNFKI × hTNFR2^ΔTregs mice demonstrated lower percentage of CCR6^+ T_reg 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 T_reg cells can affect their recruitment to the CNS during inflammation (31, 32), where T_reg cells suppress inflammation. Exacerbated EAE in hTNFKI × hTNFR2^ΔTregs

Fig. 4. Deletion of TNFR2 on T_reg cells leads to exacerbated EAE. EAE analysis of hTNFKI x hTNFR2KI (gray) and hTNFKI x hTNFR2^ΔTregs (red) mice. (*A*) Representative EAE clinical scores. (*B–F*) Analysis of CNS infiltration and peripheral immune response 2 wk postimmunization. (*B*) Total cell numbers in spleen and CNS-infiltrates. (*C*) Frequencies of FoxP3^+ cells among CD4^+TCRb^+ cells in indicated organs and mean fluorescence intensity of FoxP3 in T_reg cells. Mean fluorescence intensity of CD25 (*D*), GITR (*E*), and CTLA-4 (*F*) in T_reg cells. (*G*) Frequencies of CCR6^+ cells among FoxP3^+ cells. (*H*) Frequencies of IL-17A^+ cells among CD40L^+CD4^+T cells. Data are representative of three independent experiments with three or more mice per group in each experiment. Each point in a diagram represents a single mouse; mean ± SEM. *$P < 0,05$; **$P < 0,01$; ***$P < 0,001$; ****$P < 0.0001$; NS, nonsignificant. Two-way ANOVA (*A*) or two-tailed unpaired Student's *t* tests (*B–H*) were used.

Atretkhany et al.

pathogenic T cell responses and subsequent CNS demyelin-ation (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 T_{reg} cells, to neuro-inflammation remains incompletely understood. Despite the evidence that TNFR2 is highly expressed on T_{reg} cells, the role of TNFR2 in differentiation and maintenance of T_{reg} cells in vivo remains controversial since most reported studies were based on the adoptive transfer of T_{reg} cells. Some studies indicated that TNF has a beneficial effect for T_{reg} 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 de-letion specifically in $FoxP3^+$ cells, we directly demonstrated the loss of suppressive functions of T_{reg} cells and increased EAE severity in the absence of TNFR2. Selective ablation of TNFR2 on T_{reg} cells resulted in increased pathogenic T cell responses during EAE. Our data indicate that TNF-TNFR2 signaling in T_{reg} cells is not essential for disease initiation but is critical for the control of autoreactive Th17 responses and disease severity.

The downstream effects of TNFR2 agonism are mediated by both canonical and noncanonical NF-κB pathways (37). Through distinct combinations of NF-κB subunits, TNFR2 may regulate distinct T_{reg} features, including the initial burst in FoxP3 ex-pression and its stabilization during T_{reg} cell proliferation and differentiation (18, 38). Indeed, we found that TNFR2 deficiency in T_{reg} cells leads to reduced expression of its master regulator FoxP3 and T_{reg}-associated molecules, such as CD25, CTLA-4, and GITR, that are known to be critical for dampening the in-flammatory responses (39, 40). Interestingly, TNFR2 is highly expressed on most suppressive T_{reg} cells (17, 41), and one pos-sible role of this receptor could be its competition with other cells for TNF. Since TNFR2 has higher affinity for trans-membrane TNF than TNFR1, T_{reg} cells may bind this form of TNF more efficiently and, thus, acquire a proliferative or survival advantage. Another possible implication of TNFR2 signaling for T_{reg} cell functions is the prevention of caspase-8–mediated degradation of FoxP3 during inflammation (35). Additionally, in the absence of TNFR2 signaling, expansion of $CCR6^+$ T_{reg} cells is reduced. This subpopulation can migrate following CCL20 chemokine gradient and regulate Th17 immune re-sponses in target organs, including the CNS (32). It would be beneficial to conduct further experiments using inducible T_{reg}-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 T_{reg} cells are a characteristic trait of autoim-munity. In some human autoimmune diseases, the total numbers of T_{reg} cells are not altered, but the potent $TNFR2^+$ T_{reg} cell subpopulation is underrepresented (42). Growing evidence sug-gests that TNFR2 may define a major central switch for T_{reg} cell expansion or T_{reg} cell contraction (19, 43). Recently, a TNFR2 agonist was used to expand a stable population of human T_{reg} 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 iden-tified. Selective stimulation of TNFR2 and inhibition of TNFR1 by receptor-specific antibodies was beneficial in NMDA-induced acute neurodegeneration (46) while employing TNFR2 antago-nist inhibited proliferation of tumor-associated T_{reg} cells, sug-gesting 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 T_{reg} cells.

In summary, we have generated and validated a humanized mouse model with conditional ablation of TNFR2 in T_{reg} cells that allowed us to unambiguously demonstrate the critical pro-tective 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 × hTNFR2$^{\Delta Tregs}$ 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 She-myakin 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 dissociation 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 T_{reg} Cultures. T_{reg} cells were sorted from spleen and lymph nodes of C57BL/6 and hTNFKI × hTNFR2KI mice. T_{reg} 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 μg/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% CO_2. 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 (TY010), 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 T_{reg} Suppression Assay. T_{reg} cell suppression assay was performed as previously described (30). Briefly, naive $CD4^+$ T cells from spleen and lymph nodes of C57BL/6 mice were magnetically sorted (Miltenyi) and labeled with CellTrace Violet (CTV) at a concentration of 5 μM according to the manu-facturer's instructions. $CD4^+CD25^+$ T_{reg} cells were isolated from spleens and lymph nodes of hTNFKI × hTNFR2KI and hTNFKI × hTNFR2$^{\Delta Tregs}$ mice using a magnetic bead separation kit (Miltenyi). CTV-labeled T cells were cocultured with T_{reg} 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% CO_2. 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 MOG_{35-55} peptide (Gene Script) emulsified in complete Freund's adjuvant (CFA) supplemented with 5 mg/mL *Mycobacterium tuberculosis* (Difco), 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 stan-dard 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 × *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 and stained with luxol fast blue-periodic acid–Schiff (LFB-PAS) to assess the degree of demyelination and with antibody to amyloid precursor protein (APP) to determine axonal damage. Histological images were taken with a BZ-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 μg/mL) 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 summarized 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.

1. Maini RN, et al. (1998) Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor alpha monoclonal antibody combined with low-dose weekly methotrexate in rheumatoid arthritis. *Arthritis Rheum* 41:1552–1563.
2. van Dullemen HM, et al. (1995) Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). *Gastroenterology* 109:129–135.
3. The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group (1999) TNF neutralization in MS: Results of a randomized, placebo-controlled multicenter study. *Neurology* 53:457–465.
4. Wajant H, Pfizenmaier K, Scheurich P (2003) Tumor necrosis factor signaling. *Cell Death Differ* 10:45–65.
5. Pasparakis M, Alexopoulou L, Episkopou V, Kollias G (1996) Immune and inflammatory responses in TNF alpha-deficient mice: A critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. *J Exp Med* 184:1397–1411.
6. Pfeffer K, et al. (1993) Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. *Cell* 73: 457–467.
7. Rothe J, et al. (1993) Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes. *Nature* 364:798–802.
8. Polz J, et al. (2014) Myeloid suppressor cells require membrane TNFR2 expression for suppressive activity. *Immun Inflamm Dis* 2:121–130.
9. Chen X, Bäumel M, Männel DN, Howard OM, Oppenheim JJ (2007) Interaction of TNF with TNF receptor type 2 promotes expansion and function of mouse CD4+CD25+ T regulatory cells. *J Immunol* 179:154–161.
10. Gao H, et al. (2017) Opposing functions of microglial and macrophagic TNFR2 in the pathogenesis of experimental autoimmune encephalomyelitis. *Cell Rep* 18:198–212.
11. Madsen PM, et al. (2016) Oligodendroglial TNFR2 mediates membrane TNF-dependent repair in experimental autoimmune encephalomyelitis by promoting oligodendrocyte differentiation and remyelination. *J Neurosci* 36:5128–5143.
12. Alexopoulou L, et al. (2006) Transmembrane TNF protects mutant mice against intracellular bacterial infections, chronic inflammation and autoimmunity. *Eur J Immunol* 36:2768–2780.
13. Ruuls SR, et al. (2001) Membrane-bound TNF supports secondary lymphoid organ structure but is subservient to secreted TNF in driving autoimmune inflammation. *Immunity* 15:533–543.
14. Tsakiri N, Papadopoulos D, Denis MC, Mitsikostas DD, Kollias G (2012) TNFR2 on non-haematopoietic cells is required for Foxp3+ Treg-cell function and disease suppression in EAE. *Eur J Immunol* 42:403–412.
15. Eugster HP, et al. (1999) Severity of symptoms and demyelination in MOG-induced EAE depends on TNFR1. *Eur J Immunol* 29:626–632.
16. Steeland S, et al. (2017) TNFR1 inhibition with a nanobody protects against EAE development in mice. *Sci Rep* 7:13646.
17. Chen X, et al. (2008) Cutting edge: Expression of TNFR2 defines a maximally suppressive subset of mouse CD4+CD25+FoxP3+ T regulatory cells: Applicability to tumor-infiltrating T regulatory cells. *J Immunol* 180:6467–6471.
18. Chen X, et al. (2013) TNFR2 is critical for the stabilization of the CD4+Foxp3+ regulatory T. cell phenotype in the inflammatory environment. *J Immunol* 190:1076–1084.
19. Okubo Y, Mera T, Wang L, Faustman DL (2013) Homogeneous expansion of human T-regulatory cells via tumor necrosis factor receptor 2. *Sci Rep* 3:3153.
20. Yang S, Wang J, Brand DD, Zheng SG (2018) Role of TNF-TNF receptor 2 signal in regulatory T cells and its therapeutic implications. *Front Immunol* 9:784.
21. Kruglov AA, et al. (2011) Modalities of experimental TNF blockade in vivo: Mouse models. *Adv Exp Med Biol* 691:421–431.
22. Olleros ML, et al. (2015) Control of mycobacterial infections in mice expressing human tumor necrosis factor (TNF) but not mouse TNF. *Infect Immun* 83:3612–3623.
23. Efimov GA, et al. (2016) Cell-type-restricted anti-cytokine therapy: TNF inhibition from one pathogenic source. *Proc Natl Acad Sci USA* 113:3006–3011.
24. Ameloot P, et al. (2001) Identification of tumor necrosis factor (TNF) amino acids crucial for binding to the murine p75 TNF receptor and construction of receptor-selective mutants. *J Biol Chem* 276:37426–37430.
25. Ranges GE, Bombara MP, Aiyer RA, Rice GG, Palladino MA, Jr (1989) Tumor necrosis factor-alpha as a proliferative signal for an IL-2-dependent T cell line: Strict species specificity of action. *J Immunol* 142:1203–1208.
26. Lewis M, et al. (1991) Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific. *Proc Natl Acad Sci USA* 88:2830–2834.
27. Torrey H, et al. (2017) Targeting TNFR2 with antagonistic antibodies inhibits proliferation of ovarian cancer cells and tumor-associated Tregs. *Sci Signal* 10:eaaf8608.
28. McGinley AM, Edwards SC, Raverdeau M, Mills KHG (2018) Th17 cells, γδ T cells and their interplay in EAE and multiple sclerosis. *J Autoimmun* S0896-8411(18)30007-6.
29. Wing K, et al. (2008) CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 322:271–275.
30. Collison LW, Vignali DA (2011) In vitro Treg suppression assays. *Methods Mol Biol* 707: 21–37.
31. Yamazaki T, et al. (2008) CCR6 regulates the migration of inflammatory and regulatory T cells. *J Immunol* 181:8391–8401.
32. Villares R, et al. (2009) CCR6 regulates EAE pathogenesis by controlling regulatory CD4+ T-cell recruitment to target tissues. *Eur J Immunol* 39:1671–1681.
33. Kruglov AA, Lampropoulou V, Fillatreau S, Nedospasov SA (2011) Pathogenic and protective functions of TNF in neuroinflammation are defined by its expression in T lymphocytes and myeloid cells. *J Immunol* 187:5660–5670.
34. Leclerc M, et al. (2016) Control of GVHD by regulatory T cells depends on TNF produced by T cells and TNFR2 expressed by regulatory T cells. *Blood* 128:1651–1659.
35. Gao Y, et al. (2015) Inflammation negatively regulates FOXP3 and regulatory T-cell function via DBC1. *Proc Natl Acad Sci USA* 112:E3246–E3254.
36. Zhang Q, et al. (2013) TNF-α impairs differentiation and function of TGF-β-induced Treg cells in autoimmune diseases through Akt and Smad3 signaling pathway. *J Mol Cell Biol* 5:85–98.
37. Rauert H, et al. (2010) Membrane tumor necrosis factor (TNF) induces p100 processing via TNF receptor-2 (TNFR2). *J Biol Chem* 285:7394–7404.
38. Oh H, et al. (2017) An NF-kappaB transcription-factor-dependent lineage-specific transcriptional program promotes regulatory T cell identity and function. *Immunity* 47:450–465.e5.
39. Lu L, Barbi J, Pan F (2017) The regulation of immune tolerance by FOXP3. *Nat Rev Immunol* 17:703–717.
40. Chinen T, et al. (2016) An essential role for the IL-2 receptor in T$_{reg}$ cell function. *Nat Immunol* 17:1322–1333.
41. Chen X, et al. (2010) Co-expression of TNFR2 and CD25 identifies more of the functional CD4+FOXP3+ regulatory T cells in human peripheral blood. *Eur J Immunol* 40: 1099–1106.
42. Ban L, et al. (2008) Selective death of autoreactive T cells in human diabetes by TNF or TNF receptor 2 agonism. *Proc Natl Acad Sci USA* 105:13644–13649.
43. Chopra M, et al. (2016) Exogenous TNFR2 activation protects from acute GvHD via host T reg cell expansion. *J Exp Med* 213:1881–1900.
44. He X, et al. (2016) A TNFR2-agonist facilitates high purity expansion of human low purity Treg cells. *PLoS One* 11:e0156311.
45. Fischer R, et al. (2018) Selective activation of tumor necrosis factor receptor II induces antiinflammatory responses and alleviates experimental arthritis. *Arthritis Rheumatol* 70:722–735.
46. Dong Y, et al. (2016) Essential protective role of tumor necrosis factor receptor 2 in neurodegeneration. *Proc Natl Acad Sci USA* 113:12304–12309.
47. Mufazalov IA, Waisman A (2016) Isolation of central nervous system (CNS) infiltrating cells. *Methods Mol Biol* 1304:73–79.
48. Prinz M, et al. (2006) Innate immunity mediated by TLR9 modulates pathogenicity in an animal model of multiple sclerosis. *J Clin Invest* 116:456–464.