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1 **Tissue-restricted control of established central nervous system**
2 **autoimmunity by TNF receptor 2-expressing Treg cells**

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25

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28

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32 discussions and reviewed the manuscript.

33

34

ABSTRACT

35 CD4⁺Foxp3⁺ regulatory T (Treg) cells are central modulators of autoimmune diseases. However,
36 the timing and location of Treg cell-mediated suppression of tissue-specific autoimmunity remain
37 undefined. Here, we addressed these questions by investigating the role of TNF receptor 2 (TNFR2)
38 signaling in Treg cells during experimental autoimmune encephalomyelitis (EAE), a model of
39 multiple sclerosis. We found that TNFR2 expressing Treg cells were critical to suppress EAE at
40 peak disease in the central nervous system but had no impact on T cell priming in lymphoid tissues
41 at disease onset. Mechanistically, TNFR2 signaling maintained functional Treg cells with sustained
42 expression of CTLA-4 and Blimp1, allowing active suppression of pathogenic T cells in the
43 inflamed central nervous system. This late effect of Treg cells was further confirmed by treating
44 mice with TNF and TNFR2 agonists and antagonists. Our findings are the first to show that
45 endogenous Treg cells specifically suppress an autoimmune disease by acting in the target tissue
46 during overt inflammation. Moreover, they bring a mechanistic insight to some of the adverse
47 effects of anti-TNF therapy in patients.

48

49

SIGNIFICANCE STATEMENT

50 Regulatory T (Treg) cells have been highlighted for their central function in limiting the severity
51 of autoimmune diseases such as multiple sclerosis (MS). To date, the anatomical location and
52 timing of this Treg cell-mediated suppression are unknown. In this report, in a mouse model of
53 MS, we demonstrate that Treg cells inhibit the pathogenic process directly in the central nervous
54 system during established disease, rather than in the pre-symptomatic phase. This protective
55 function requires the surface expression of TNF receptor 2 by Treg cells, as its genetic ablation or
56 antibody-mediated blockade worsens disease symptoms. Our data reveal a unique function of Treg
57 cells in autoimmunity and highlight TNFR2 as a promising therapeutic target.

INTRODUCTION

58
59
60 CD4⁺Foxp3⁺ regulatory T (Treg) cells play a pivotal role in the control of immune responses. In
61 particular, the effector Treg cell subset, controlled in part by the master transcription factor Blimp-
62 1 (1-3), exerts a critical role in the protection against autoimmune diseases by inhibiting
63 autoreactive cells. Perturbations in Treg cell numbers or function trigger or exacerbate autoimmune
64 diseases in mice and humans, such as type 1 diabetes, rheumatoid arthritis or experimental
65 autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS) (4, 5). In line
66 with this latter observation, Treg cell depletion increased EAE symptoms (6), while transfer of
67 polyclonal or myelin-reactive Treg cells limited the disease (7, 8). However, it is still unknown
68 whether Treg cells suppress the priming of pathogenic conventional T (Tconv) cells in the draining
69 lymph nodes (dLN) at disease onset, and/or inhibit their function directly in the central nervous
70 system (CNS) in the phase of ongoing inflammation.

71 Our group and others have reported that TNF increased the proliferation of Treg cells via TNF
72 receptor 2 (TNFR2) while maintaining or enhancing their suppressive function in vitro and in
73 different immune-pathologies (9-13). This intriguing immune-regulatory facet of TNF is clearly
74 exemplified in EAE. Mice deficient for TNFR2 exhibited aggravated symptoms of EAE, which
75 was associated with lower Treg cell proportions in the CNS (14). In the same line, mice genetically
76 engineered to express the human version of TNF, and in which TNFR2 was ablated in Treg cells,
77 developed severe EAE symptoms (15). These studies suggested that the TNF/TNFR2 axis is central
78 in Treg cell-mediated suppression of autoimmunity. However, the location and timing of this Treg
79 cell-mediated suppression are currently unknown. Here, we addressed these questions by using
80 constitutive and inducible ablation of TNFR2 in mature Treg cells. We showed that TNFR2

81 expressing Treg cells suppressed EAE locally in the inflamed CNS on established disease, without
82 affecting pathogenic T cell priming in the dLN.

83

RESULTS

TNFR2 expression by hematopoietic cells is required to limit EAE severity and promotes Treg cell-intrinsic expansion in the CNS

Since TNFR2 is up-regulated in Treg cells upon activation (9), we analyzed its expression after EAE induction. The receptor was highly and preferentially expressed in Treg cells at all time points in dLN and from day 10 in the inflamed CNS (Fig. S1). To study the role of the TNF/TNFR2 axis in Treg cells during EAE, we first performed experiments with mice carrying germline deletion of TNFR2 (*Tnfrsf1b*^{-/-}, hereafter named 'KO'). As previously observed (16-18), KO mice exhibited normal CD4⁺ T cell numbers but had reduced Treg cell numbers in the spleen at steady state (Fig. S2). These mice developed more severe EAE than wild type (WT) controls, which was associated with reduced Treg cell accumulation in the inflamed CNS (Fig. S3A and B). We then generated bone marrow chimeric mice to assess the role of TNFR2 expression by hematopoietic and non-hematopoietic cells. Ablation of TNFR2 in the immune system, but not in the non-hematopoietic compartment, led to exacerbated EAE and reduced Treg cell numbers in the CNS (Fig. S3 C-F). This role of TNFR2 in Treg cell expansion was a cell-autonomous mechanism, as revealed by their reduced numbers in the competitive environment of mixed bone marrow chimeras (Fig. S3G).

TNFR2 signaling in Treg cells mediates disease suppression during overt CNS inflammation.

To further delineate the cell-autonomous role of TNFR2 in Treg cell biology, we generated mice with conditional ablation of TNFR2 in Treg cells by crossing mice expressing the CRE-recombinase in Treg cells (*Foxp3*^{Cre}) with mice carrying floxed *Tnfrsf1b* alleles (*Tnfrsf1b*^{fl}). It was critical to compare these *Foxp3*^{Cre}*Tnfrsf1b*^{fl} (hereafter named conditional knock-out or 'cKO') mice to their proper controls, the *Foxp3*^{Cre} mice, because of putative toxic effect of Cre expression, as recently reported (19, 20). As expected, Treg cells of cKO mice had complete ablation of TNFR2

108 expression (Fig. S4A). However, a partial decreased TNFR2 expression was also observed on
109 Tconv cells, which was likely due to leakiness of Cre expression in non-Treg cells in *Foxp3^{Cre}*
110 mice, previously reported in other mouse models (21, 22). cKO mice had no sign of spontaneous
111 autoimmunity and displayed normal body weight (Fig. S4B). Treg cell proportion, numbers and
112 expression of Foxp3, CD25 and CTLA-4 in lymphoid tissues were unaltered (Fig. S4 C and D).
113 Also, the *in vitro* suppressive capacity of Treg cells was not or slightly reduced by TNFR2 ablation
114 (Fig. S4 E and F). Therefore, TNFR2 is mainly dispensable for Treg cell homeostasis at steady
115 state.

116 Next, we explored the cell-autonomous function of TNFR2 in Treg cells during CNS inflammation.
117 Strikingly, compared to controls, cKO mice developed a very severe EAE leading to death in
118 almost half of the mice by day 15 (Fig. 1A). This was not associated with increased total leukocytes
119 infiltration in the CNS or dLN (Fig. S5A). Treg cell proportions were only transiently reduced at
120 day 10 in the CNS of cKO mice (Fig. 1B, and Fig. S5B) and the overall level of Foxp3, Ki67, CD25
121 or Helios was unaltered (Fig. S5D). Also, TNFR2-deficient Treg cells did not acquire the capacity
122 to produce pathogenic cytokines, such as IFN γ , IL-17A or GM-CSF after phorbol 12-myristate 13-
123 acetate (PMA) ionomycin stimulation in the CNS (Fig. S5 E and F). However, further examination
124 showed that these Treg cells in the CNS of cKO mice exhibited lower expression of CTLA-4,
125 Blimp-1 and ICOS (Fig. 1 C and D and Fig. S5C), which are markers of effector Treg cells (1, 23,
126 24). Interestingly, these quantitative and phenotypic Treg cell alterations in the CNS were not
127 observed in the spleen or dLN. To further investigate whether TNFR2⁺ Treg cell-mediated
128 suppression of EAE occurs during the priming or effector phase, we used an inducible Cre system
129 allowing TNFR2 ablation in Treg cells upon tamoxifen treatment. We thus generated *Foxp3^{Cre-}*
130 *^{ERT2}Tnfrsf1b^{fl}* (hereafter named induced conditional KO or ‘icKO’) mice. Importantly, in most of

131 these mice, tamoxifen administration induced TNFR2 ablation in Treg cells as efficiently as in
132 cKO mice but not at all in Tconv cells (Fig. S6A). This icKO model is thus of great interest since
133 TNFR2 ablation is Treg cell-specific contrary to cKO mice. To assess whether EAE control by
134 TNFR2-expressing Treg cells is taking place in the CNS after disease onset, we administered
135 tamoxifen from day 7 to 14 after disease induction. Remarkably, EAE severity was dramatically
136 increased in icKO when compared to *Foxp3^{Cre-ERT2}* control animals, similarly to what we observed
137 with cKO mice (Fig. 2A). At day 14, total leukocyte numbers, Treg cell proportions and numbers
138 as well as their Foxp3 expression remained unaltered in CNS and dLN of icKO mice (Fig. 2B and
139 Fig. S6 B-D). Treg cell proportion was also normal at day 10 (Fig. S6E). The proportion of activated
140 CD44^{high} CD62L^{low} Treg cells was unchanged in dLN and CNS between icKO and control mice
141 (Fig. S7A). Also, Treg cells specific for the myelin oligodendrocyte glycoprotein 35-55 peptide
142 (MOG)₃₅₋₅₅, the immunizing antigen in EAE, were present in similar proportions in icKO and
143 control mice in both dLN and CNS (Fig. S7B). However, TNFR2-deficient Treg cells expressed
144 lower levels of CTLA-4 and Blimp-1 in the CNS (Fig. 2 C and D), as observed in cKO mice. Once
145 again, this altered Treg cell phenotype was not observed in the dLN, which is compatible with a
146 disease control by TNFR2⁺ Treg cells in the inflamed CNS rather than in the dLN. Moreover, this
147 suggests that TNFR2 was not involved in the initial activation steps but rather in the acquisition of
148 an optimal immunosuppressive state by Treg cells after day 7.

149 To further address the mechanism of EAE exacerbation, we performed RNA-sequencing on Treg
150 cells from the CNS and dLN of icKO and control mice at day 14. In the inflamed CNS, a number
151 of important genes exhibited altered expression in mutant Treg cells (Fig. 2 E and F). For instance,
152 the expression of several genes associated with the highly suppressive effector Treg cell subset,
153 such as *Myb*, *Ccr8* and *Cd177* (25), was down-regulated in TNFR2-deficient Treg cells.
154 Surprisingly, mRNA expression of *Ctla4* and *Prdm1* (Blimp-1) remained unchanged, suggesting

155 that TNFR2 ablation in Treg cells may lead to post-transcriptional modifications. Conversely,
156 genes usually associated with Tconv cell effector function, such as granzymes and perforin, and
157 even *Cd8a* and *Cd8b* genes, displayed augmented expression in mutant Treg cells. Gene set
158 enrichment analyses (GSEA) revealed an enrichment of the CD8⁺ Tconv cell signature in mutant
159 Treg cells (Fig S8A). This was not due to cell contamination of the samples as mutant Treg cells
160 expressed normal amounts of *Cd4* and *Zbtb7b* (ThPOK), as well as *Tbx21*, *Runx1* or *Runx3*,
161 compared to WT Treg cells (Fig. S8B). To confirm this data, some of the DEG were analyzed at
162 protein level in the CNS. *CD8a*, *Thy1* (coding for CD90) and *Eomes* that had higher level of mRNA
163 in TNFR2-deficient Treg cells (Fig. 8C), also displayed increased protein expression (Fig. S8D).
164 Importantly, the increased CD8 α expression was due to a slight shift of the whole Treg cell
165 population and not to the presence of few cells expressing high level of CD8, definitively ruling
166 out the hypothesis of the presence of CD8⁺ Tconv cell contaminants. Interestingly, expression of
167 the long non-coding RNA *Flicr*, which was described to negatively regulate *Foxp3* expression and
168 Treg cell function (26), was increased in the absence of TNFR2. GSEA further confirmed that
169 TNFR2-deficient Treg cells were less activated and did not acquire the full identity of Treg cells
170 from the inflamed CNS, when compared with control Treg cells (Fig. 2G). Importantly, the
171 differential expression of these genes in TNFR2-deficient Treg cells was not seen in dLNs, in which
172 only subtle decrease in genes involved in homing such as *Ccr1*, *Itga5* or *Itgam* was observed (Fig.
173 S9 A and B). Altogether, these results show that the loss of TNFR2 expression by Treg cells induces
174 alteration of their activation and identity during EAE, specifically in the CNS and not in the dLN.
175
176 **TNFR2 expression by Treg cells limits the activation and pathogenic function of Tconv cells**
177 **in the CNS.**

178 We then characterized the pathogenic profile of CD4 Tconv cells. Quite surprisingly, they acquired
179 a similar CD44^{high} CD62L^{low} activated phenotype in icKO and control mice and expressed
180 equivalent amounts of the pathogenic GM-CSF after strong polyclonal PMA ionomycin re-
181 stimulation in dLN and CNS (Fig. S10 *A and B*). Also, MOG₃₅₋₅₅ specific Tconv cells were present
182 in similar proportions in both types of mice in dLN and CNS (Fig. S10C). More informative data
183 were obtained when we performed transcriptomic analyses at day 14. In line with the aggravated
184 EAE symptoms and the impairment of the Treg cell gene expression profile in icKO mice,
185 transcriptomic analyses of CD4⁺ Tconv cell counterparts in the CNS showed major alterations. The
186 expression of 185 and 229 genes was respectively down- and up-regulated in Tconv cells isolated
187 from mutant animals, compared to controls (Fig. 3A). Tconv cells from icKO mice displayed a
188 highly activated phenotype. Notably, mRNA of cytokines and chemokines (*Csf2, Il22, Ifng, Tnf,*
189 *Il2, Lta, Ccl3, Ccl4*) and cytokine receptors and chemokine receptors as well as other activation
190 markers (*Il2ra, Il12r, Ifngr, Ccr2, Ccr5, Icos, Fasl, Ctla4, Gzmb*) were significantly increased in
191 these cells (Fig. 3B and Fig. S9C). They also expressed increased levels of genes of signaling
192 pathways (such as MAPK and NF-κB), interferon signature (*Ifit, Isg*) and transcription factors
193 (*Prdm1, Bhlhe40, Rora, Egr1, Fosl2, Junb*), known to be up-regulated in activated T cells.
194 Accordingly, the expression of genes characterizing resting T cells were down-regulated in Tconv
195 cells from the CNS of icKO mice, such as *Sell* (CD62L), *Ly6c1, Ccr7, Klf2, Bach2, Tcf7* or *Lef1*
196 (Fig. 3B). Among genes up-regulated in Tconv cells of icKO mice, network analysis of putative
197 protein-protein interactions connected two nodes, one regrouping genes coding for activation
198 markers with the other one belonging to an interferon signature. Interestingly, these two nodes were
199 connected by the *Tnf* and *Ifng* genes (Fig. S9D). The metabolic profile icKO Tconv cells further
200 supports their activated status with increased expression of most genes of the glycolytic pathway
201 as well as of *Slc2a1* (Glut1), the main glucose transporter in T cells, and *Hif1a*, a master positive

202 regulator of glycolysis (Fig. 3C and Fig. S9C). GSEA analysis further confirmed that these icKO
203 Tconv cells had an activated status (Fig. 3D). Not only these Tconv cells have an activated status,
204 but also their transcriptome suggests a Th1/Th17 pathogenic profile with increased expression of
205 *Csf2* (GM-CSF), *Il22*, *Ifng*, *Ifngr* or *Il12r*. We confirmed some of these results at the protein level
206 by measuring cytokines produced by CNS-infiltrating cells isolated at day 14 by ELISA. GM-CSF,
207 which is the major pathogenic cytokine produced by Tconv cells in EAE (27), was significantly
208 increased in icKO mice compared to control mice after both anti-CD3 and immunizing myelin
209 antigen stimulation (Fig. 3E). IL-17A and IFN- γ production were slightly increased in icKO Tconv
210 cells as well but not significantly. All these modified expression patterns (gene expression and
211 cytokine expression) were not observed -or at a much lower extend- in the dLN (Fig. S9 E-G).
212 Altogether, these data strongly suggest that TNFR2 expression by Treg cells is involved in the local
213 suppression of activated and pathogenic Tconv cells in the CNS after disease onset, but not in their
214 initial priming in lymphoid tissues.

215

216 **TNF/TNR2 signaling in Treg cells enables local suppression of EAE in the inflamed CNS.**

217 We then conducted experiments to further confirm the CNS-restricted role of TNFR2-expressing
218 Treg cells. In the course of active EAE, the disease is initiated in dLN and spleen. Then, from day
219 5, pathogenic Tconv cells migrate to the CNS where they are re-activated, leading to their
220 progressive accumulation and increased inflammation, perpetuating locally the disease process
221 (28). We first analyzed the phenotype of T cells during priming in lymphoid tissues to evaluate
222 their capacity to acquire pathogenic features. We assessed expression of CD11a (LFA-1 α chain),
223 CXCR3, CCR6 and CD49d (VLA-4 α chain) because of their involvement in T cell migration from
224 lymphoid tissues to inflamed CNS during EAE (29, 30). Ten days after EAE induction, these

225 molecules were expressed at the same levels in control and cKO mice, suggesting unaltered
226 migration capacity into the CNS (Fig. S11). To more directly quantify priming of MOG-reactive
227 T cells, we transferred T-cell receptor-transgenic Tconv cells specific for this antigen in cKO and
228 control mice. Then, mice were immunized with MOG₃₅₋₅₅ peptide and we measured the activation
229 of donor cells in dLN and spleen 3 and 7 days later. The level of T-cell proliferation and expansion
230 was high and comparable in the 2 groups of mice (Fig. 4A and Fig. S12 A and B). Donor MOG-
231 specific T cells expressed similar levels of CD11a and CD49d and of the IFN γ , IL-17A and GM-
232 CSF pathogenic cytokines after PMA-ionomycine stimulation in cKO mice compared to controls
233 (Fig. S12 C and D). Together with the unchanged proportions of MOG-reactive T cells we detected
234 in icKO animals (Fig. S10C), these data suggest a normal priming of MOG-specific Tconv cells in
235 lymphoid tissues in both cKO and icKO mice. To further assess the pathogenicity of polyclonal
236 MOG-specific T-cells primed in dLN, we measured their capacity to induce EAE after adoptive
237 transfer in naive mice. Remarkably, cells primed in cKO and control mice, and re-stimulated ex
238 vivo, induced similar passive EAE (Fig. 4B). Thus, all these data concur to show that exacerbation
239 of EAE in cKO mice was not due to enhanced initial priming of MOG-specific T cells in spleen
240 and dLN. Then, to further confirm that TNFR2-expressing Treg cells selectively controlled EAE
241 severity within the inflamed CNS, we transferred WT pathogenic T cells to cKO or control naive
242 mice. In this setting, injected cells rapidly migrated into the CNS to damage the neural tissue during
243 passive EAE, without being primed in the dLN. Strikingly, EAE was much more severe in cKO
244 than control recipients (Fig. 4C). We obtained similar findings when we transferred pathogenic T-
245 cells in WT recipients that were treated with an anti-TNF blocking mAb (Fig. 4D). Taken together,
246 these results confirm our earlier findings that TNFR2 signaling in Treg cells is critical to control
247 EAE within the inflamed CNS, rather than limiting the priming of pathogenic T-cells in lymphoid
248 tissues.

249

250 **Systemic modulation of the TNF/TNFR2 axis affects EAE severity.**

251 The protective facet of TNF in CNS autoimmunity relied originally on observations made in
252 patients. Indeed, anti-TNF therapies are formally contra-indicated in MS patients because of
253 disease exacerbation (31, 32). The mechanism of this long-term conundrum, well known to
254 clinicians, remains essentially unexplained. To investigate if our earlier findings could bring a
255 mechanistic insight to this adverse effect of anti-TNF treatments, we assessed the effect of this
256 therapy on EAE severity and Treg cell phenotype in EAE. WT mice were treated with a blocking
257 anti-TNF mAb from day 10 - a time when they developed the first clinical signs - until day 18. As
258 observed in MS patients, these mice exhibited an aggravated form of EAE compared to isotype-
259 control treated mice (Fig. 5A). Similar findings were obtained when using the clinically approved
260 soluble TNFR2-Fc fusion receptor (Etanercept) to block TNF (Fig. 5B). Interestingly, TNF-
261 blockade at earlier time points (from day 0) did not significantly modify the course of the disease
262 (Fig. 5C), highlighting once more the protective role of TNF during established disease and not
263 during Tconv cell priming. We next analyzed the Treg cell compartment in the inflamed CNS of
264 these mice. In anti-TNF treated mice, Treg cell proportion was significantly reduced in the CNS
265 compared to control animals, whereas it was unchanged in dLN. These observations are similar to
266 the ones we previously made in TNFR2 conditional KO mice and, which further support the CNS-
267 restricted role of TNF on Treg cells (Fig. 5D). This protective effect of TNF was most likely due
268 to TNFR2 triggering since blocking this receptor with a specific mAb worsened EAE (Fig. 5E),
269 similarly to anti-TNF mAb treatment or TNFR2 ablation in Treg cells. Finally, in a therapeutic
270 perspective, we investigated whether stimulating TNFR2 signaling in the course of the disease
271 could improve disease outcome. Remarkably, the use of a TNFR2 specific agonist from day 4 to
272 18 significantly reduced EAE severity (Fig. 5F). This therapeutic effect was lost in icKO animals,

273 suggesting that this agonist functions by stimulating TNFR2-expressing Treg cells (Fig. S13).
274 Collectively, these data are in line with our previous findings, highlighting the protective role of
275 TNF in CNS during EAE and providing a mechanistic explanation for the deleterious events
276 following anti-TNF administration in patients with MS.

277

278

279

DISCUSSION

280 The protective properties of Treg cells in autoimmune diseases are clearly established. However,
281 when and where do endogenous Treg cells control these diseases remain unknown. Some published
282 works provided indirect evidence that Treg cells may suppress an autoimmune disease in the target
283 organ. Indeed, late accumulation of Treg cells was observed in the inflamed CNS during EAE (6,
284 33, 34). Systemic Treg cell deletion precipitated established autoimmune diseases but at the
285 expense of massive and multifocal inflammation, precluding a proper evaluation of physiological
286 function of Treg cells (6, 35). Administration of Treg cells specific for the target tissue had a
287 therapeutic efficacy in organ-specific autoimmune diseases, suggesting a capacity to suppress
288 pathogenic cells locally (36, 37). However, these studies did not address the role of endogenous
289 Treg cells during the natural course of the disease. Here, we found that TNFR2 ablation in Treg
290 cells after EAE induction had no systemic impact, but led to increased CNS inflammation and
291 disease severity. This suggested that Treg cells suppressed EAE at peak of disease in the inflamed
292 CNS and not during T-cell priming in the dLN. This hypothesis was further supported by the
293 following cumulative proofs. (i) Treg cell phenotype, and their proportion in some models, were
294 altered in the CNS but not in the dLN in cKO mice, icKO mice or WT mice treated with anti-TNF
295 drugs. (ii) Increased expression of activation molecules and pathogenic GM-CSF by Tconv cells
296 could be observed in the CNS of icKO mice but not in dLN. The increased production of GM-CSF
297 in icKO mice was no longer observed after PMA-ionomycin stimulation, probably because this
298 strong and non-physiological activation could outweigh the difference observed with more
299 physiological T cell receptor stimulation. (iii) Activation and expansion in lymphoid tissues and
300 migration into the CNS of polyclonal and MOG-specific T cells appeared normal in cKO and icKO
301 mice, as compared to control mice. (iv) Blocking TNF in WT mice from day 10, but not from day
302 0, after disease induction led to EAE exacerbation, suggesting that the early autoimmune process

303 that was primed in dLN was not altered by the absence of TNFR2 in Treg cells. In the same line,
304 disease exacerbation following TNFR2 ablation in Treg cells was obtained when this deletion
305 obtained by tamoxifen administration was performed 7 days after disease induction. (v) Cell
306 transfer experiments showed that the activation of myelin-specific T-cells in spleen and dLN, as
307 well as their capacity to induce passive EAE, were largely similar in cKO and control mice.
308 However, it remains possible that the ex vivo re-stimulation protocol, which is required to induce
309 disease, may have skewed the potential differences between Tconv cells from the two strains. (vi)
310 Finally, pathogenic T cells from WT mice that were re-activated in the CNS after their injection
311 induced a more severe disease in cKO than in control recipients. Thus, the control of EAE by
312 TNFR2-expressing Treg cells is regulated in the inflamed CNS and not in the dLN. Together, our
313 work strongly supports the concept that endogenous Treg cells suppress an autoimmune disease in
314 the target tissue.

315 Our findings also provide some insight into the mechanism of EAE control by Treg cells in the
316 CNS. Indeed, TNFR2 expression by Treg cells appears to control their function rather than their
317 numbers. We did find a decreased proportion of Treg cells in the CNS at day 10 in cKO mice but
318 this was no more observed earlier (day 7) or later (day 15) in these mice or in the icKO animals.
319 More importantly, TNFR2-deficient Treg cells have lower mRNA expression of several Treg cell-
320 signature genes and increased expression of genes normally expressed by Tconv cells. Also,
321 TNFR2-deficient Treg cells expressed lower level of *Myb* that was shown to be essential for
322 differentiation of effector Treg cells (25). The lack of TNFR2 expression in Treg cells seems to
323 have an additional impact on protein expression, as observed for CTLA-4 and Blimp-1, whose
324 protein levels were decreased in CNS of icKO mice. This may alter Treg cell suppressive activity,
325 as CTLA-4 is one of the major mechanisms of Treg cell-mediated suppression and Blimp-1 is
326 critical to promote IL-10 production (1, 23, 24). Also, Blimp-1 is expressed by the fraction of

327 highly suppressive effector Treg cells and is up-regulated in CNS Treg cells during EAE.
328 Moreover, its ablation in Treg cells led to exacerbated EAE, reduced Treg cell identity and
329 increased expression of inflammatory cytokines, such as IL-17 (2, 3). Consequently, TNFR2-
330 deficient Treg cells would be deficient in suppressing Tconv cells in the CNS, leading to massive
331 Tconv cell activation, as observed at the level of activation markers, cytokines and chemokines,
332 signaling molecules, transcription factors and glucose metabolism. These highly activated Tconv
333 cells produced increased level of pathogenic cytokines such as GM-CSF, precipitating EAE. In
334 conclusion, our data demonstrate that TNFR2 expression by Treg cells is essential to limit EAE
335 severity by promoting their transient expansion and by increasing their suppressive function in the
336 CNS, thereby limiting pathogenic Tconv cell activation. Thus, we reveal here a non-redundant
337 function of TNF in the control of EAE in the inflamed CNS. Recent findings emphasized the
338 critical role of IL-33 in the accumulation of Treg cells residing in the intestine or adipose tissues
339 (38-40). Thus, depending on the tissue and type of inflammation, Treg cells may rely on different
340 environmental cues (IL-33, TNF) for their homeostasis and function.

341 Besides this non-redundant function of TNFR2 in Treg cells, our data also bring a mechanistic
342 explanation to the deleterious effect of TNF-blockade in patients with MS. A series of pre-clinical
343 studies in mice conducted in the nineties concluded that blocking TNF was beneficial in EAE
344 (reviewed in (41)). These findings led to initiate two clinical trials in MS patients that had to be
345 rapidly stopped because of disease aggravation (31, 32). Here, we revisited this question using
346 reagents that selectively blocks TNF, whereas older studies used drugs blocking both TNF and
347 lymphotoxin- α , which is an issue since the latter cytokine is pathogenic in EAE (42). We found
348 that blocking TNF at disease's peak induced EAE exacerbation, as in MS patients who were also
349 treated during advanced disease progression. Interestingly, when TNF was blocked at earlier times,
350 EAE was not exacerbated, and even slightly delayed. Thus, at disease initiation, TNF might be

351 pathogenic by activating antigen-presenting cells via TNFR1, whereas it would regulate the disease
352 afterwards, by activating Treg cells via TNFR2 in the inflamed CNS. In this line, blocking TNFR1
353 at disease induction attenuated EAE (43), whereas we showed that blocking TNFR2 at day 10
354 induced disease exacerbation, similarly to late TNFR2 ablation in Treg cells. Furthermore, we
355 demonstrated that stimulation of TNFR2 signaling reduced EAE severity.

356 In conclusion, our data reveal that the TNF/TNFR2 axis is critical to reach an optimal Treg cell
357 function specifically in the CNS during EAE, thereby preventing excessive inflammation and
358 controlling disease severity. Moreover, our results bring new insights in the mechanism of
359 autoimmune disease control by Treg cells and provide an explanation for the failure of anti-TNF
360 therapy in MS patients, paving the way to the development of more specific treatments aiming at
361 the selective blockade of TNFR1 and/or selective stimulation of TNFR2.

362

METHODS

363
364
365 **Mice.** C57BL/6J (WT) mice were purchased from Janvier Labs (France). *Tnfrsf1b*^{tm1Mwm/J}
366 (*Tnfrsf1b*^{-/-}), *Foxp3*^{tm9(EGFP/cre/ERT2)Ayr/J} (*Foxp3*^{Cre-ERT2}) and C57BL/6
367 Tg(Tcra2D2,Tcrb2D2)1Kuch/J (2D2) T cell receptor transgenic mice, specific for myelin
368 oligodendrocyte glycoprotein, were purchased from the Jackson Laboratory. *Cd3*^{etm1Mal} (*Cd3*^{-/-}),
369 CD45.1 and CD90.1 mice were provided by the Cryopreservation Distribution Typing and animal
370 Archiving department (Orléans, France). B6.129(Cg)-*Foxp3*^{tm4(YFP/cre)Ayr/J} (*Foxp3*^{Cre}) mice were a
371 gift from Pr. Alexander Rudensky. C57BL/6-*Tnfrsf1b*^{<tm1c(EUCOMM)Wtsi>/Ics} (*Tnfrsf1b*^{fl}) mice were
372 obtained from the EMMA consortium. All mice were on a C57BL/6J background or have been
373 backcrossed at least 10 times to C57BL/6J mice. Mice were housed under specific pathogen-free
374 conditions and were studied at 7–14 weeks of age or two months after bone marrow transplanted.
375
376 **TNF- and TNFR2-targeting biological.** Anti-TNF (XT3-11) and anti-TNFR2 (TR75-54.7) mAb
377 were purchased from BioXCell and were injected at a dose of 500 µg by intraperitoneal route every
378 other day for 8 or 14 days. Etanercept (TNFR2-Fc) was provided by Wyeth and was administered
379 at a dose of 1 mg by intraperitoneal route every other day for 8 days. Purification and functional
380 characterization of the TNFR2-specific agonist STAR2 has been described elsewhere (10).
381
382 **Bone marrow transplantation.** Bone marrow cells were isolated from tibia and femur of donor
383 mice. Recipient mice were lethally irradiated (10.5 Gray) and transplanted intravenously with 10
384 x 10⁶ bone marrow cells. EAE was induced at least 8 weeks after transplantation.
385

386 **EAE induction.** For active EAE, mice were injected subcutaneously in the flanks with 100 µg of
387 MOG₃₅₋₅₅ peptide (Polypeptide) emulsified in 100 µl of complete Freund adjuvant (Sigma-Aldrich)
388 supplemented with 50 µg of heat-killed *Mycobacterium tuberculosis* H37Ra (BD Biosciences).
389 Animals were additionally injected intravenously with 200 ng of *Bordetella pertussis* toxin (Enzo)
390 at the time of immunization and two days later. For the passive EAE model, we first induced active
391 EAE in donor mice as described above to generate pathogenic cells. Ten days post-immunization,
392 cells from spleen and dLN were cultured in complete RPMI (Gibco) medium with 10% fetal calf
393 serum at 5×10^6 cells/ml with 20 µg/ml of MOG₃₅₋₅₅ peptide, 10 µg/ml of anti-IFN γ (XMG1.2,
394 BioXCell) and 5 ng/ml of IL-23 (R&D Systems). After 3 days, dead cells were removed with a
395 Ficoll gradient and 2×10^6 cells were injected intravenously to recipient mice to induce passive
396 EAE. The clinical evaluation was performed on a daily bases by a 6-point scale ranging: 0, no
397 clinical sign; 1, limp tail; 2, limp tail, impaired righting reflex, and paresis of one limb; 3, hind
398 limb paralysis; 4, hind limb and forelimb paralysis; 5, moribund/death. A score of 5 was
399 permanently attributed to dead animals.

400
401 **Preparation of cell suspensions.** For isolation of CNS-infiltrating leukocytes, mice were
402 anesthetized with a xylazine/ketamine solution and perfused with cold PBS. Spinal cords were
403 removed by intrathecal hydrostatic pressure. Brain and spinal cords were cut into small pieces and
404 digested in RPMI 1640 (Gibco) supplemented with 1 mg/ml collagenase type IV (Sigma), 100
405 µg/ml DNase I (Sigma) and 1 µg/ml TLCK for 30 min at 37°C followed by mechanical
406 desegregation. Single cell suspensions were washed once and re-suspended in Percoll 40%. For
407 FACS analysis, cells were laid on a Percoll 80% solution, centrifuged for 20 min at 2,000 rpm at
408 room and mononuclear cells were collected from the interface of the 40:80% Percoll gradient and
409 were washed two times in a PBS-3% fetal calf serum buffer. For FACS cell sorting, cells were

410 centrifuged for 10 minutes at 400g and mononuclear cells were collected from the pellet of the
 411 monolayer Percoll gradient. Cells from thymus, spleen and LN were obtained after mechanical
 412 dilacerations.

413
 414 **Antibodies and flow cytometry analysis.** The mAb and fluorescent reagents used in this study
 415 are listed below in the table. Intracellular staining was performed using the Foxp3/Transcription
 416 Factor Staining Buffer Set kit and protocol from eBioscience. Identification of MOG-specific T
 417 cells was performed using the I-A(b) mouse MOG 38-49 GWYRSPFSRVVH APC-labeled
 418 tetramer, obtained from the NIH Tetramer Facility, according to their protocol. Intracellular
 419 cytokine staining (Fig S5, S10 and S12) was assessed after 4 hours of PMA (25 ng/ml) ionomycin
 420 (1 mg/ml) stimulation in RPMI 1640 containing 10% FCS and Golgi plug (BD Biosciences).
 421 Cells were acquired on a BD LSRII cytometer and analyzed using the FlowJo software.

mAb or reagent	Clone	Vendor	Reference	Dilution
BV510 anti-mouse CD4	RM4-5	BD Biosciences	563106	1/500
BUV496 anti-mouse CD4	GK1.5	BD Biosciences	564667	1/400
Alexa Fluor® 700 anti-mouse CD8a	53-6.7	BD Biosciences	557959	1/400
BUV805 anti-mouse CD8a	53-6.7	BD Biosciences	564920	1/400
PerCP-C5.5 anti-mouse CD11a	2D7	BD Biosciences	562809	1/100
Biotin anti-mouse CD25	7D4	BD Biosciences	553070	1/300
PE-Cy7 anti-mouse CD44	IM7	Invitrogen/eBioscience	25-0441-82	1/400
BUV395 anti-mouse CD45	30-F11	BD Biosciences	564279	1/400
PE-CF594 anti-mouse CD45	30-F11	BD Biosciences	562420	1/1000
PE anti-mouse CD45.1	A20	BD Biosciences	561872	1/100
Biotin anti-mouse CD45.1	A20	Miltenyi	130101902	1/10
PE anti-mouse CD49d	R1-2	BD Biosciences	553157	1/100
AF700 anti-mouse CD62L	MEL-14	BD Biosciences	560517	1/100
APC anti-mouse CD90.1	OX-7	BD Biosciences	561409	1/400
PE-Cy7 anti-mouse ICOS	7E.17G9	Invitrogen/eBioscience	25-9942-82	1/400
PE anti-mouse CTLA-4 (CD152)	UC104F10-11	BD Biosciences	553720	1/200
Alexa Fluor® 647 anti-mouse Blimp1	5E7	BD Biosciences	563643	1/100
PE-Cy7 anti-mouse Eomes	Dan11mag	Invitrogen/eBioscience	25-4875-80	1/100
BV421 anti-mouse TNFR2 (CD120b)	TR75-89	BD Biosciences	564088	1/200

FITC anti-mouse GITR	DTA-1	BD Biosciences	558139	1/200
PE anti-mouse V β 11	RR3-15	BD Biosciences	553198	1/200
BV421 anti-mouse CCR6	140706	BD Biosciences	564736	1/20
APC anti-mouse CXCR3	CXCR3-173	BD Biosciences	562266	1/100
APC anti-human/mouse Foxp3	FJK-16s	Invitrogen/eBioscience	17-5773-82	1/200
FITC anti-human/mouse Foxp3	FJK-16s	Invitrogen/eBioscience	11-5773-82	1/200
PE-e610 anti-huma/mouse Foxp3	FJK-16s	Invitrogen/eBioscience	61-5773-82	1/100
eF450 anti mouse/human Ki67	SOLA15	Invitrogen/eBioscience	48-5698-82	1/400
AF647 anti-mouse IFN γ	XMG1.2	BD Biosciences	557735	1/100
APC-Cy7 anti-mouse IL-17A	TC11-18H10	BD Biosciences	560821	1/100
PE anti-mouse GM-CSF	MP1-22E9	BD Biosciences	554406	1/100
BV421 Streptavidin		BD Biosciences	563259	1/400
PE-Cy7 Streptavidin		Invitrogen/eBioscience	25-4317-82	1/200
e450 Cell trace		Life technologies		1/2000
e506 Fixable viability dye		Invitrogen/eBioscience	65-0866-14	1/1000

422

423 **Treg and Tconv cell purification.** For suppression assay, colitis and transcriptomic analyses, cell
424 suspensions from the spleen, LN or the CNS were stained with e780 viability dye, BV510 anti-
425 CD4 (RM4-5) and PE-CF594 anti-CD45 (30F11) mAbs. Purified Tconv cells (CD4⁺YFP⁻ or
426 CD4⁺GFP⁻) and Treg cells (CD4⁺YFP⁺ or CD4⁺GFP⁺) were obtained using a BD FACSAria II. For
427 transcriptomic analyses, cells were sorted twice with the cytometer leading to over 99.8% purity.
428

429 **Cell culture.** Culture medium was composed of RPMI 1640 (Gibco) supplemented with 10% fetal
430 calf serum. For suppression assay, purified Tconv cells (2.5 x 10⁴ cells/well), labeled with
431 CellTrace Violet (Proliferation Kit, Life technologies), and purified Treg cells were stimulated by
432 splenocytes from *Cd3^{-/-}* mice (7.5 x 10⁴ cells/well) and soluble anti-CD3 mAb (0.05 μ g/ml 2C11,
433 BioXCell) in 96-well plate at different Treg/Tconv cell ratios. At day 3, CellTrace dilution was
434 assessed by flow cytometry. To assess TNFR2 expression, whole splenocytes (3 x 10⁶ cells/well)
435 were stimulated by soluble anti-CD3 (5 μ g/ml 2C11, BioXCell) in 96-well plate. For ELISA studies
436 (Fig. 3, S9), cells from 3.5 x 10⁶ LN cells in 2 ml in 24-well plates or 1 x 10⁵ CNS cells in 200 μ l

437 in 96-round bottom well plates were stimulated with 10 or 100 $\mu\text{g/ml}$ of MOG or with 5 $\mu\text{g/ml}$ of
438 anti-CD3 mAb (2C11) for 24h (CNS cells) or 72h (LN cells) before performing ELISA on the
439 supernatant using kits from Invitrogen.

440
441 **Adoptive transfer of 2D2 T cells.** LN and spleen cells from 2D2 CD90.1 or 2D2 CD45.1 mice
442 were stained incubated with anti-CD19 (6D5), anti-CD11b (M1/70), anti-CD11c (N418), anti-CD8
443 (53-6.7) and anti-CD25 (7D4) biotin-labeled mAbs and then were coated with anti-biotin
444 microbeads (Miltenyi Biotec). After magnetic sorting, cells of the CD4⁺ enriched negative fraction
445 were labeled with CellTrace Violet Proliferation Kit and were intravenously injected in naive mice
446 (10^6 cells/mouse). The following day, mice were immunized with MOG₃₅₋₅₅ peptide as for EAE
447 induction. Donor cells, identified by their CD4⁺CD90.1⁺V β 11⁺ (T cell receptor transgene) or
448 CD45.1⁺CD3⁺ phenotypes, were analyzed by flow cytometry in dLNs and spleen 3 or 7 days later.

449
450 **RNA-sequencing and bioinformatics analyses.** RNA was extracted from highly purified Treg
451 and Tconv cells using the NucleoSpin RNA XS kit from Macherey-Nagel, quantified using a ND-
452 1000 NanoDrop spectrophotometer (NanoDrop Technologies) and purity/integrity was assessed
453 using disposable RNA chips (Agilent High Sensitivity RNA ScreenTape) and an Agilent 2200
454 TapeStation (Agilent Technologies, Waldbrunn, Germany). mRNA library preparation was
455 performed following manufacturer's recommendations (SMART-Seq v4 Ultra Low Input RNA Kit
456 TAKARA). Final-17 samples pooled library prep was sequenced on Nextseq 500 ILLUMINA with
457 HighOutput cartridge (2x400Millions of 75 bases reads), corresponding to 2 times 23×10^6 reads
458 per sample after demultiplexing. Poor quality sequences have been trimmed or removed with
459 Trimmomatic software to retain only good quality paired reads. Star v2.5.3a (44) has been used to

460 align reads on reference genome mm10 using standard options. Quantification of gene and isoform
461 abundances has been done with rsem 1.2.28 (45) prior to normalization on library size with
462 DESeq2 bioconductor package. Finally, differential analysis has been conducted with edgeR
463 bioconductor package. Multiple hypothesis adjusted p-values were calculated with the Benjamini-
464 Hochberg procedure to control FDR. For Treg cells, data were obtained from a biological triplicate.
465 For Tconv cells, data were obtained from two independent experiments with biological
466 quadruplicate, after exclusion of two outliers. GSEA has been done with fgSEA bioconductor R
467 package (v1.12.0) on pre-ranked list. Genes in our data set ([GSE165821](#)) were ranked according
468 to the signed fold-change multiplied by the $-\log_{10}$ p-value of the differential analysis. **Gene sets**
469 **were extracted from [GSE165821](#) (Fig. 2G, right panel), [GSE146135](#) (Fig. 2G, left panel) and**
470 **[GSE11057](#) (Fig. 3D).**

471
472 **Statistics.** Statistical analyses were performed using GraphPad Prism Software v8.3.1. For EAE
473 scores and 2-groups comparisons, statistical significance was determined using the two-tailed
474 unpaired nonparametric Mann–Whitney U-test, excluding D0 to D5 as all scores were 0. For
475 survival curves, Log-Rank (Mantel-Cox) test was used. For ELISA assays, 2-way unpaired
476 ANOVA test with Sidak correction for multiple comparisons was used. $*p<0.05$, $**p<0.01$,
477 $***p<0.001$, $****p<0.0001$.

478
479 **Study approval.** All experimental protocols were approved by the “Comité d’éthique en
480 expérimentation animal Charles Darwin N°5” under the number 02811.03 and are in compliance
481 with European Union guidelines.

482

483 **Data Availability Statement:** The RNA-Seq data reported in this paper have been deposited in
484 the Gene Expression Omnibus (GEO) database, <https://www.ncbi.nlm.nih.gov/geo> (accession no.
485 GSE165821).

486

487
488

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501

502

DECLARATION OF INTERESTS

503 The authors declare no competing interests.

504

505

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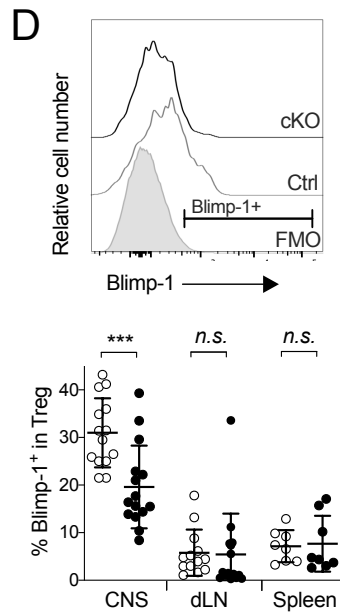
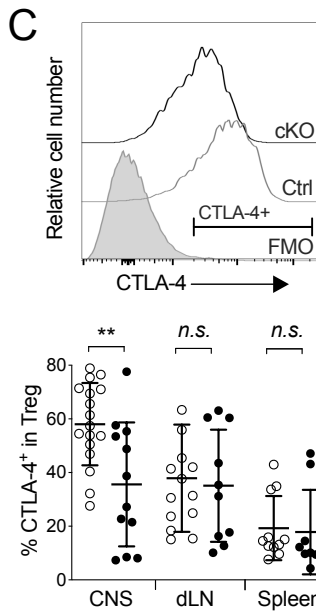
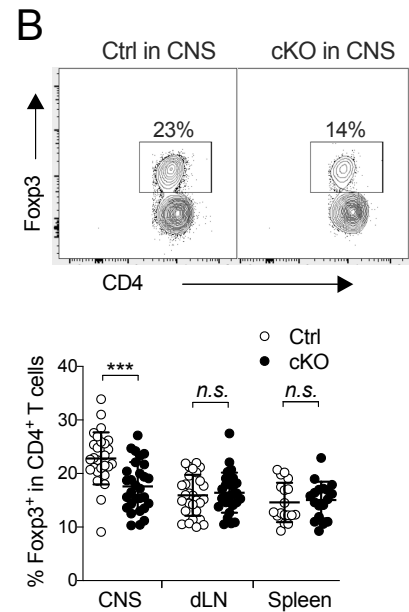
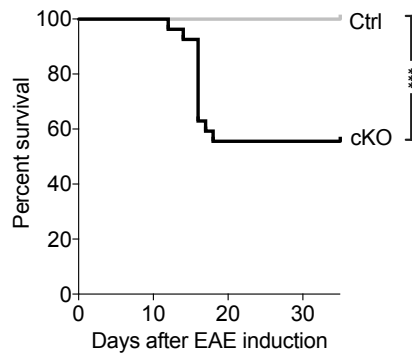
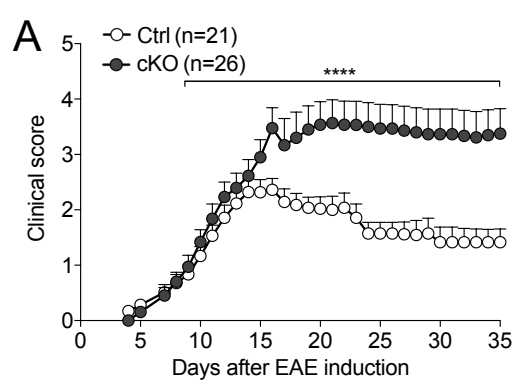
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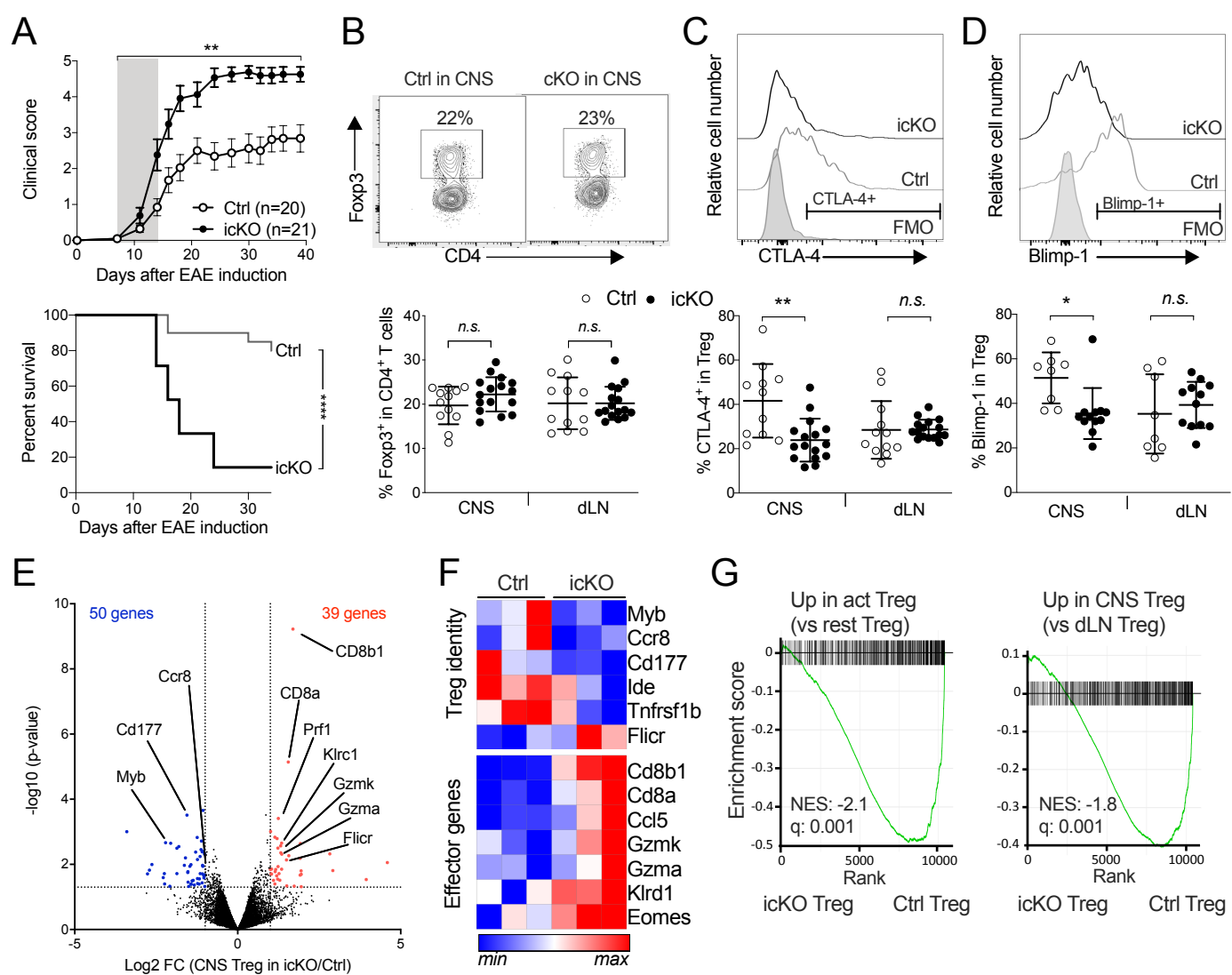
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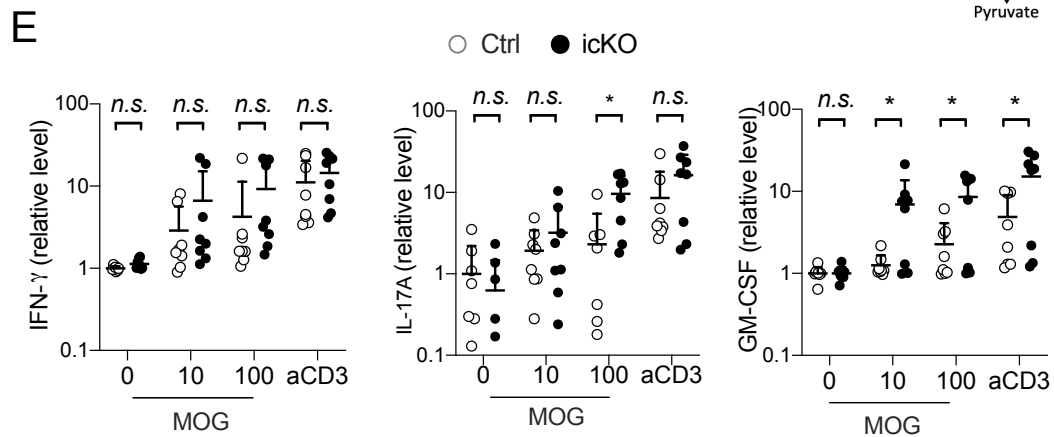
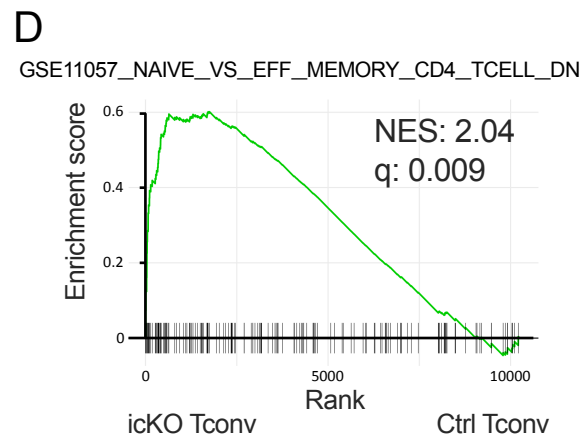
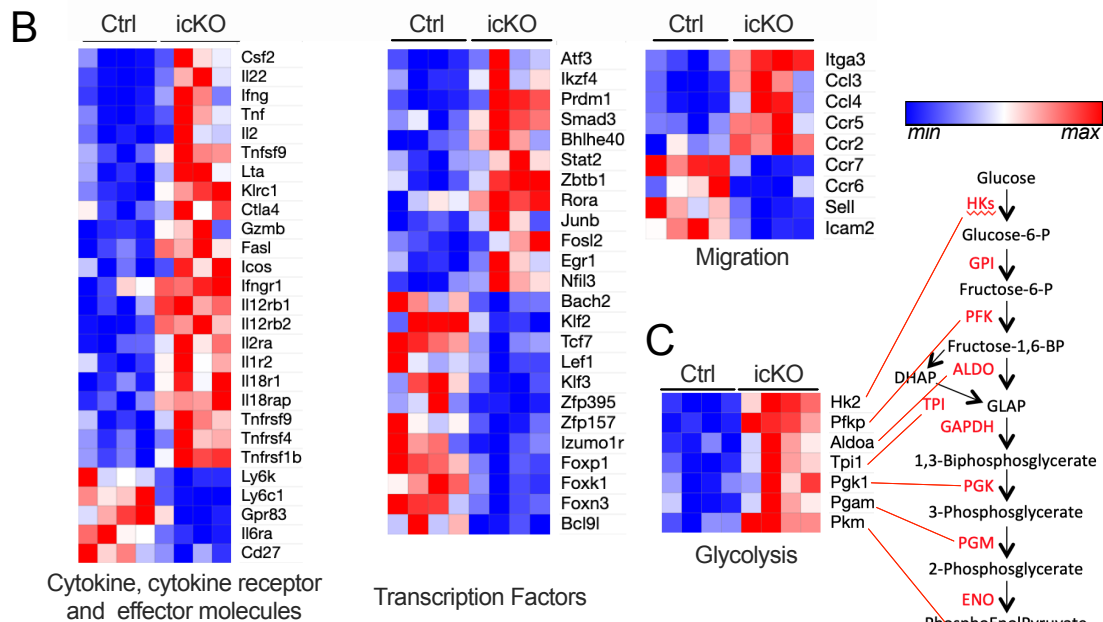
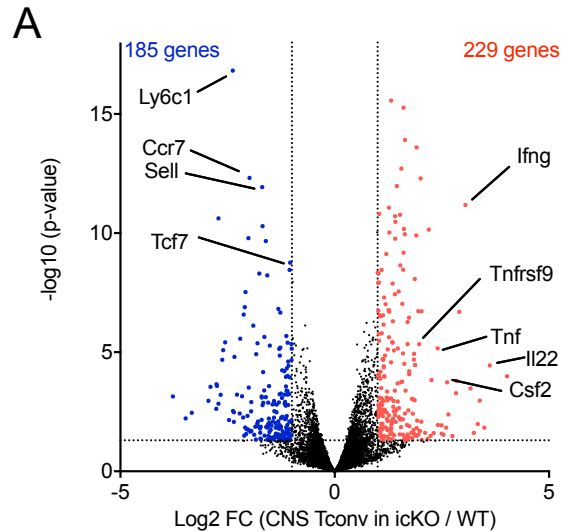
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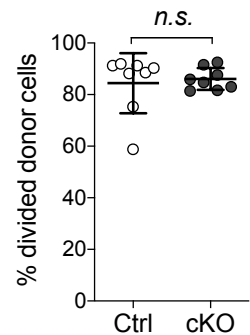
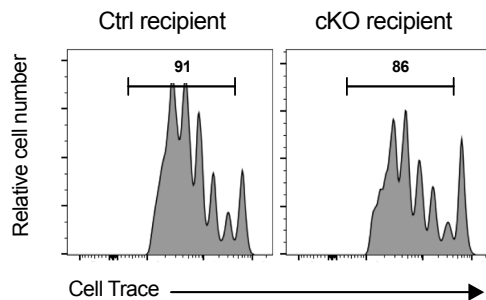
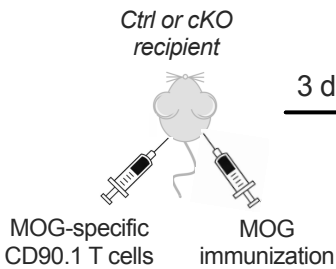
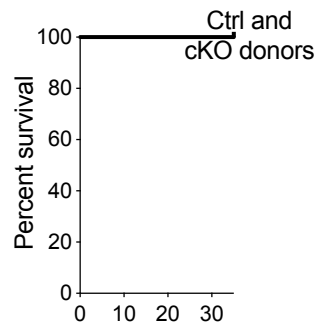
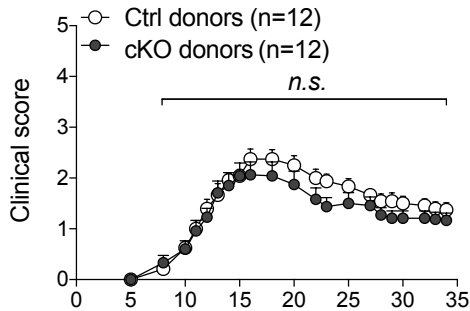
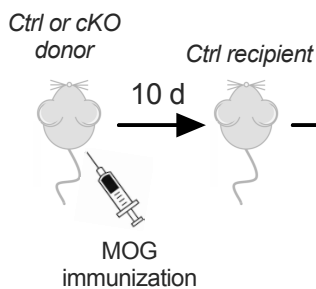
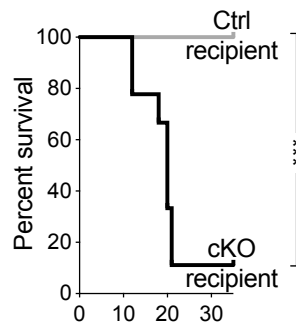
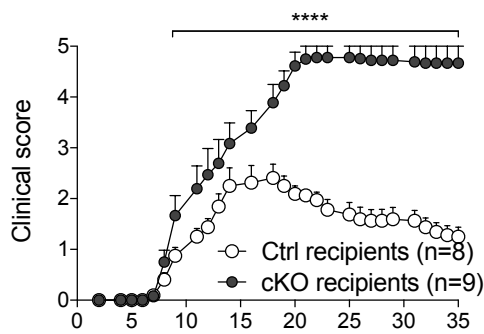
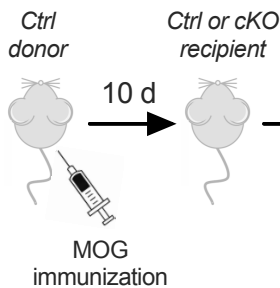
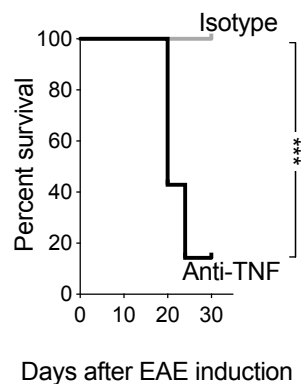
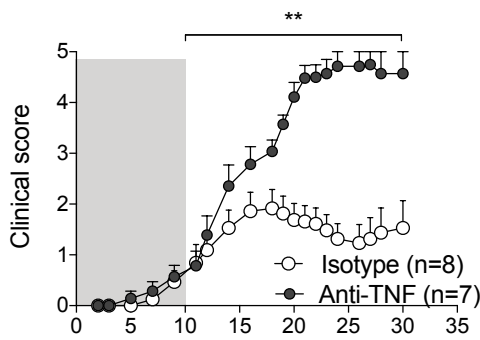
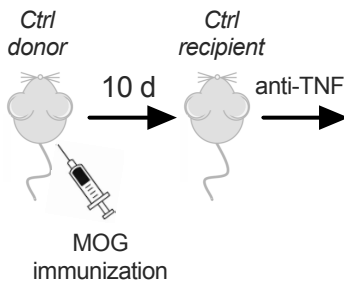
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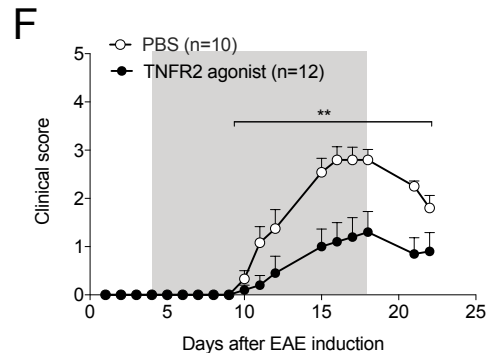
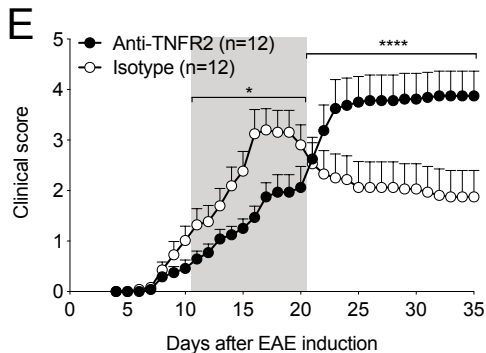
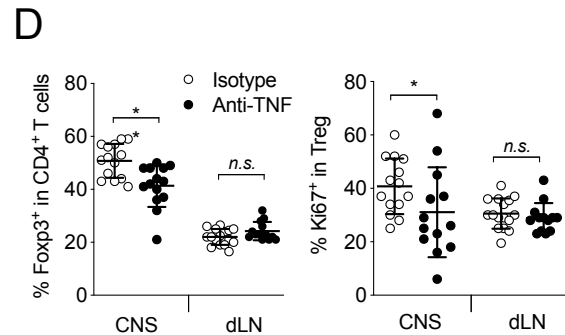
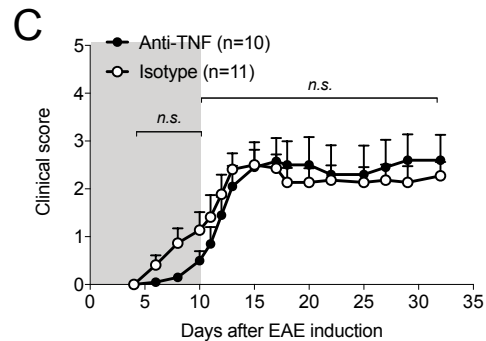
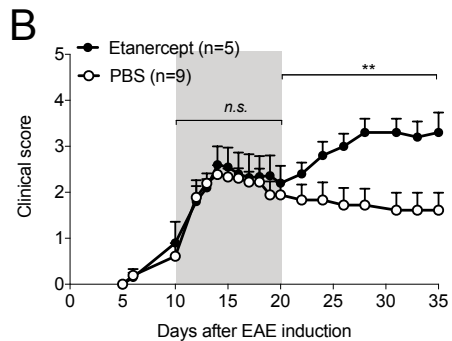
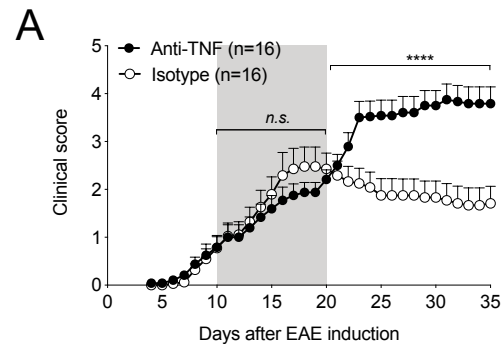
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Supplementary Information for

Tissue-restricted control of established central nervous system autoimmunity by TNF receptor 2 expressing Treg cells

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This PDF file includes:

Figures S1 to S13

SI References

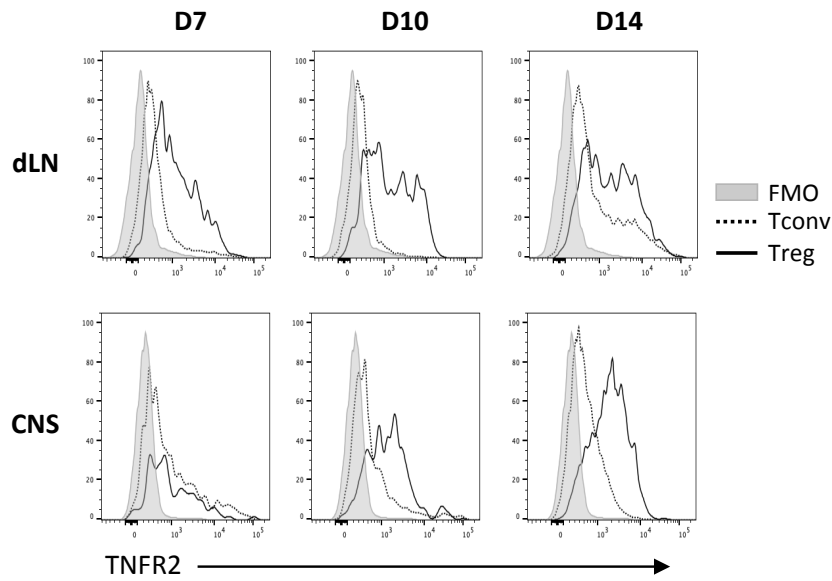


Figure S1. TNFR2 is highly expressed by Treg cells over the course of EAE. TNFR2 expression on Tconv and Treg cells, freshly isolated from dLN and CNS of C57BL/6 mice at day 7, 10 and 14 after EAE induction. Representative data from 2 independent experiments.

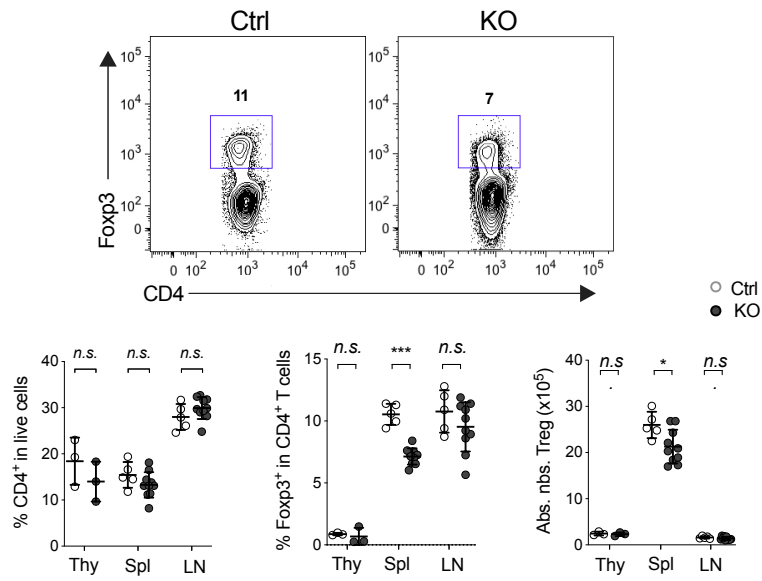


Figure S2. Partial defect of Treg cells in the spleen of *Tnfrsf1b*^{-/-} (KO) mice. Proportions and absolute numbers of total CD4⁺ and Treg cells in KO and wild type (Ctrl) mice in the thymus (Thy), spleen (Spl) and LN. Representative flow cytometry plots in the spleen (upper panels). Each dot represents a mouse from 2 independent experiments (lower panels). Mean (+/-S.D.) is shown. Two-tailed, unpaired Mann-Whitney tests were used; ****p<0.0001, n.s.: not significant.

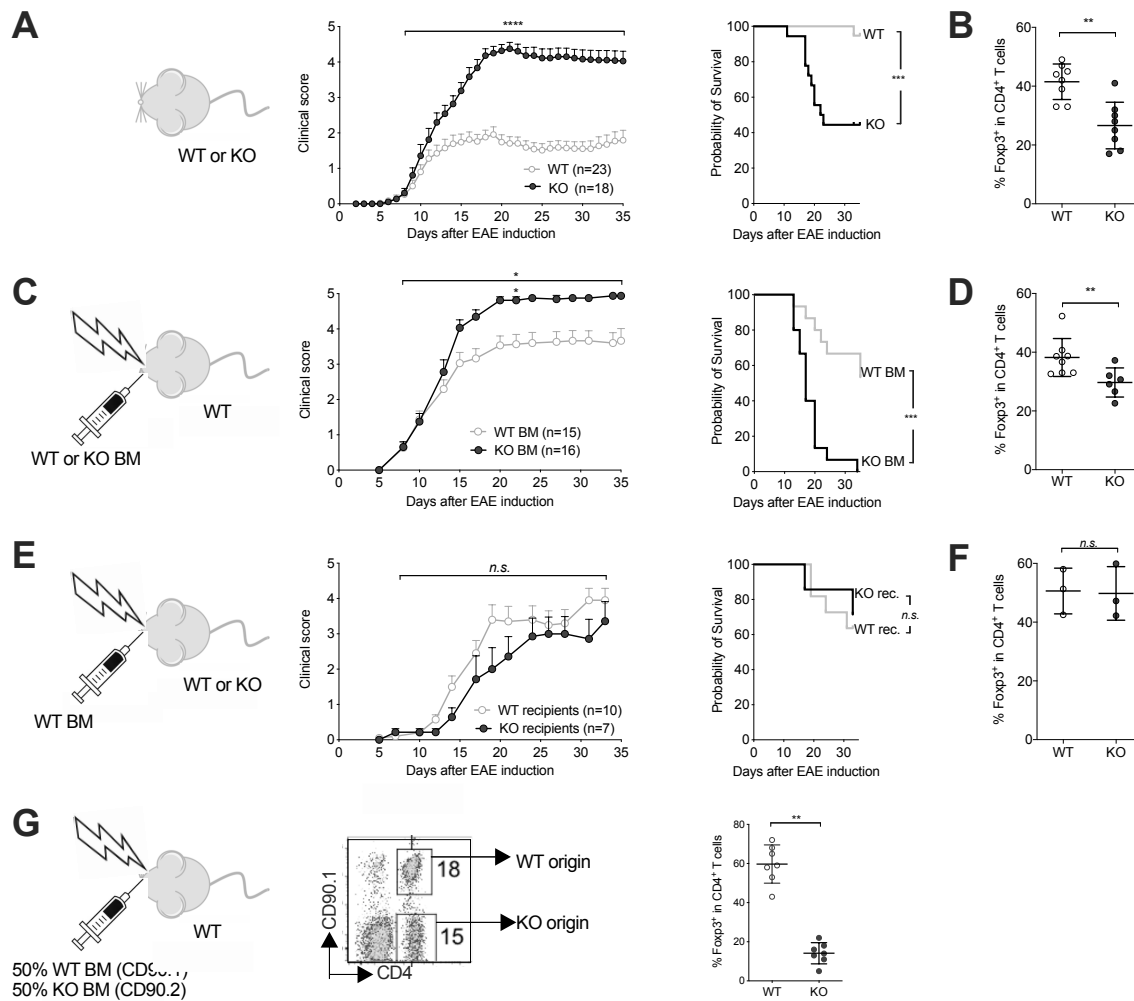


Figure S3. TNFR2 expression by hematopoietic cells is required to limit EAE severity and promotes Treg cell-intrinsic expansion in the CNS. EAE was induced in *Tnfrsf1b*^{-/-} (KO) or WT littermate control mice (A, B), in WT recipients reconstituted with KO or WT bone marrow cells (C, D), in KO or WT recipients reconstituted with WT bone marrow cells (E, F), and in WT recipients reconstituted with 1:1 ratio of a mix of KO CD90.2⁺ and WT CD90.1⁺ bone marrow cells (G). EAE clinical score (Mean + SEM) and disease survival (A, C, E). Treg cell proportion in the CNS at day 10-15 (B), day 16-19 (D) and day 20 (F) and their proportion from WT or KO origins at day 20 (G). Data were collected from 4 (A, B) or 2 (C-G) independent experiments. Two-tailed, unpaired Mann-Whitney (for EAE scores and FACS analyses) and Log-Rank (Mantel-Cox, for survival curves) tests were used. *p<0.05, **p<0.01, ***p<0.0001, n.s. : not significant.

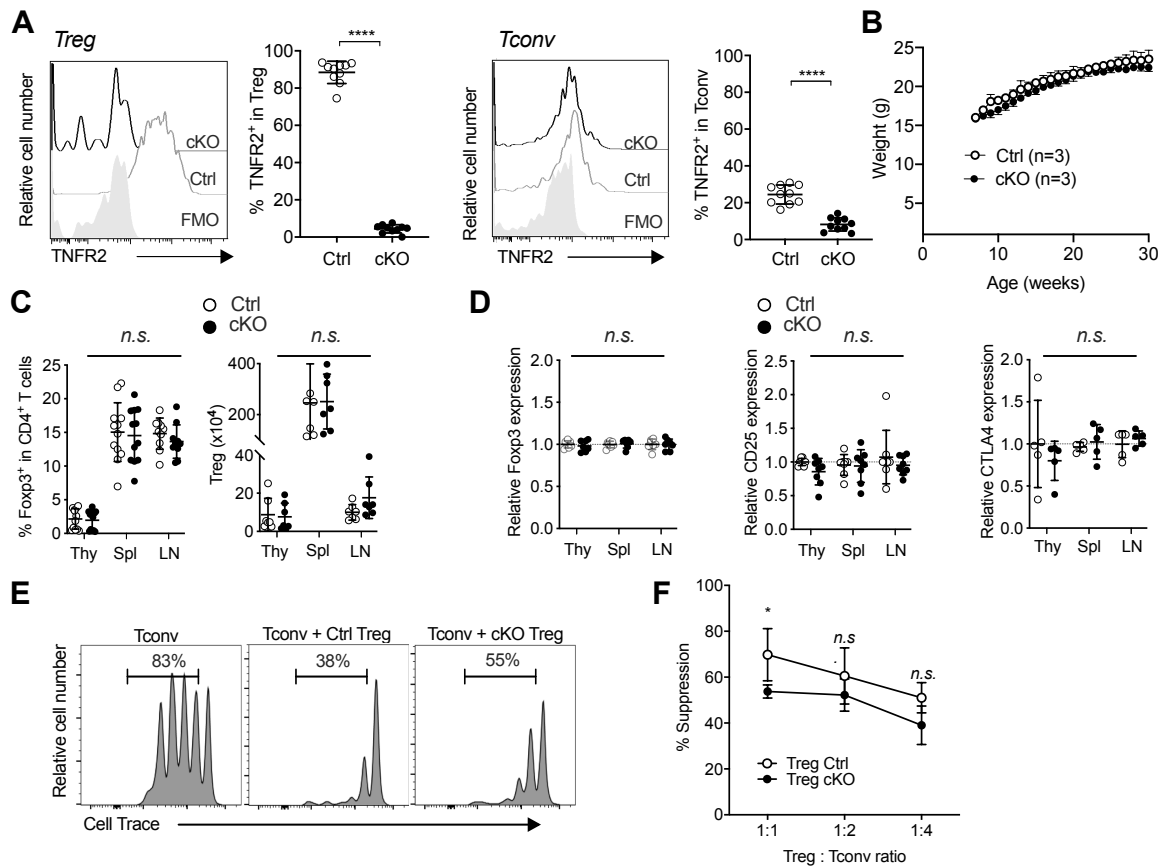


Figure S4. TNFR2 does not play a major role in Treg cell biology at steady state. (A) TNFR2 expression in *in vitro* activated Treg and Tconv cells, isolated from LN of *Foxp3^{Cre}Tnfrsf1b^{fl/fl}* (cKO) and *Foxp3^{Cre}* control (Ctrl) mice. Representative data (histograms) and mean (+/- S.D.) from 3 independent experiments with each symbol representing individual mice (graphs). (B) Weight curves of unmanipulated females. Mean +/- S.D. is shown. (C) Proportion and absolute number of Treg cells in cKO and Ctrl mice in the thymus (Thy), spleen (Spl) and LN. Mean (+/- S.D.) of 5 independent experiments; each dot represents a mouse. (D) Relative mean fluorescent intensity (MFI) of Foxp3, CD25 and CTLA-4 expression among Treg cells of cKO and Ctrl mice in the thymus, spleen and LN. The MFI of Treg cells from cKO mice divided by the average MFI of control mice is shown. Mean (+/-S.D.) is shown; each symbol represents the value of an individual mouse from 5 independent experiments. (E, F) *In vitro* suppressive activity of Treg cells from cKO and ctrl mice. Representative Tconv cell proliferation at the 1:2 Treg:Tconv cell ratio (E) and mean (+/- S.D.) at different Treg:Tconv cell ratios from 3 independent experiments (F). Two-tailed unpaired Mann-Whitney (a), two-way ANOVA (C, D) and paired t-tests (F) were used. *p<0.05, ***p<0.001, ****p<0.0001, n.s.: not significant.

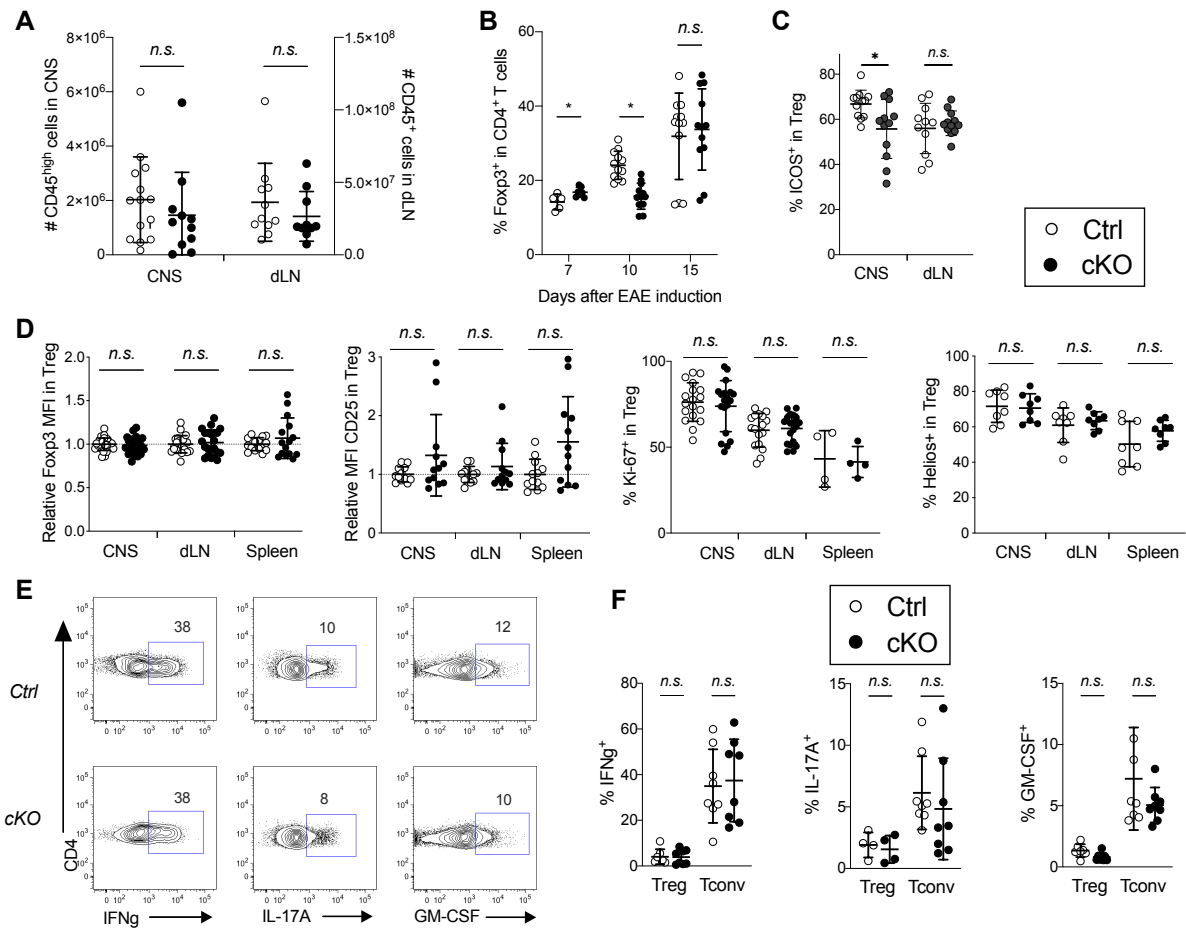


Figure S5. The decrease in Treg cell proportion in *Foxp3^{Cre}Tnfrsf1b^{fl/fl}* (cKO) mice is only transient and not associated with changes in Foxp3, Ki67, CD25 and Helios expression or cytokine production by Treg and Tconv cell subsets. EAE was induced in *Foxp3^{Cre}Tnfr2^{fl/fl}* (cKO) and *Foxp3^{Cre}* control (Ctrl) mice. (A) Absolute numbers of CD45^{high} and CD45⁺ cells in the CNS and dLN at day 10. (B) Treg cell proportion in the CNS of cKO and Ctrl mice at day 7, 10 and 15. Proportion of ICOS⁺ (C), Ki67⁺ and Helios⁺ among Treg cells and relative MFI of Foxp3 and CD25 among Treg cells (D), calculated as in Fig. S4D, at day 10. Representative flow cytometry plots in Tconv cells (E) and proportions in Treg or Tconv cells (F) of IFN γ ⁺, IL-17A⁺ and GM-CSF⁺ cells after PMA-ionomycin stimulation in the CNS of Ctrl and cKO mice at day 10. Mean (+/-S.D.) is shown. Each symbol represents the value of an individual mouse from 4 (A), 2 (B, day 7), 4 (B, day 10), 5 (B, day 15), 5 (C) and 2 to 5 (D, F) independent experiments. Two-tailed, unpaired Mann-Whitney tests were used; *p<0.05, n.s.: not significant.

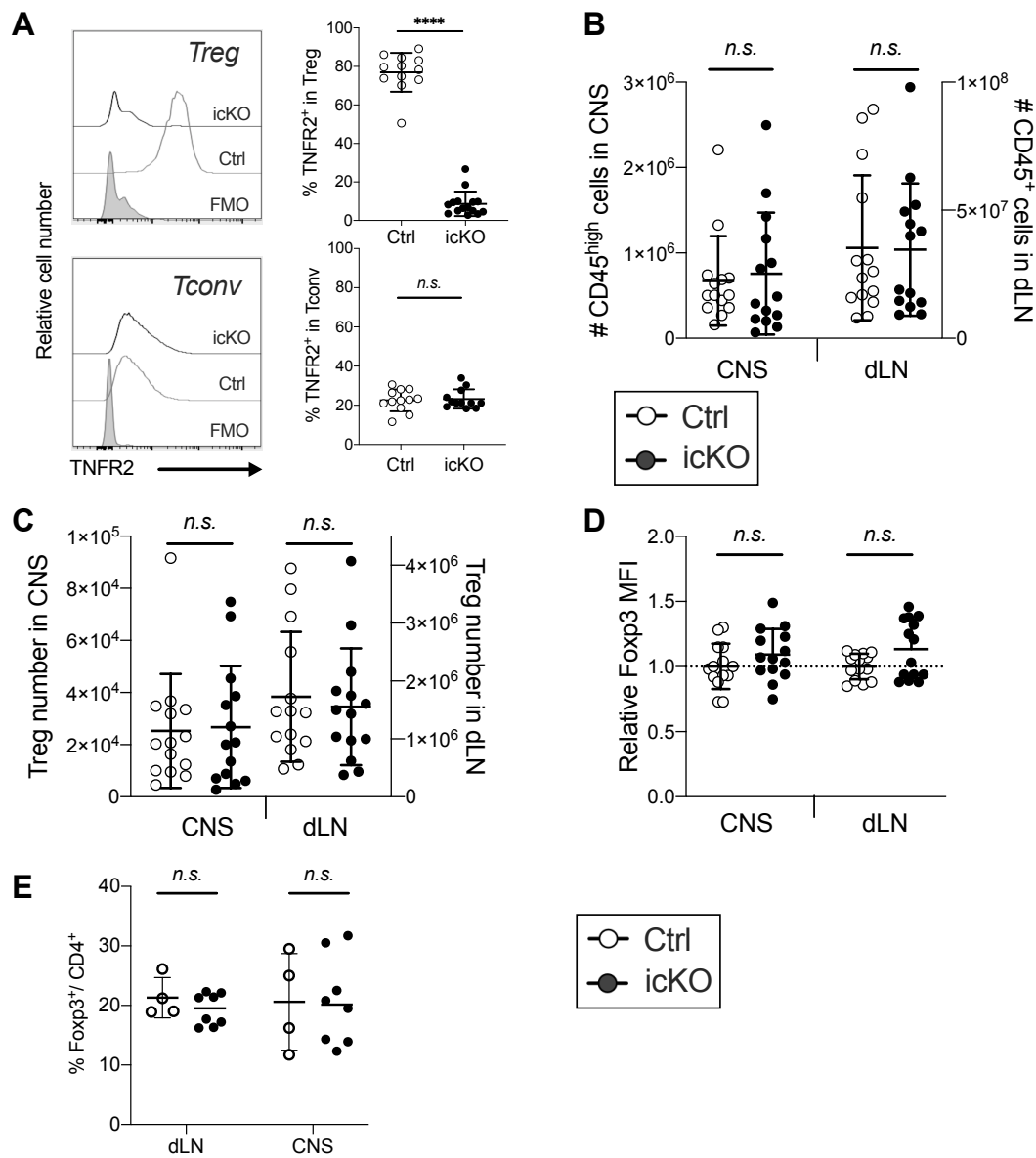


Figure S6. TNFR2 deletion in $Foxp3^{Cre-ERT2}Tnfrsf1b^{fl}$ (icKO) mice is Treg specific and does not impact on their number or Foxp3 expression during EAE. $Foxp3^{Cre-ERT2}Tnfrsf1b^{fl}$ (icKO) and $Foxp3^{Cre-ERT2}$ (Ctrl) mice were immunized to induce EAE at day 0, treated with tamoxifen from day 7 to 14 and analyzed at day 14 (A-D) or day 10 (E). (A) TNFR2 expression in *in vitro* activated Treg and Tconv cells, isolated from LN. Representative data (left panels) and mean (+/- S.D.) from 3 independent experiments with each symbol representing individual mice (right panels). Number of leukocytes (B), Treg cells (C) and Foxp3 expression level in Treg cells calculated as in Fig. S4D (D) in the CNS and dLN. (E) Treg cell proportion in dLN and CNS at day 10. Means (+/- S.D.) were obtained from 5 (B-D) or 2 (E) independent experiments. Two-tailed, unpaired Mann-Whitney tests were used ; n.s.: not significant, ****p<0.0001

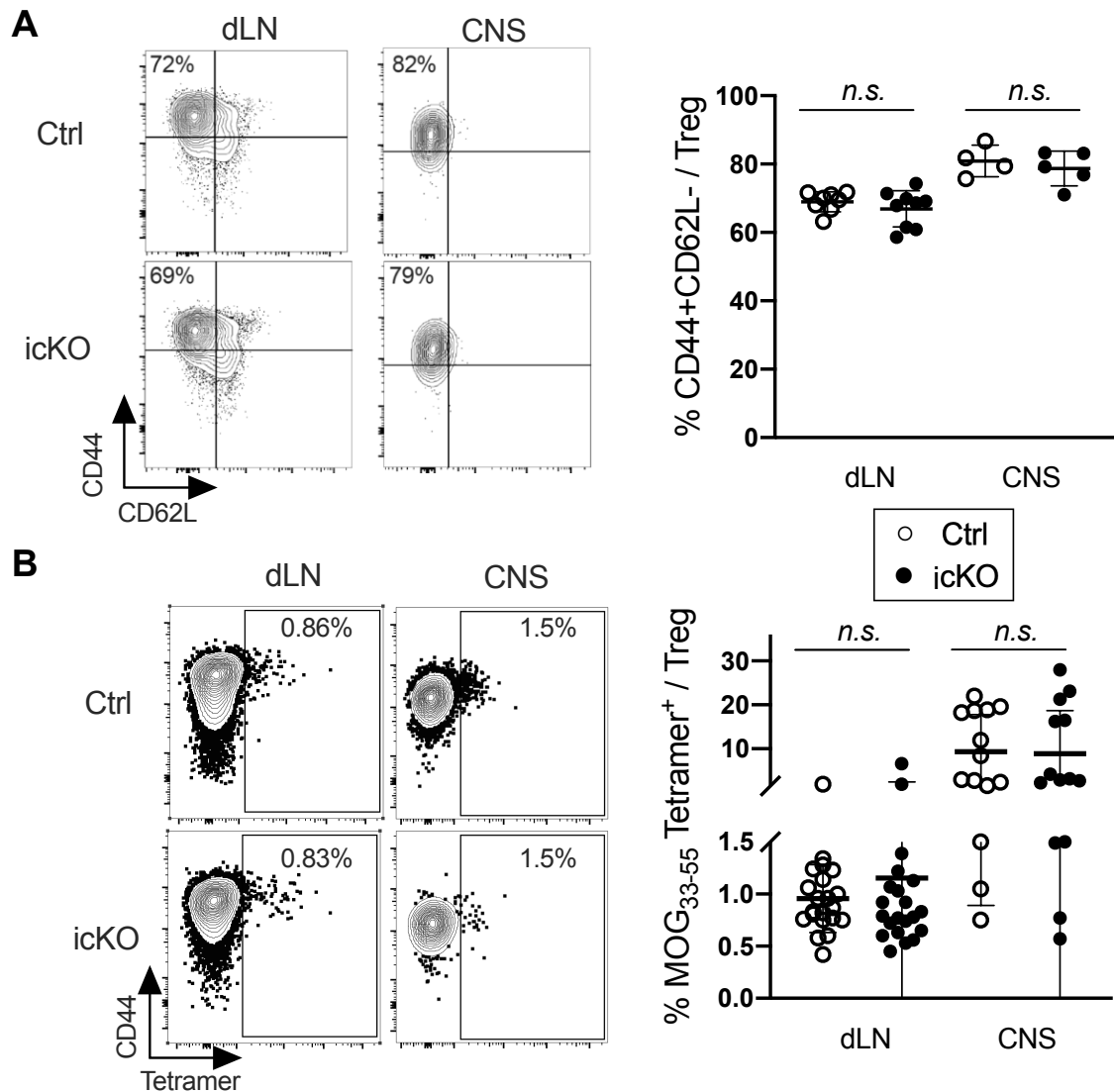


Figure S7. Similar proportions of activated and MOG-specific Treg cells in *Foxp3^{Cre-ERT2}Tnfrsf1b^{fl}* (icKO) and control mice. *Foxp3^{Cre-ERT2}Tnfrsf1b^{fl}* (icKO) and *Foxp3^{Cre-ERT2}* (Ctrl) mice were immunized to induce EAE at day 0, treated with tamoxifen from day 7 to 14 and analyzed at day 14. Representative flow cytometry plots (left panels) and proportions (right panels) of CD44⁺CD62L⁻ (A) and MOG₃₅₋₅₅-specific (Tetramer⁺) (B) cells among Treg cells in the dLN and CNS. Mean (+/-S.D.) is shown. Each symbol represents the value of an individual mouse from 2 or 3 independent experiments. Two-tailed, unpaired Mann-Whitney tests were used; *n.s.*: not significant.

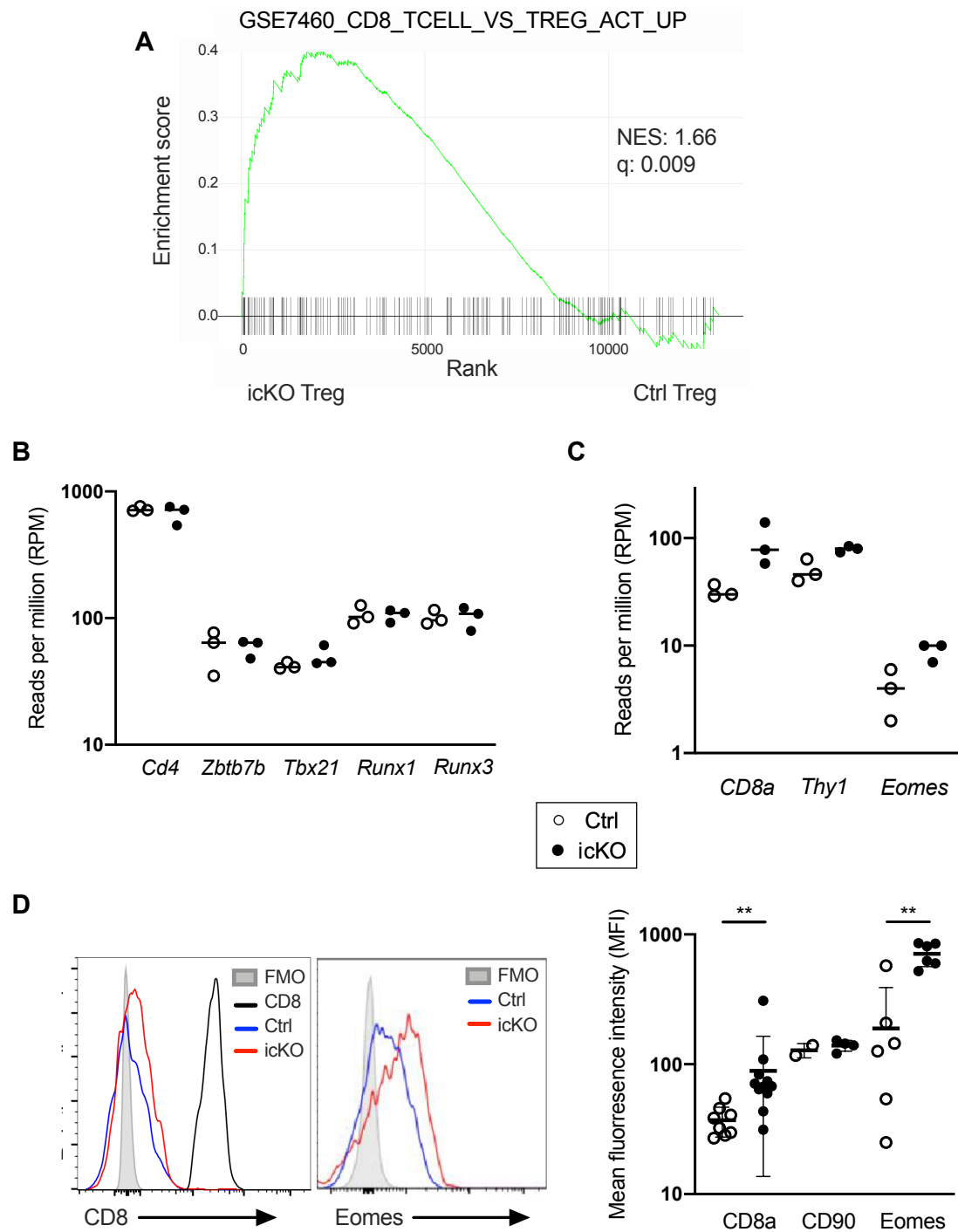


Figure S8. Validation of RNA expression at the protein level in CNS Treg cells. *Foxp3^{Cre-ERT2}Tnfrsf1b^{fl}* (icKO) and *Foxp3^{Cre-ERT2}* (Ctrl) mice were immunized to induce EAE at day 0, treated with tamoxifen from day 7 to 14, and CNS Treg cells were analyzed at day 14. (A) GSEA analysis showing an enrichment of the CD8 Tconv cell signature by TNFR2-deficient compared to Ctrl Treg cells. (B, C) Expression levels of *Cd4*, *Zbtb7b*, *Tbx21*, *Runx1*, *Runx3* (B) and *CD8a*, *Thy1*, *Eomes* (C) genes, as determined from RNA-seq data. (D) Representative histograms (left panels) and mean (+/-S.D., right panel) of expression level of CD8a, CD90 (encoded by *Thy1*) and Eomes proteins analyzed by flow cytometry. Each symbol represents the value of an individual mouse from 2 to 3 independent experiments. Two-tailed, unpaired Mann-Whitney tests were used; **p<0.01.

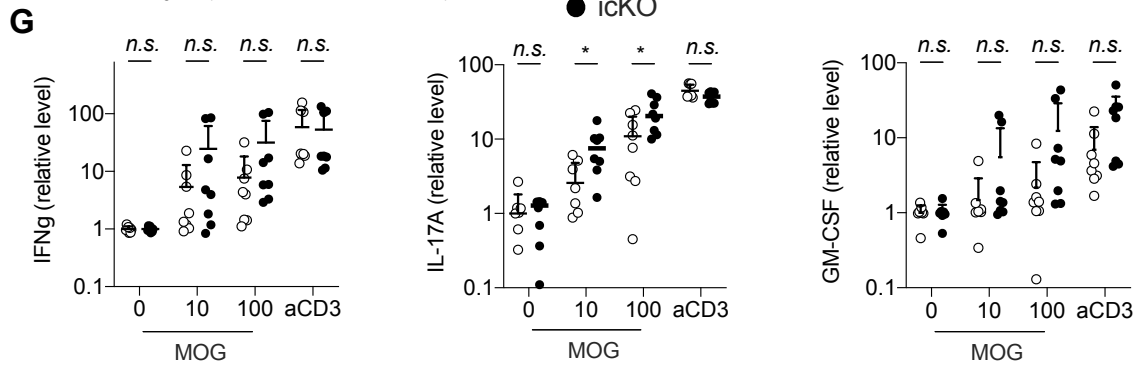
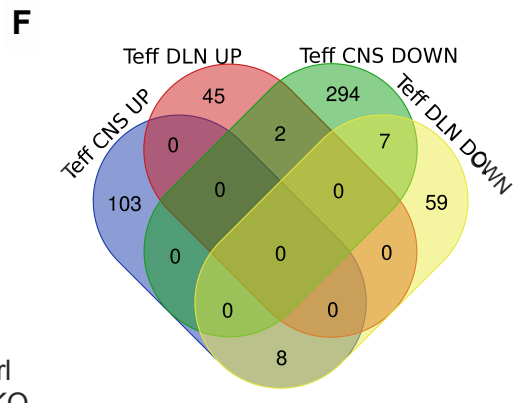
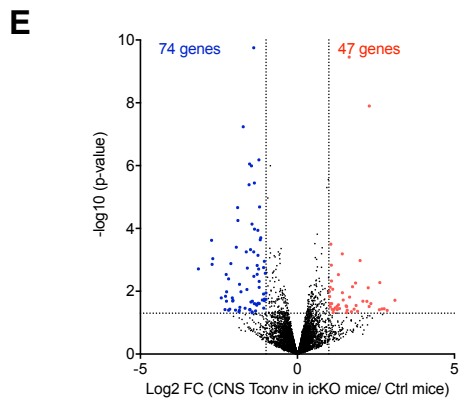
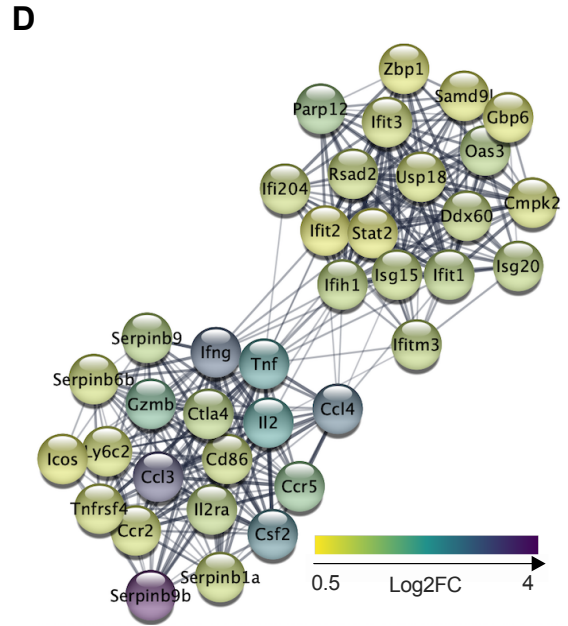
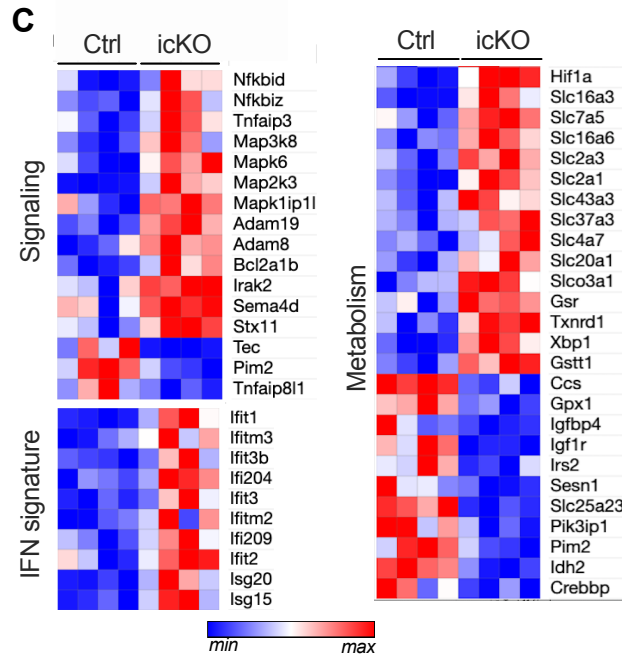
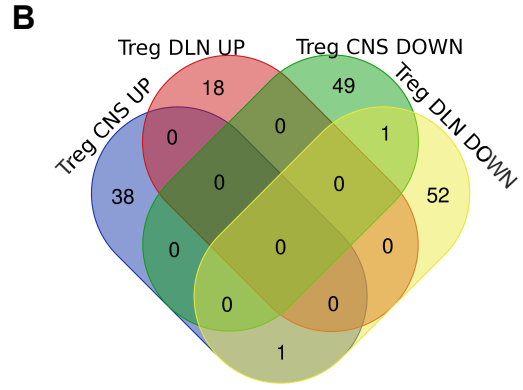
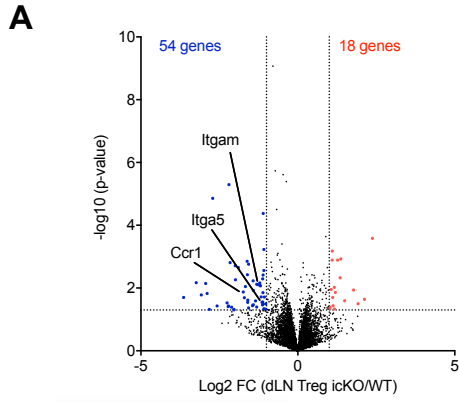


Figure S9. Gene expression and cytokine production by dLN- and CNS- derived Treg cells and Tconv cells. EAE was induced in *Foxp3^{Cre-ERT2}Tnfrsf1b^{fl}* (icKO) and *Foxp3^{Cre-ERT2}* (Ctrl) mice, treated with tamoxifen as in Fig. 5 and analyzed at day 14. Volcano plot of DEG in DLN-Treg cells (A) and DLN-Tconv cells (E). Venn diagrams showing the comparison of DEG (Log2FC<-1 or >1, p<0.05) between CNS and dLN isolated Treg cells (B) and Tconv cells (F) using <http://bioinformatics.psb.ugent.be/webtools/Venn/> and Cytoscape v3.7. (C) Heatmaps showing expression of selected genes (FDR<0.05) belonging to the signaling, IFN signature and metabolic (excluding enzymes of the glycolysis) pathways, in CNS-Tconv cells. (D) Network analysis of putative protein-protein interaction of genes up-regulated (FDR<0.05 Log2FC>0.5) in CNS-Tconv cells of icKO mice relative to controls using the STRING application in Cytoscape v3.7 (1, 2). (G) Cytokines produced by dLN cells re-stimulated ex-vivo by the immunizing MOG antigen or an anti-CD3 mAb were measured in the supernatants by ELISA. Graphs show the fold change concentration over control un-stimulated cells. Pool of 2 independent experiments with each symbol representing an individual mouse. 2-way unpaired ANOVA test with Sidak correction for multiple comparisons. *p<0.05, *n.s.* : *not* significant.

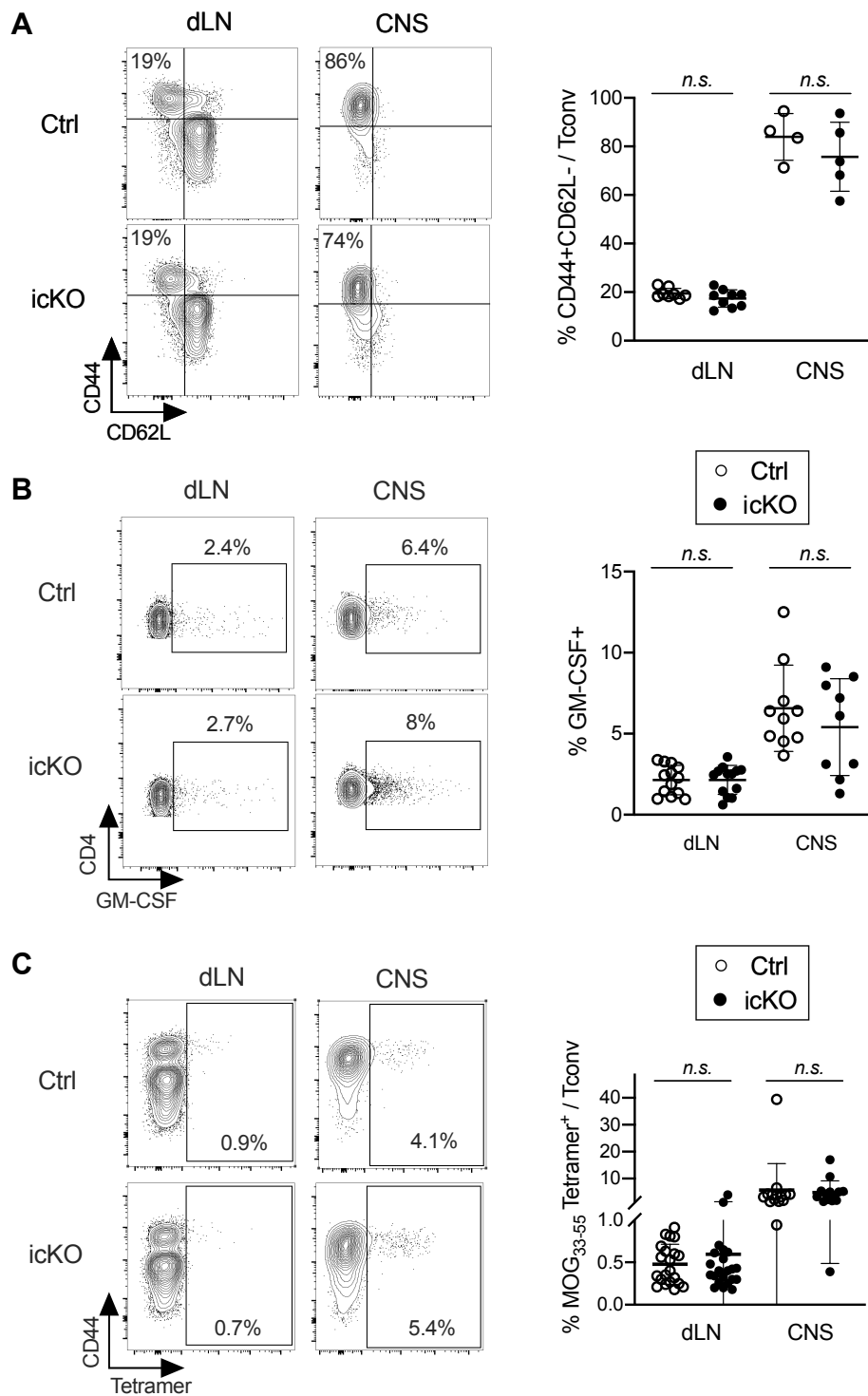


Figure S10. Similar activation and specificity of Tconv cells in $Foxp3^{Cre-ERT2}Tnfrsf1b^{fl}$ (icKO) and control mice. $Foxp3^{Cre-ERT2}Tnfrsf1b^{fl}$ (icKO) and $Foxp3^{Cre-ERT2}$ (Ctrl) mice were immunized to induce EAE at day 0, treated with tamoxifen from day 7 to 14 and analyzed at day 14. Representative flow cytometry plots (left panels) and proportion (right panels) of $CD44^+CD62L^-$ (A), $GM-CSF^+$ (B) and MOG-specific cells (Tetramer) (C) cells among Treg cells in the dLN and CNS. For cytokine detection, cells were stimulated by PMA-ionomycin. Mean (+/-S.D.) is shown. Each symbol represents the value of an individual mouse from 2 or 3 independent experiments. Two-tailed, unpaired Mann-Whitney tests were used; n.s.: not significant.

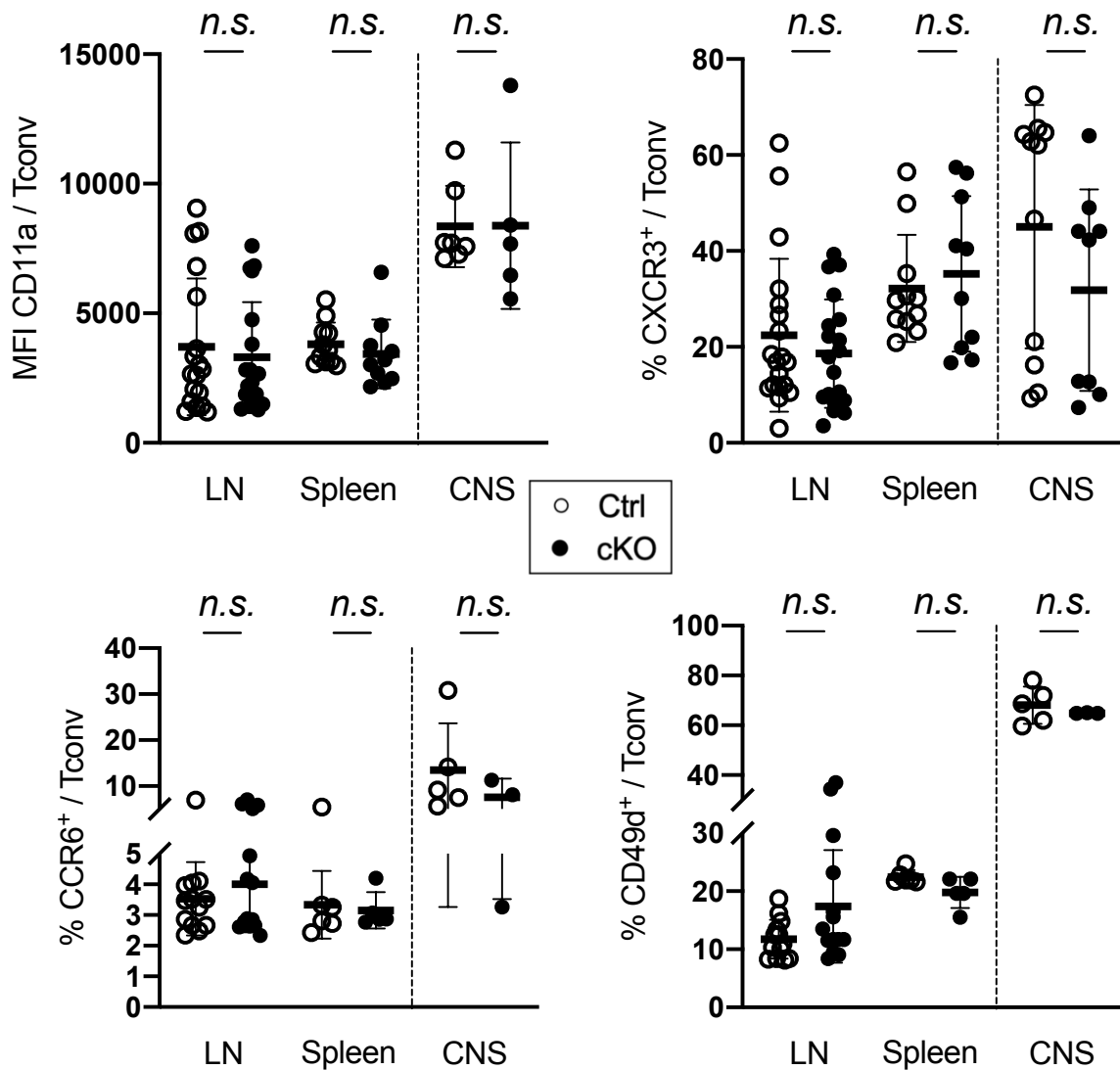


Figure S11. Tconv cells of *Foxp3^{Cre}Tnfrsf1b^{fl}* (cKO) mice exhibit normal expression of molecules involved in migration to the inflamed CNS. *Foxp3^{Cre}Tnfrsf1b^{fl}* (cKO) and *Foxp3^{Cre}* control (Ctrl) were immunized to induce EAE at day 0 and analyzed at day 10. Expression level of CD11a and proportions of CXCR3⁺, CCR6⁺ and CD49d⁺ cells among Tconv cells in the LN, spleen and CNS analyzed by flow cytometry. Mean (+/-S.D.) is shown. Each symbol represents the value of an individual mouse from 3 independent experiments. Two-tailed, unpaired Mann-Whitney tests were used; *n.s.*: not significant.

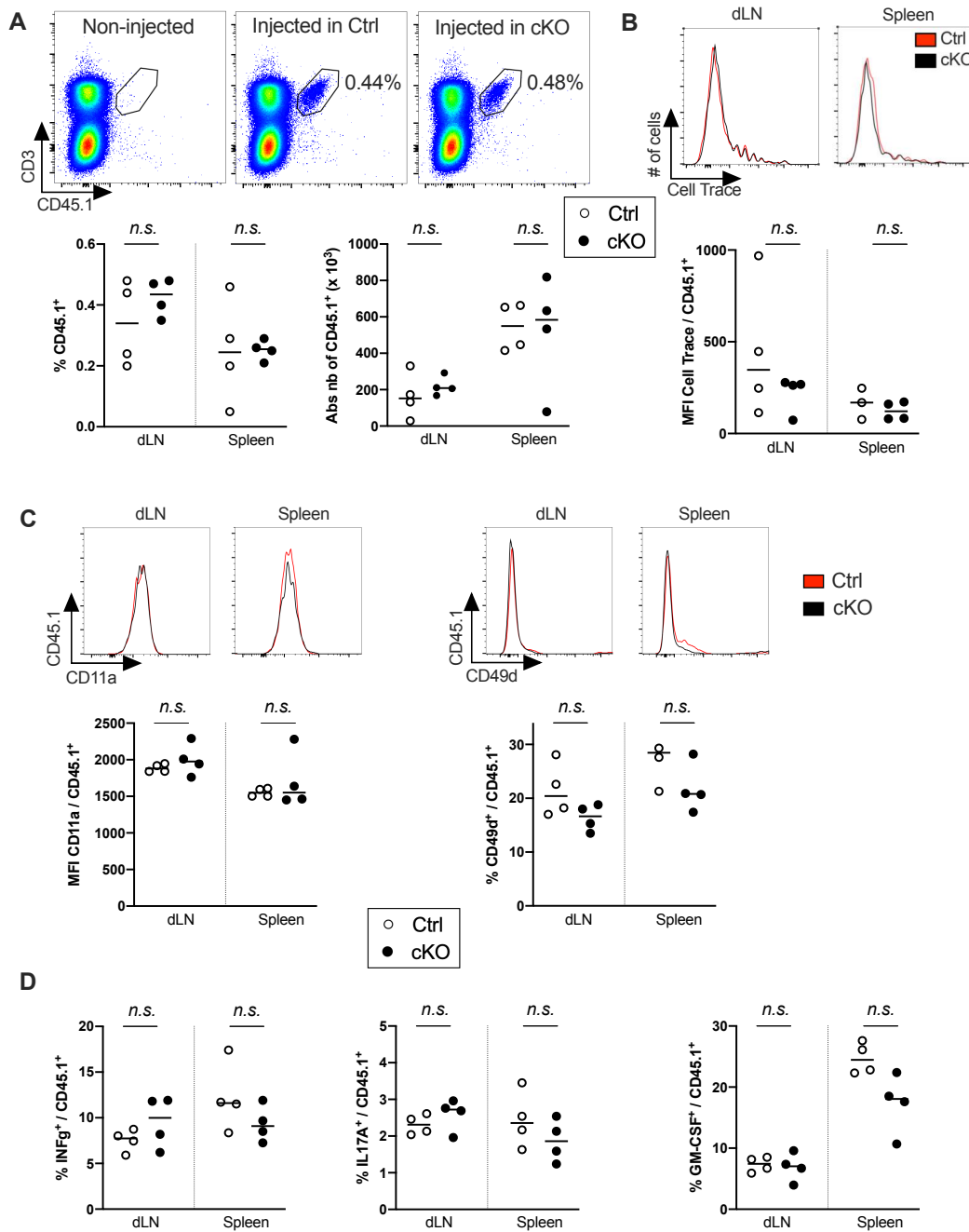


Figure S12. MOG-specific Tconv cells are primed similarly in *Foxp3^{Cre}Tnfrsf1b^{fl/fl}* (cKO) and *Foxp3^{Cre}* control (Ctrl). MOG-specific Tconv cells collected from 2D2 CD45.1 mice were adoptively transferred in *Foxp3^{Cre}Tnfrsf1b^{fl/fl}* (cKO) and *Foxp3^{Cre}* control (Ctrl) mice, which were immunized for EAE induction the day after and analyzed 7 days later in the dLN and spleen. (A) Representative flow cytometry plots (dLN, top panels) and proportions and numbers (bottom panel) of MOG-specific donor Tconv cells (CD3⁺CD45.1⁺). (B) Representative data (histograms) and MFI of cell trace dilution in injected MOG-specific donor Tconv cells. (C) Representative data (histograms), expression level of CD11a and proportion of CD49d⁺ cells in injected MOG-specific donor Tconv cells. (D) Proportions of IFNγ⁺, IL-17A⁺ and GM-CSF⁺ in injected MOG-specific donor Tconv cells after PMA-ionomycin stimulation. Mean (+/-S.D.) is shown. Each symbol represents the value of an individual mouse from 2 independent experiments. Two-tailed, unpaired Mann-Whitney tests were used; n.s.: not significant.

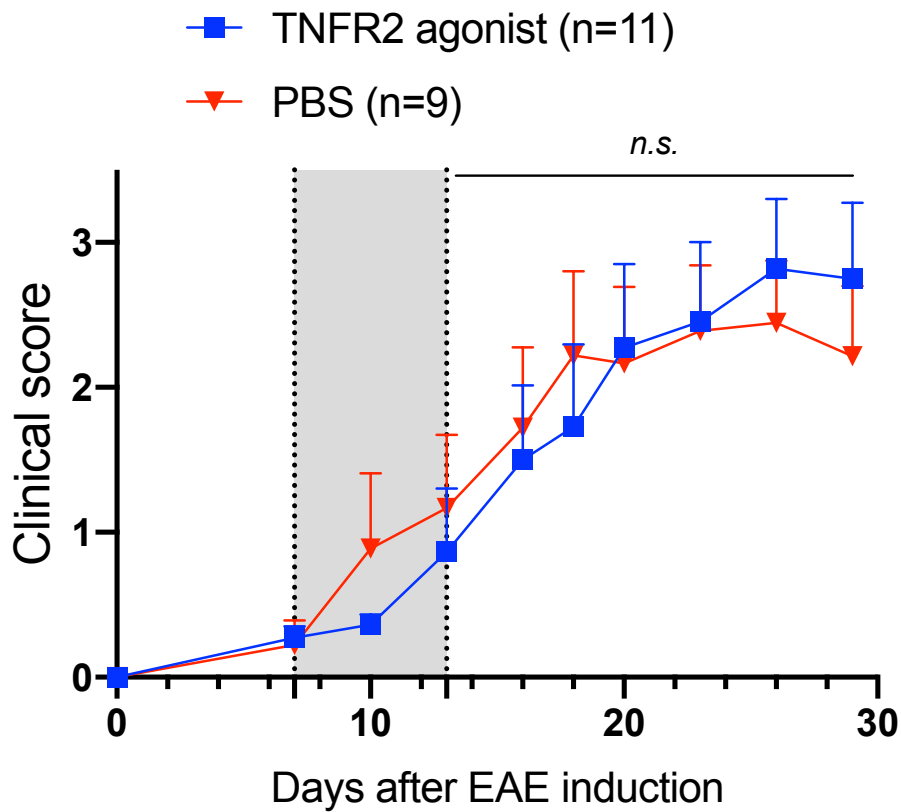


Figure S13. Therapeutic effect of a TNFR2 agonist is dependent on TNFR2 expression by Treg cells. EAE clinical score of *Foxp3^{Cre-ERT2}Tnfrsf1b^{fl}* (icKO) mice that were immunized to induce EAE at day 0, treated with tamoxifen from day 7 to 14, and with PBS or a TNFR2 agonist from day 4 to 18. Mean (+ SEM) from 2 independent experiments is shown. Two-tailed unpaired Mann-Whitney test was used. *n.s.*: not significant.

SI References (for Figure S9)

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