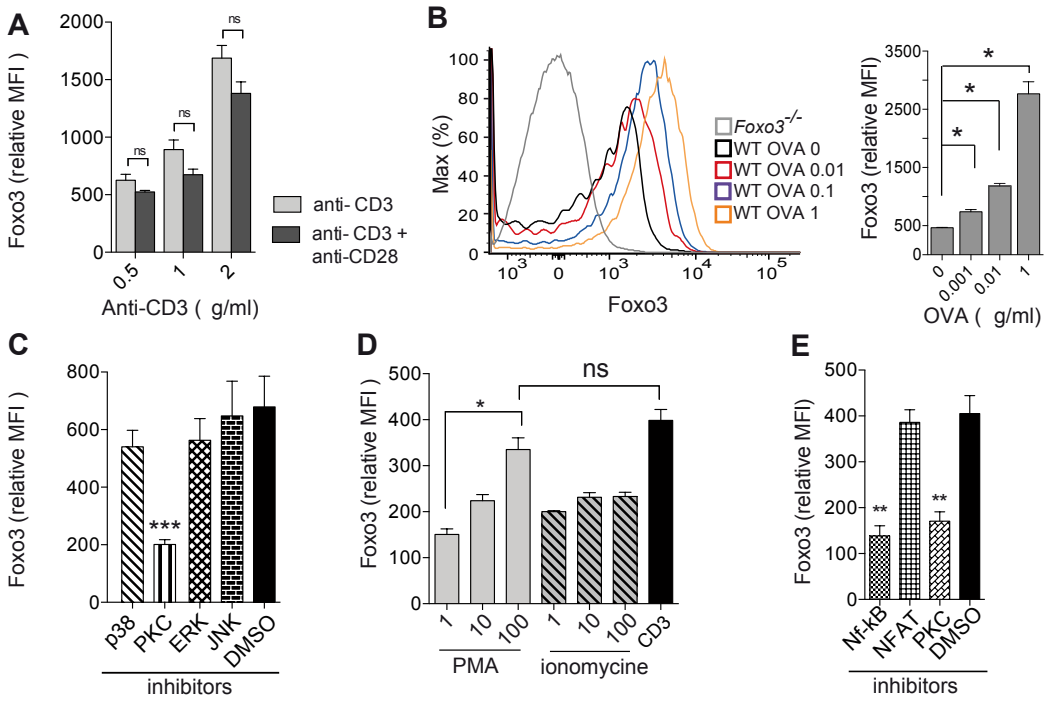
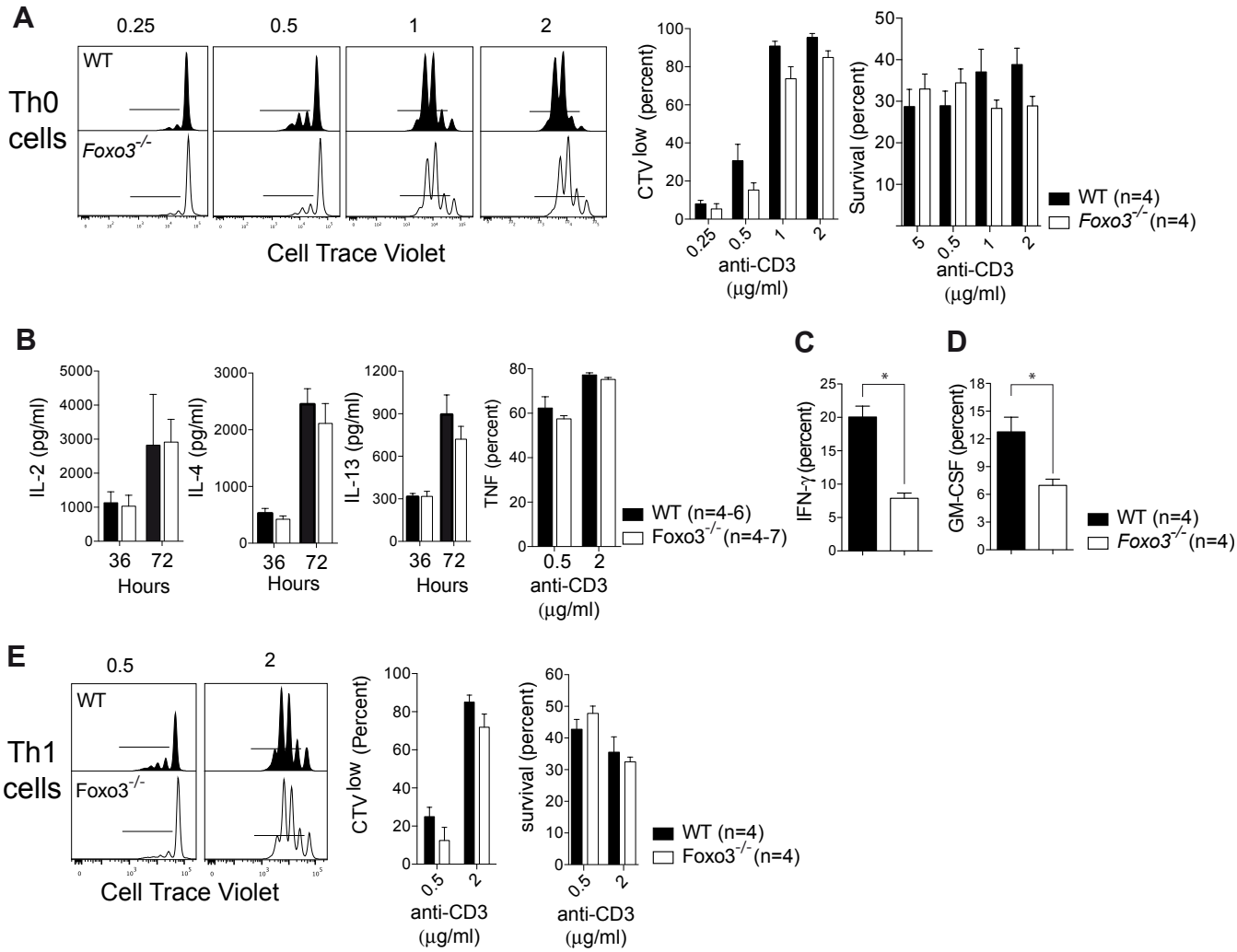
Figure S1 related to Figure 1



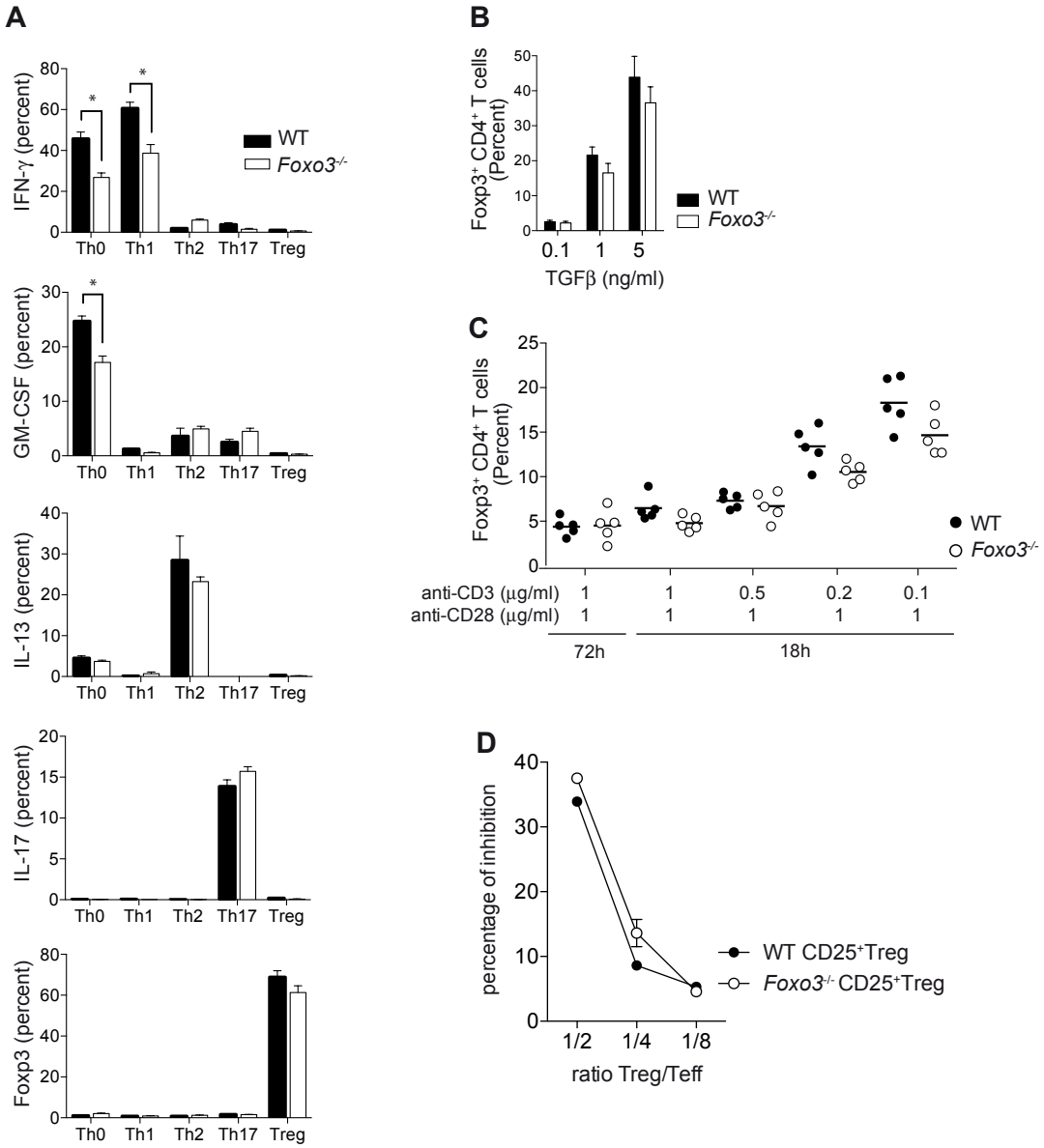
Stienne et al. Figure S1

Figure S2 related to Figure 2



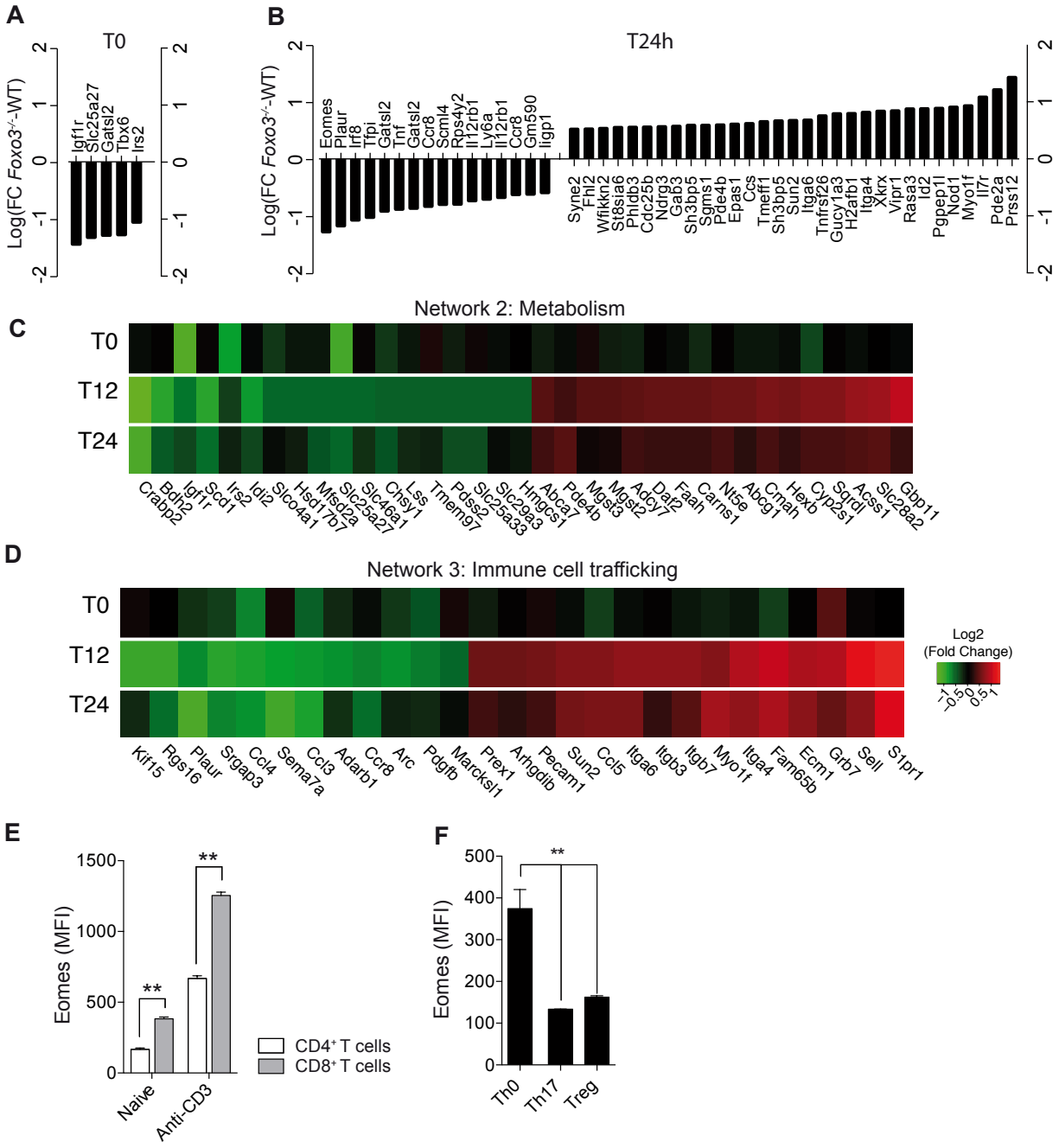
Stienne et al. Figure S2

Figure S3 related to Figure 2



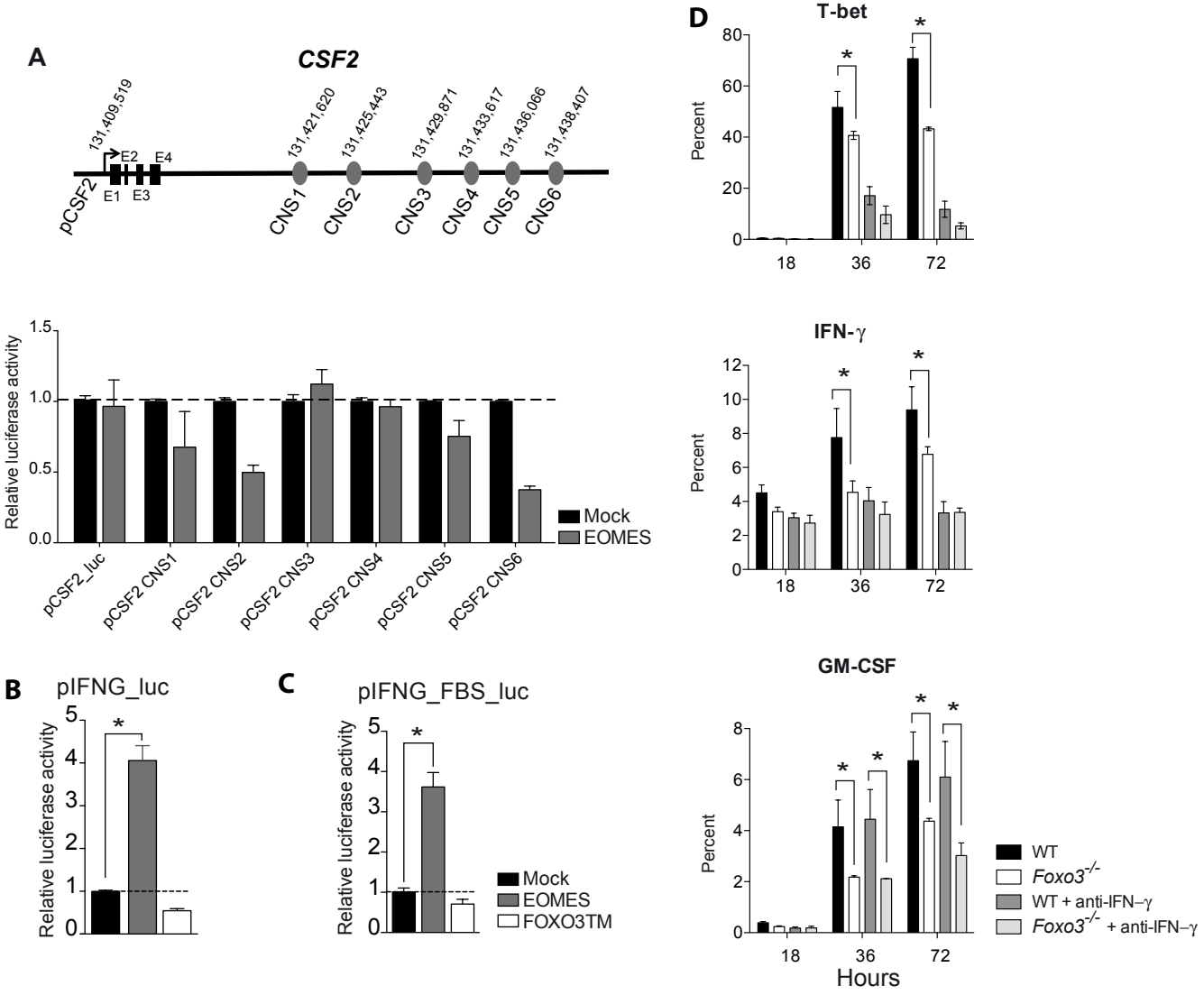
Stienne et al. Figure S3

Figure S4 related to Figure 3



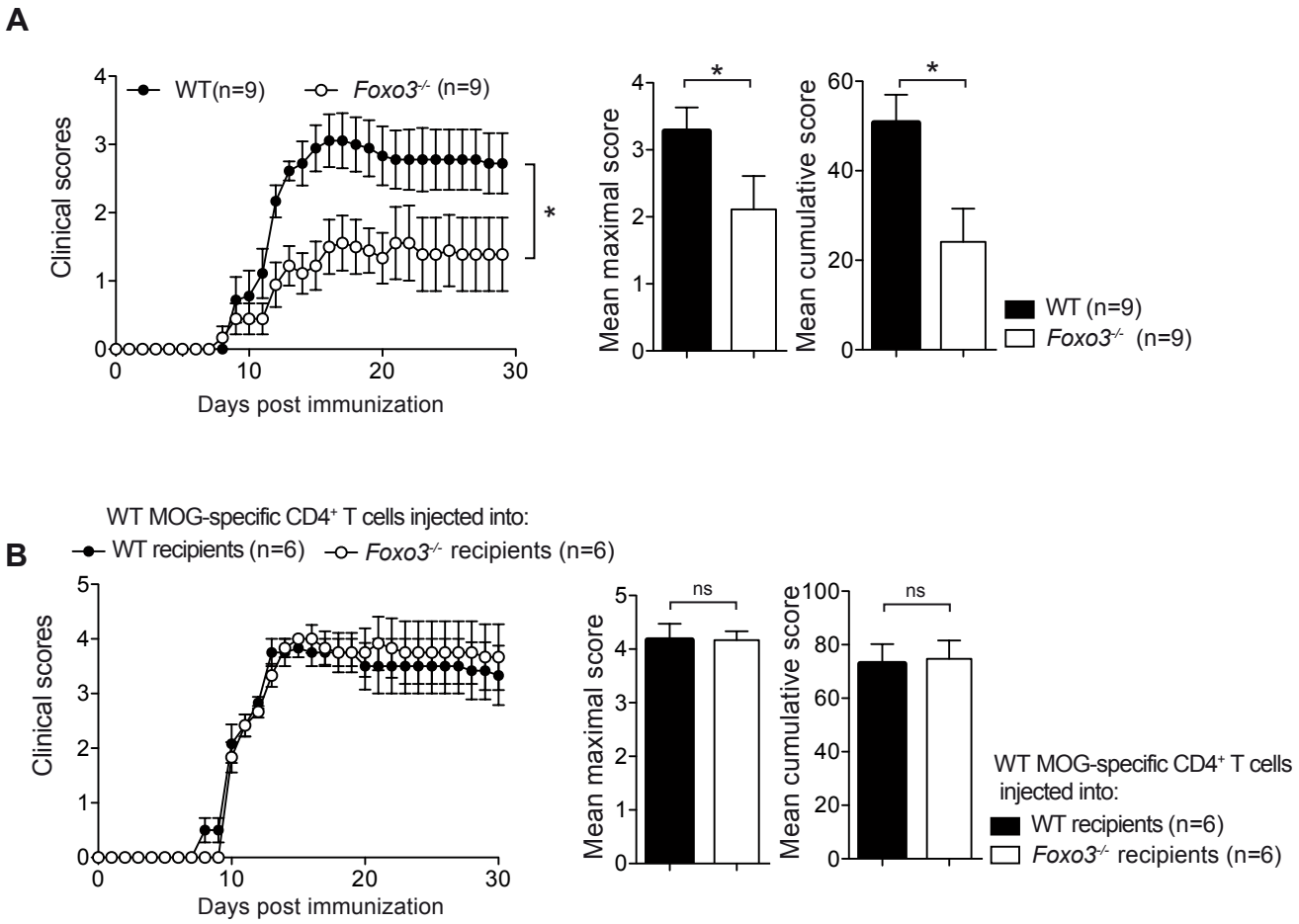
Stienne et al. Figure S4

Figure S5 related to Figure 4 and 5



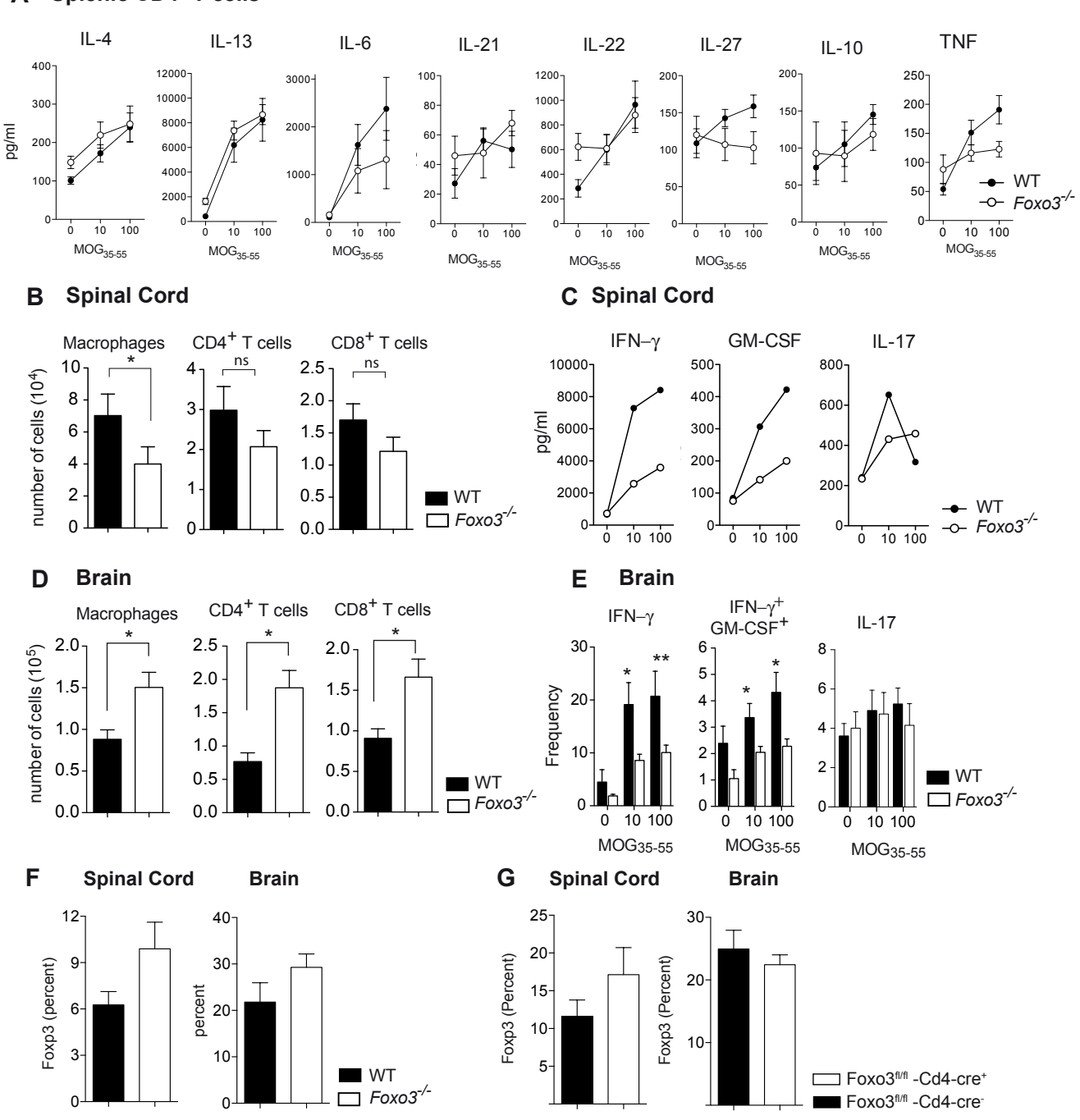
Stienne et al. Figure S5

Figure S6 related to Figure 6



Stienne et al. Figure S6

Figure S7 related to Figure 7



Stienne et al. Figure S7

Table S1 related to Experimental Procedures

ChIP

Region	Forward/Reverse	Sequence
FBS1	Forward:	CGGGGTTTGTTTTCTTGCG
	Reverse:	GATTGTAGGTGCCCCTTTCCT
FBS2	Forward:	AAGATCAAGGTCTGGGAACCCG
	Reverse:	GTGGGGAGTGTAAACAAGCCG
FBS3	Forward:	CACCGATAAACAAGCCTCCATT
	Reverse:	GGGTTCCCAGACCTTGATCTTTAT
3105	Forward:	AGAGACTTCGGGTCGCTCTC
	Reverse:	GTCTACCCACGGCATAGGAA

Plasmids

Target	Forward/Reverse	Sequence
csf2 promoter	Forward:	CTCTGGTACCGCCGAGTCAAGGTCACACCA
	Reverse:	CTCTGCTAGCCATCCTCCAGAGAACTTTAG
CNS1-csf2	Forward:	CTCTGGATCCACTGGGGCCTCCTCTTCCTC
	Reverse:	CTCTGTCGACTCATGGTCCTGCTGTGTCTG
CNS2-csf2	Forward:	CTCTGGATCCATTGTGTCTTTATTCTTGTCAAAAT
	Reverse:	CTCTGTCGACAGTCTGAACCACTTGGGCAT
CNS3-csf2	Forward:	CTCTAGATCTTTGGCCTTTAGACCGCAAGT
	Reverse:	CTCTGTCGACCAAAGGCCCTGGAGCTGGGA
CNS4-csf2	Forward:	CTCTGGATCCGCTGTCAAAGATGTCTCATG
	Reverse:	CTCTGTCGACTGGAAGAAGAATCATCAGCA
CNS5-csf2	Forward:	CTCTGGATCCCCCATGCCATGATACACATT
	Reverse:	CTCTGTCGACTCCCTTAGAAAGGGAGAATT
CNS6-csf2	Forward:	CTCTGGATCCTGGACTAAGAACTGGCAGAG
	Reverse:	CTCTGTCGACGAAGTGGAAACAGAGATGCA
Foxo-binding-Site pIFNG	Forward:	AGCTTTCTCTCGGCAAAGAGCAGTAACTTGG TGGGTTGTTTTCTTCTTAGTCACCTGATAGGATTTCCCAGGAC
	Reverse	AAGAGAGCCGTTTCTCGTCATTGAACCACCC AACAAAAGAAGAATCAGTGGACTATCCTAAAGGGTCCTGAGCT

Stienne et al. Table S1

Supplementary Figure legends

Figure S1 related to Figure 1: (**A**) Intracellular staining of Foxo3 expression by naive CD62L$^+$ CD25$^-$ CD4$^+$ T cells stimulated with the indicated doses of anti-CD3 mAbs alone (light grey bars) or anti-CD3 and anti-CD28 mAbs (dark grey bars). (B) Intracellular staining of Foxo3 expression by naive WT OT2 CD4$^+$ T cells stimulated with APC and indicated amount of $OVA_{323–339}$ peptide for 72 hours. Mean and SEM of MFI of Foxo3 (n=4 mice per genotype). (**C**) Intracellular staining of Foxo3 expression by naive CD62L$^+$ CD25$^-$ CD4$^+$ T cells stimulated with 2μg/ml of anti-CD3 mAbs in the presence of a series of inhibitors to block p38 (1μM, Calbiochem, ref. 506121), JNK (1μM, Calbiochem, ref. 420119), total PKCs (1μg/ml, Sigma, ref. R136-1MG) or ERK (1μM, Calbiochem, ref. 420119) pathways. (**D**) Intracellular staining of Foxo3 expression by naive CD62L$^+$ CD25$^-$ CD4 T cells stimulated with PMA alone (1, 10 or 100 ng/ml), ionomycin alone (1, 10 or 100 ng/ml) or anti-CD3 mAbs (2μg/ml). (**E**) Intracellular staining of Foxo3 expression by naive CD62L+ CD25- CD4 T cells stimulated with 2μg/ml of anti-CD3 mAbs in the presence of specific inhibitors of PKC total (Sigma ref. R136-1MG), NFAT (1μg/ml, Calbiochem, ref. 480401-1MG) or NF-kB (1μg/ml, IKK inhibitor, Sigma, Bay 11-7082). Mean and SEM of the relative MFI of Foxo3 expression was calculated by subtracting the WT MFI from the *Foxo3*$^{-/-}$ MFI. (n=4-5 mice per genotype). See Also Figure 1

Figure S2 related to Figure 2: (**A**) Proliferation (CellTrace Violet dilution) and survival (Viability Dye) of WT (black histogram/bars, n=4) or *Foxo3*$^{-/-}$ (open histograms/bars, n=4) naive CD4$^+$ T cells stimulated with the indicative amount of anti-CD3 mAbs under non-polarizing condition for 72 hours. (**B**) The secretion of IL-2, IL-4, IL-13 or TNF was assessed by ELISA. (**C**) Mean and SEM of the frequency of IFN-γ and (**D**) GM-CSF producing CD4$^+$ T cell from WT (black bars, n=4) or *Foxo3*$^{-/-}$ (open bars, n=4) mice stimulated with 2 μg/mL of anti-CD3 mAbs and 1 μg/ml anti-CD28 under non-polarizing condition for 72 hours. (**E**) Proliferation and survival of WT (black histogram/bars, n=4) or *Foxo3*$^{-/-}$ (open histograms/bars, n=4) naive CD4$^+$ T cells stimulated with the indicative amount of anti-CD3 mAbs under Th1 polarizing condition for 72 hours. See also Figure 2

Figure S3 related to Figure 2: (**A**) Naive CD62L$^+$ CD25$^-$ CD4$^+$ T cells purified from WT (black bars) or *Foxo3*$^{-/-}$ (open bars) were stimulated with plate-bound anti-CD3 mAbs (10μg/ml) and anti-CD28 mAbs (1μg/ml) for 6 days in neutral (Th0) or Th1, Th2, Th17 or Treg cell polarizing conditions. (**B**) Naive CD62L$^+$ CD25$^-$ CD4$^+$ T cells purified from WT (black bars) or *Foxo3*$^{-/-}$ (open bars) were stimulated with plate-bound anti-CD3 mAbs (2μg/ml) and increased concentration of TGF-β for 72 hours. Percentage of Foxp3$^+$ cells was assessed by Flow cytometry. (C) Naive CD62L$^+$ CD25$^-$ CD4$^+$ T cells purified from WT (black symbols) or *Foxo3*$^{-/-}$ (open symbols) were stimulated with the indicated amount of anti-CD3 mAbs for 18 hours. After this, the cells were either further stimulated with anti-CD3 mAbs and anti-CD28 mAbs for 54 h (72h) or maintained without TCR stimulation for 54 h (18h). The induction of Foxp3 was then examined by intracellular staining in lived cells. (**D**) CD4$^+$ CD62L^{high} CD25^{high} Treg cells were purified from WT (black symbols) or *Foxo3*$^{-/-}$ (open symbols) splenocytes and cultivated in presence of CTV-labeled WT CD4$^+$ T effector cells

stimulated with APC and anti-CD3 mAbs. Treg suppressive function was assessed and presented as percentage of inhibition. See also Figure 2

Figure S4 related to Figure 3: Gene expression microarray experiments comparing WT (n=4) versus *Foxo3*$^{-/-}$ (n=4) CD62L^{high} CD25^{-} CD4^{+} T cells unstimulated (**A**) or after 24h of stimulation with 2 μg/mL of anti-CD3 mAbs (**B**). Data are expressed as Log2 (Fold Change *Foxo3*$^{-/-}$-WT) of the top most significantly regulated genes (FDR ≤ 0.05 and fold change >2 or <2). (**C**) Gene expression fold changes expressed as Log2(FC *Foxo3*$^{-/-}$-WT) of the top most significantly regulated (FDR ≤ 0.05 and fold change > 1.5) genes within the "Metabolism" and (**D**) "Immune cell trafficking" pathways shown as a Heatmap of over-(red) or under-(green) expressed genes in naive *Foxo3*$^{-/-}$ CD4^{+} T cells stimulated for 0, 12 or 24 hours with anti-CD3 mAbs. (**E**) Intracellular staining of Eomes expression by WT naive CD4^{+} T (open bars) or CD8^{+} T cells (light gray bars) either unstimulated or stimulated with 2 μg/mL anti-CD3 mAbs for 72 hours. (**F**) Intracellular staining of Eomes expression by WT CD4^{+} T cells stimulated under Th0, Th17 or Treg cell polarizing conditions for 6 days. See also Figure 3

Figure S5 related to Figure 4 and 5: (**A**) Top panel: Schematic structure of the CSF2 gene, the arrow represents the transcriptional start site, the black boxes represent exon position (E1 to E4), grey dots represent CNS position (CNS1 to 6, numbers represent CNS positions). Bottom panel: HEK293 T cells were co-transfected with reporter plasmids containing either the human promoter region of CSF2 (2kbs) cloned into the pGL3-Basic vector (pCSF2_luc) or the promoter of CSF2 with addition of the CNS listed bellow (pCSF2_CNS 1 to 6). All constructs were co-transfected with plasmids coding for EOMES (EOMES, dark grey bars) or an empty vector (Mock, black bars). (**B**) HEK293 T cells were co-transfected with reporter plasmids containing the first 3.6kbs of the human IFNG promoter cloned into the pGL3-Basic vector (pIFNG_luc) or (**C**) the human IFNG promoter with the previously described Foxo binding site (pIFNG_FBS_luc). Both constructs were co-transfected with plasmids coding for EOMES (EOMES, dark grey bars), the constitutively active FOXO3a mutant (FOXO3TM, white bars), or an empty vector (Mock, black bars). All luciferase activities were normalized to the expression of the co-transfected Renilla luciferase. (**D**) Frequency of T-bet, IFN-γ^{+} or GM-CSF^{+} producing cells in naive WT or *Foxo3*$^{-/-}$CD4^{+} T cells stimulated with anti-CD3 mAbs for 18, 36 or 72 hours in absence or presence of neutralizing anti- IFN-γ mAbs. See also Figure 4 and 5

Figure S6 related to Figure 6: (**A**) Male *Foxo3*$^{-/-}$ (open circles) and WT littermate mice (black circles) were immunized with 50μg of peptide MOG_{35-55} emulsified in CFA at day 0 and 200 ng of pertussis toxin was given on day 0 and day 2. Clinical scores were assessed on a daily basis over a 30-day period. Mean with SEM of maximal and cumulative clinical scores for WT (black bars) and *Foxo3*$^{-/-}$ (open bars) were calculated. (**B**) Naive C57BL/6 mice were immunized with 50μg of peptide MOG_{35-55} emulsified in CFA. Ten days after immunization, cells from draining LN and spleen were cultured in the presence of 20μg/mL of MOG_{35-55}, in presence of IL-23 and anti-IFN-γ. After three days, CD4^{+} T cells were purified and $5x10^{6}$ CD4^{+} T cells were injected into WT (black circles,) or *Foxo3*$^{-/-}$ mice (open circles). Clinical scores were assessed on a daily basis over a 30-d period. Mean with SEM of the maximal and

cumulative clinical scores were calculated (n=6 mice per genotype). Data are from a pool of 3 independent experiments with a total of 9 mice per genotype (A) and are representative of at least two independent experiments (B). See also Figure 6

Figure S7 related to Figure 7: (**A**) Total *Foxo3*$^{-/-}$ (open circles, n=8) and WT littermate mice (black circles, n=8) were immunized with MOG_{35-55} peptide emulsified in CFA. At day 9 post-immunization, $CD4^+$ T cells were purified from spleen and restimulated in vitro with WT APC and increasing concentrations of MOG_{35-55} peptide, the secretion of cytokines was analyzed in the supernatant after 3 days of culture by Flowcytomix. (**B**) Spinal cords-infiltrating cells were isolated from WT (black bars) and *Foxo3*$^{-/-}$ (open bars) mice on day 12 after immunization and analyzed by flow cytometry (n=8 mice per genotype) or (**C**) re-stimulated *in vitro* with increasing concentrations of MOG_{35-55} peptide. The production of IFN-γ, GM-CSF and IL-17 in the supernatant was analyzed by ELISA (pool of 4 mice per genotype). (**D**) Brain-infiltrating cells were isolated from WT (black bars) and *Foxo3*$^{-/-}$ (open bars) mice on day 12 after immunization and analyzed by flow cytometry (n=8 mice per genotype) or (**E**) re-stimulated in vitro with increasing concentrations of MOG_{35-55} peptide. The frequency of IFN-γ^+, GM-CSF^+ and IL-17^+ producing cells was analyzed by ICS (n=6-8 mice per genotype). (**F**) Frequency of $Foxp3^+$ $CD4^+$ T cells infiltrating the spinal cords and brain from total *Foxo3*$^{-/-}$ mice (open bars) or WT littermates (black bars) on day 12 after immunization. (**G**) Frequency of $Foxp3^+$ $CD4^+$ T cells infiltrating the spinal cords and brain from *Foxo3*$^{fl/fl}$-Cd4-cre^+ mice (open bars) or *Foxo3*$^{fl/fl}$-Cd4-cre^- littermates (black bars) on day 12 after immunization. See also Figure 7

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice deficient for Foxo3 (*Foxo3*$^{-/-}$) were generated using embryonic stem cell clones (Omnibank ES cell library) by gene trap insertion in exon 1 of *Foxo3a* gene (Lexicon Genetic group). These mice were provided by Steve Hedrick in 2010 (UCSD, California, USA) and maintained in the breeding facility of PreCREFRE (Toulouse UMS06; Anexplo platform) under SPF conditions. 2D2 mice, which express transgenic TCR specific for $MOG_{35-55,}$ were obtained from Vijay Kuchroo (Boston, USA). *Foxo3*$^{-/-}$ mice were crossed with either 2D2 mice or *Rag2*$^{-/-}$ mice in the animal facility UMS06 (Toulouse, France) to generate 2D2-*Foxo3*$^{-/-}$ mice and *Rag2*$^{-/-}$-*Foxo3*$^{-/-}$ mice. The derivation of mice carrying floxed *Foxo3* alleles has been described elsewhere (Paik et al., 2007). Mice carrying floxed *Foxo3* alleles were bred with *Cd4-cre* mice to generate T-cell-specific Foxo3-deficient (*Foxo3*$^{fl/fl}$-*Cd4-cre*) mice. All animal procedures were conducted in accordance with institutional guidelines on Animal Experimentation and were under a French Ministry of Agriculture license.

Active and passive encephalomyelitis autoimmune experimental (EAE)

For 2D2-WT and 2D2-*Foxo3*$^{-/-}$ mice, EAE was induced by a single injection of 150 ng/mL of pertussis toxin as described in (Bettelli et al., 2003). For passive EAE, naive C57BL/6 mice were immunized with MOG_{35-55} emulsified in CFA. At day 10 after immunization, cells from spleen and lymph nodes were cultured with 20 μg/mL of MOG_{35-55} peptide, 5 ng/mL of IL-23 (R&D) and 10 μg/mL of anti-IFN-γ for 3 days. $CD4^+$ T cells were then purified and 5.10^6 of $CD4^+$ T cells were injected intravenously into WT and Foxo3$^{-/-}$ mice. For RAG-deficient mice, $2x10^4$ WT MOG_{35-55} specific 2D2 $CD4^+$ T cells plus $4x10^6$ total WT $CD4^+$ T cells were transferred into *Rag2*$^{-/-}$-*Foxo3*$^{+/+}$ and *Rag2*$^{-/-}$-*Foxo3*$^{-/-}$ mice. The next day, mice were immunized with 50μg MOG_{35-55} emulsified in CFA and injected i.v. with 100ng of pertussis toxin. Clinical score were evaluated on a five-stage scale from 0 to 5.

Fractionation assay

Naive $CD4^+$ T cells were stimulated with anti-CD3 mAbs during 36 h and the subcellular fractionation was performed with NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific). After nuclear and cytoplasm separation, the protein concentrations were determined with a Bradford protein

assay kit and equal amount of total proteins were analysis by Western Blot using anti-Foxo3 (Cell signaling), anti-PLC-γ1 (1249-Santa Cruz) and anti-TFIID (58C9-Santa Cruz).

Immunofluorescence staining

Naive $CD4^+$ T cells from WT and *Foxo3*$^{-/-}$ mice were stimulated with anti-CD3 antibody during 48 h. Cells were washed and incubated 20 minutes with "Foxp3 staining buffer" for fixation and permeabilization. After incubation Fc-Block (2.4G2), cells were incubated 2 h at room temperature with primary antibody Foxo3 (Cell signaling) and 1h at room temperature in secondary antibody. Then, cells were disposed on chamber slides (Lab-Teck II-Nunc) and slides were mounted with mounting media (Vectashield containing DAPI) and examined on laser scanning confocal microscope 710 (Zeiss).

Microarray gene expression study

For each samples, Cyanine-3 (Cy3) labeled cRNA was prepared from 200 ng of total RNA using the One-Color Quick Amp Labeling kit (Agilent) according to the manufacturer's instructions, followed by Agencourt RNAClean XP (Agencourt Bioscience Corporation, Beverly, Massachusetts). 600 ng of Cy3-labelled cRNA were hybridized on the microarray slides following the manufacturer's instructions. Immediately after washing, the slides were scanned on Agilent G2505C Microarray Scanner using Agilent Scan Control A.8.5.1 software and fluorescence signal extracted using Agilent Feature Extraction software v10.10.1.1 with default parameters. Microarray data were analyzed using R (www.r-project.org, R v. 3.0.1), using Bioconductor packages (www.bioconductor.org, v 2.12, (Gentleman, Carey et al. 2004)) as described in GEO entry GSE86287. Raw data (median of pixels intensity) were log2 transformed and normalized using quantile method (Bolstad, Irizarry et al. 2003). Quality controls on raw data lead us to exclude 2 samples from the data matrix (1 Foxo3$^{-/-}$ at T0 and 1 Foxo3$^{-/-}$ at T24, getting 22 samples out of 24. A model was fitted using the limma lmFit function (Smyth, 2004). A correction for multiple testing was then applied using the Storey procedure (*q* value) for the False Discovery Rate (FDR) control (Storey and Tibshirani, 2003). Probes with FDR≤0.05 were considered to be differentially expressed between conditions.

Plasmid constructs

Eomes luciferase reporter containing the 1000bp upstream EOMES transcriptional start site was kindly provided by Pr. Bin Li (Li et al., 2013b). The pEomes_p3'E_luc was obtained by insertion of the sequence containing the two putatives FBS 5'-AGCTTCTACTGTGGCTATTTGTTCGGAGATAAATTTATTTCTAGTTCCCGAGCCGCGGGCCAAAC AAATGTAAGAGGCGGC-3' into the Eomes_Luc plasmid by using *BamHI and XhoI* restriction sites. FOXO3TM and FOXO3TM-A32A253Nter were gifts from C. Charvet (Charvet et al., 2003). Briefly, the cDNA encoding HA-tagged-FOXO3TM was subcloned from pECE constructs (Brunet et al., 1999) into pCDNA3 using HindIII and XbaI digestion. Nt fragment were amplified from pCDNA3 HA-FOXO3-TM by PCR using the following primers: 50FOXO3a-Nt: 5'-ACCATGGCAGAGGCACCG-3' and 3'FOXO3a-Nt: 50-CAGCTCATCACTGCTGCGTGACGTGGG-3'. The amplified fragments were directly ligated into the V5-tagged pCDNA3.1 vector (Invitrogen, Groningen, Netherlands). FOXO3TM-H212R plasmid was obtained by mutation of FOXO3TM on his DNA-Binding Domain (H212R) using QuickChange Lightning Site-Directed Mutagenesis Kit according to manufacturer's recommendations (Agilent technologies). Primers used from the mutagenesis are forward: 5'-AAGAACTCCATCCGGCGCAACCTGTCACTGCAT-3' and reverse: 5'-ATGCAGTGACAGGTTGCGCCGGATGGAGTTCTT-3'

Lentiviral vector production

To produce the lentiviral particles, 10 T150 flasks plated with 1.8 x10^7 HEK-293T cells each were co-transfected with the two packaging plasmids, psPAX2 and pMD2.G (Addgene, France), and either the pWPXLd-IRES-GFP (control vector) or the pWPXLd-Eomes-IRES-GFP vector. Plasmids were then mixed with 100 µl of GeneJuice (Merck, France). Culture medium was removed the next day and replaced by warm OptiMEM medium (Gibco, France). Supernatants containing either the control lentiviral particles (LV-GFP) or the eomes lentiviral particles (LV-Eomes) were collected 48h and 72h post-transfection, pulled together, cleared by low-speed centrifugation and filtered using a 0.45 µm filter. Lentiviral particles (LV-GFP or LV-Eomes) were then purified by ultracentrifugation through a 20% sucrose cushion (25,000 rpm, 2 h, 4°C; SW32Ti rotor, Beckman Coulter). Pellets were resuspended in ice-cold PBS under gentle agitation overnight at 4°C, aliquoted and stored at -80°C.

ELISA

Antibodies used for ELISA were 11B11 (anti-IL-4), AN18 (anti-IFN-γ), purified anti-mouse IL-17A, purified anti-mouse GM-CSF, Biotin anti-mouse IFN-γ (XMG1.2), Biotin anti-mouse IL-17A, Biotin anti-mouse GM-CSF. All antibodies were purchased from BD Biosciences except the BVD6-24G2 (anti-mouse IL-4 Biotin) which is from e-Biosciences. Enzyme immunoassays were used to measure cytokines in culture supernatants. Briefly, 96 well plates were coated for 2h at 37°C with anti-IFN-γ, anti-IL-17 or anti-GM-CSF in carbonate buffer 0.05 M pH 9.6. Culture supernatants or standards were incubated 2h at 37°C. The plates were then incubated 1h30 with a secondary biotinylated antibody specific for each cytokine, followed by 20 min of incubation with streptavidin-phosphatase alkaline at 37°C. Finally, plates were revealed by phosphatase alkaline substrate and absorbance was measured at 450/540 nm.

$CD4^+$ T cell differentiation:

For Th1 cell polarization, naïve $CD4^+$ T cells were stimulated with plate bound anti-CD3 mAbs (2μg/ml) in presence of IL-12 (10ng/ml) and IL-2 (1ng/ml). For Th2 cells, naïve $CD4^+$ T cells were cultured in presence of anti-IFN-γ (10μg/ml), IL-4 (50ng/ml) and CD28 (2μg/ml). For Th17 cells, naïve $CD4^+$ T cells were cultured in presence of anti-IFN-γ (10μg/ml), anti-IL-4 (10μg/ml), IL-6 (20ng/ml) TGF-β (3ng/ml) and anti-CD28 (2μg/ml). For iTreg cell differentiation, cells were stimulated in presence of TGF- β (5ng/ml), IL-2 (1ng/ml) and anti-CD28 (2μg/ml). After 6 days of stimulation cells were then activated with PMA plus ionomycin (0.5μg/ml each) plus Golgiplug (1/1000) for 4h and stained for IFN-γ, GMCSF, IL-13, Eomes, IL-17 and Foxp3.

Luciferase reporter assay construct

Human CSF2 promoter region was amplified by PCR using the high fidelity Phusion DNA polymerase (Thermo Fisher F530S) with the primers listed in table S1 and inserted into the pGL3_Basic vector (Promega) using NheI and KpnI restriction sites to create the pCSF2_Luc reporter plasmid. All CNS (1 to 6) were also amplified by PCR using primers listed in table S1 and each CNS was subcloned into the pCSF2 reporter plasmid using BamHI and SalI restriction sites to create pCSF2_CNS plasmids. pIFNG_luc promoter was obtained from addgene (Plasmid #17598). The 74bp containing Foxo-Binding Site was subcloned into the pIFNG_Luc reporter plasmid using XhoI and HindIII restriction

sites to create pIFNG_FBS_Luc plasmid. All luciferase experiments were performed as for the promoter of EOMES

Analysis of spinal cords and brains infiltration:

Mice were anesthetized with Ketamine and perfused with cold PBS. Brain and spinal cord were collected separately and were homogenized and digested with collagenase D (2.5 mg/ml, Roche Diagnostics), Dnase I (10 µg/ml) and TLCK (1 µg/ml, Roche, Basel, Switzerland) for 30min at 37 ºC. Cells were then washed, suspended in 37% Percoll, and layered on 70% Percoll. After a 20-minute centrifugation at 2000 rpm, the mononuclear cells were collected from the interface, washed and resuspended in culture medium. Isolated cells were counted using a hematometer and then stained in order to analyze the presence of different cell populations by flow cytometry. $3x10^5$ CNS infiltrated cells were stimulated overnight with different concentrations of MOG_{35-55} (0, 10 and 100 µg) to analyze the cytokine expression by $CD4^+$ T cells using intracellular staining and ELISA. Similarly draining lymph nodes cells and splenocytes were stimulated with different concentrations of MOG_{35-55} (0,10 and 100 µg) for 48 and 72 hours to investigate the cytokine expression using intracytoplasmic staining and ELISA.