Supplementary information for

Antigen receptor-engineered Tregs inhibit CNS autoimmunity in cell therapy using non-redundant immune mechanisms in mice

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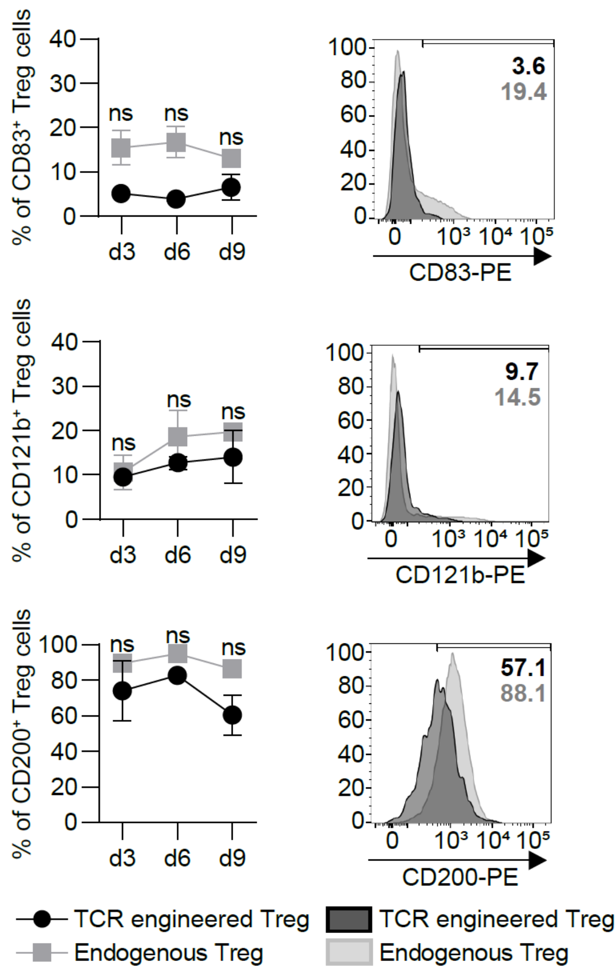
Supplementary information



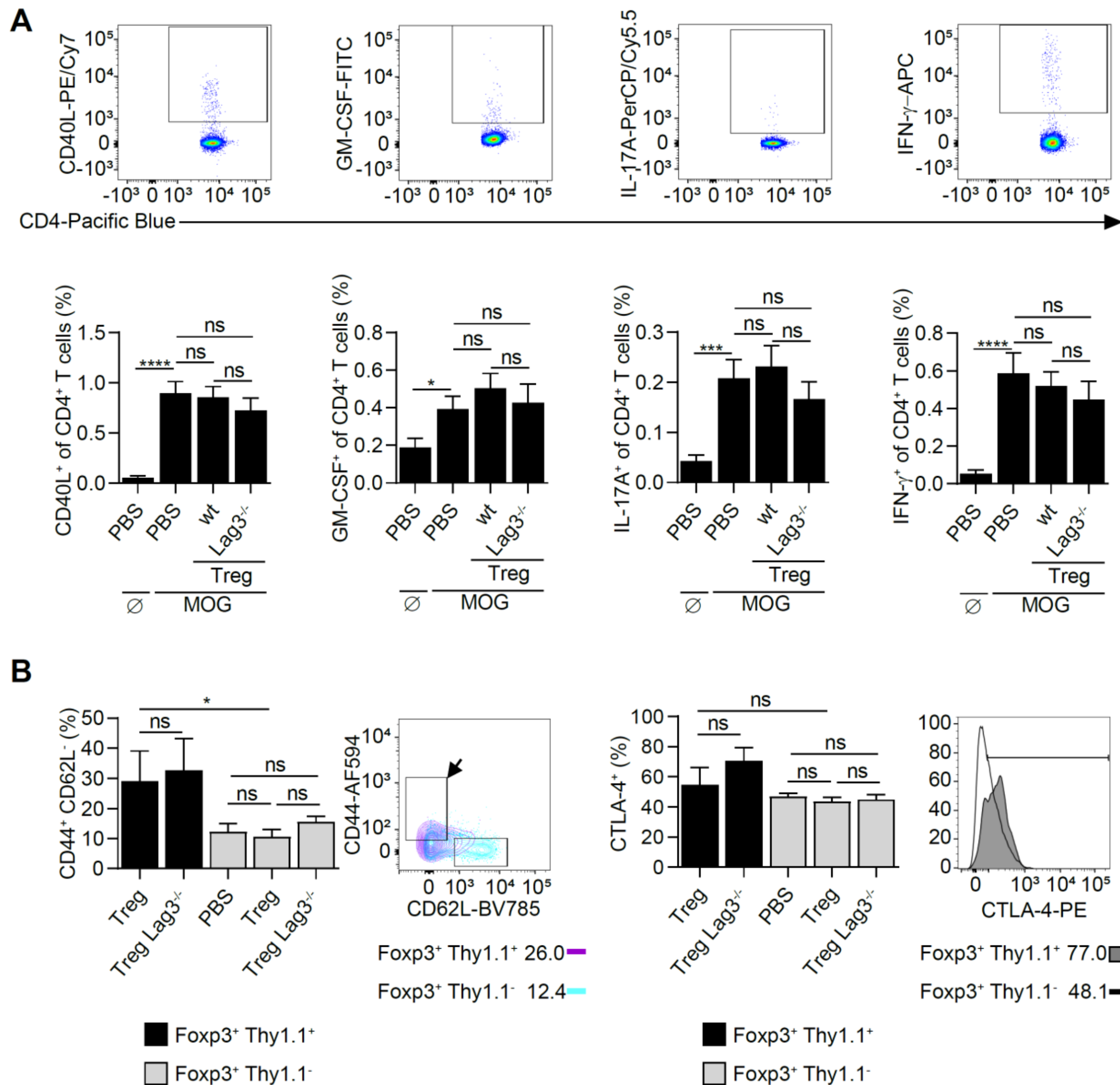
Supplementary Figure 1 (related to Fig. 1): Surface expression of CD83, CD121b, and CD200 does not differ significantly between engineered and endogenous Tregs.

Polyclonal Tregs were isolated from C57BL/6 mice and transduced with high-avidity MOG reactive TCR. $1x10^6$ engineered Tregs were injected into C57BL/6 recipient mice one day before immunization with MOG(35-55). On days 3, 6 and 9 p.i. cells from draining LN (inguinal and popliteal) were harvested and stained for CD83, CD121b (IL-1R2), and CD200 surface markers to determine their expression on the cell surface of endogenous and engineered MOG-reactive Tregs.

In each experiment, three mice were sacrificed on the selected days and cells from all LN were pooled. Data are pooled from two experiments. Black circles correspond to engineered Tregs, and grey squares to endogenous Tregs. Graphs show mean ± SEM. Representative flow cytometry plots show

the percentage of cells with high expression of selected surface markers on day 3. Dark histograms correspond to TCR-engineered Tregs and light grey one's to endogenous Tregs.

ns – not significant.



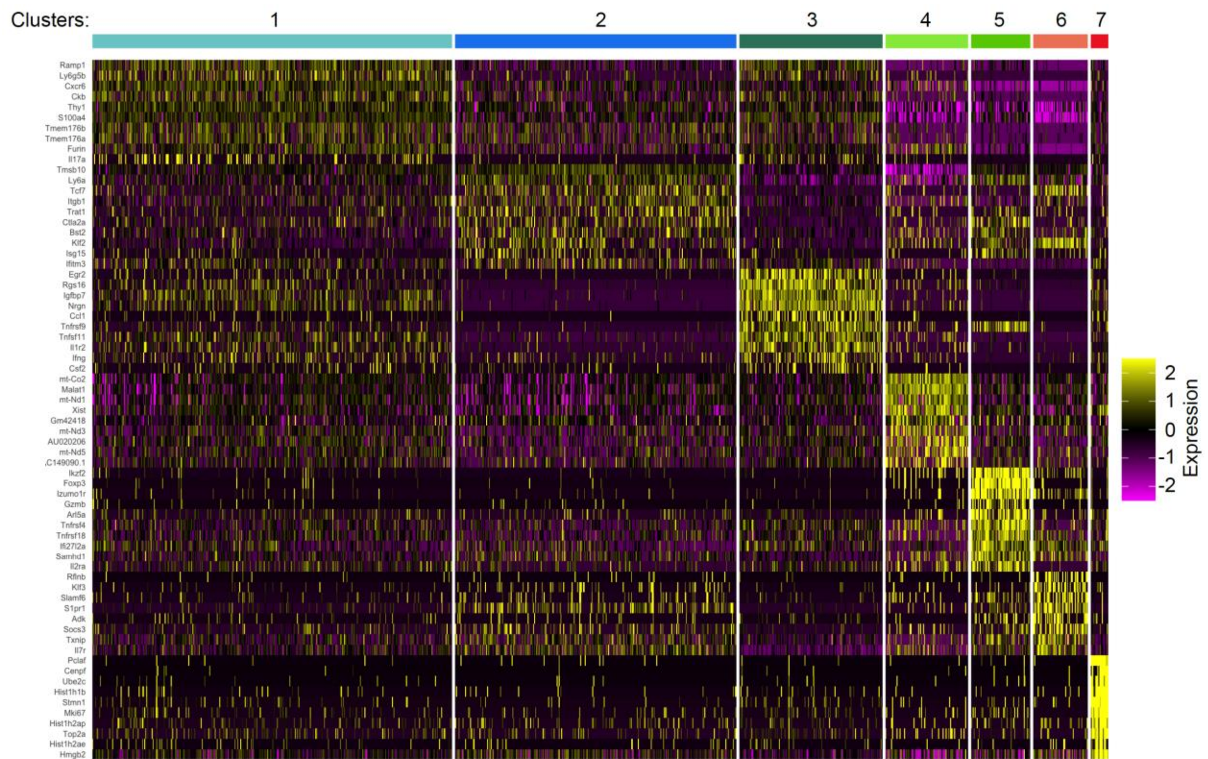
Supplementary Figure 2, related to Fig. 2: LAG-3 contributes to the protective function of engineered MOG-reactive Tregs.

Engineered Tregs were prepared from WT or *Lag3*-deficient mice and transduced with MOG-reactive TCR of high avidity. 2 x 10^5 engineered Tregs were adoptively transferred into C57BL/6 mice on day -1. Control groups were treated only with PBS. On day 0, mice were immunized with MOG(35-55) to induce EAE. At the end of the experiment (day 35 p.i.), mice were sacrificed and spleens collected. Data show mean + SEM and are a compilation of 3 independent experiments (n=14-17 per group).

(A) Splenocytes were left untreated (Ø) or stimulated with MOG(35-55) for 6 hours. The expression of immune molecules (CD40L, GM-CSF, IL-17A, and IFN-γ) was analyzed by intracellular cytokine staining. Representative flow cytometry plots showing the expression of immune molecules in $CD4^+$ T cells (top).

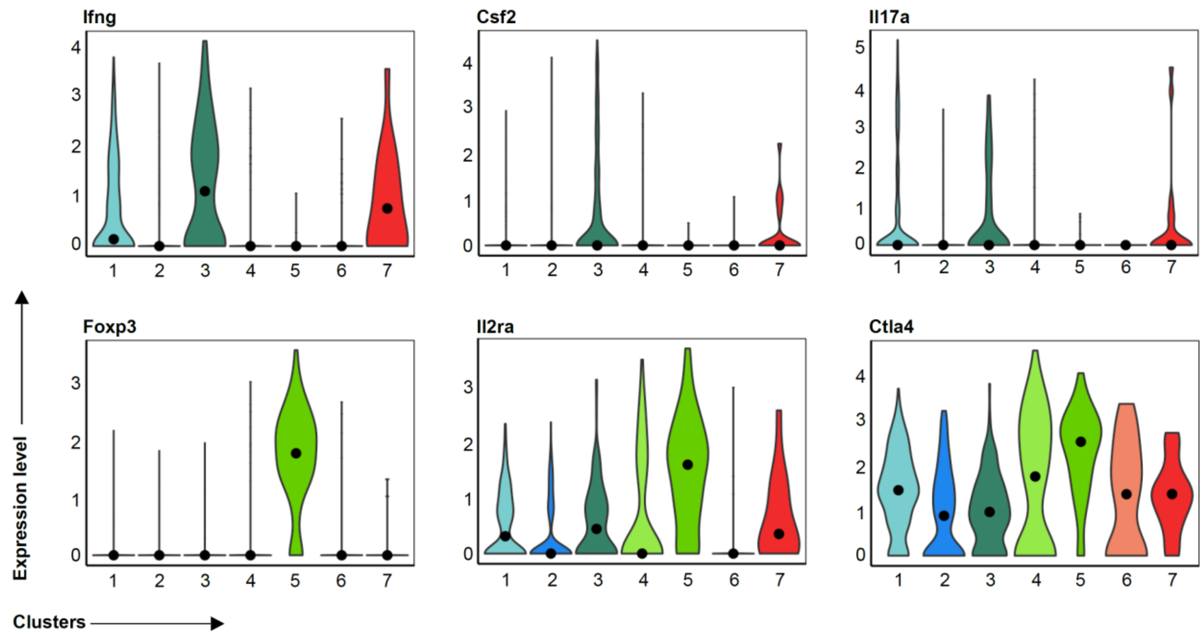
(B) Splenocytes were stained for CD44, CD62L and CTLA-4. Percentage of $CD44^+CD62L^-$ and CTLA-4^+ cells was compared between engineered ($Foxp3^+Thy1.1^+$) and endogenous ($Foxp3^+Thy1.1^-$) Tregs. Representative flow cytometry plot shows the expression of CD44, CD62L and CTLA-4 by engineered ($Foxp3^+Thy1.1^+$, magenta) and endogenous ($Foxp3^+Thy1.1^-$, cyan) Tregs. The arrow indicates "activated" $CD44^{hi}CD62L^{lo}$ cells.

****p<0.0001, ***p<0.001, **p<0.01, *p<0.05, ns – not significant.



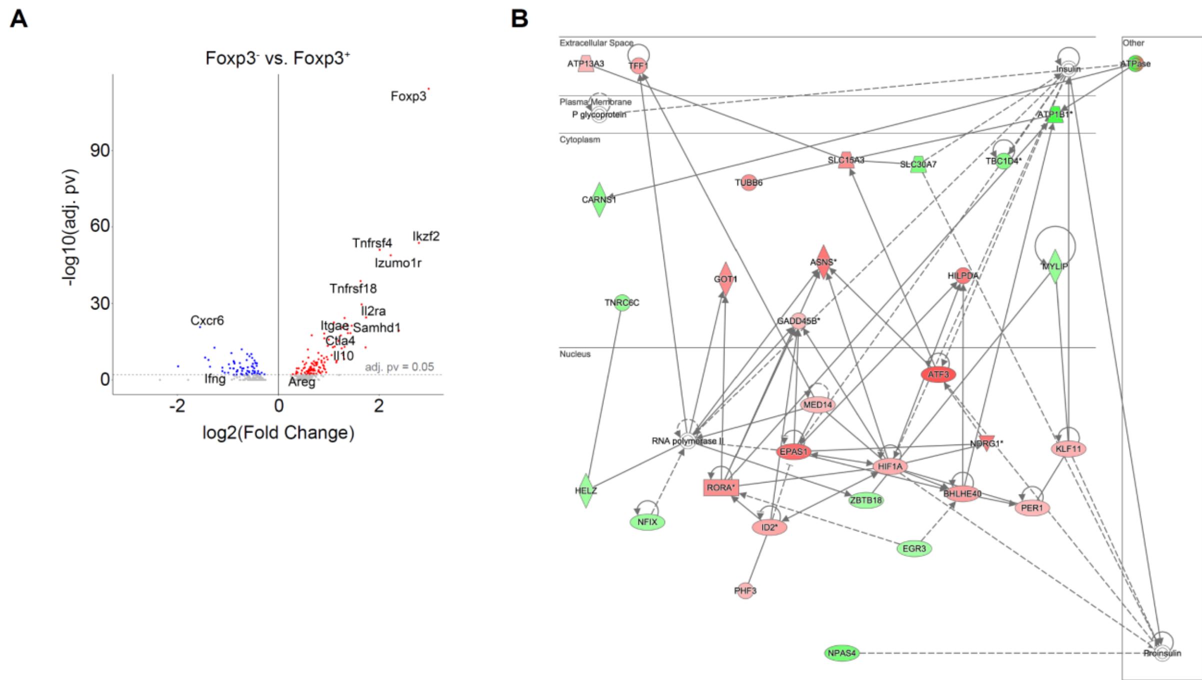
Supplementary Figure 3, related to Fig. 3: Transcriptome characteristics of CNS Tregs

CD45^{+} cells were sorted from the CNS of C57BL/6 mice at the peak of EAE (day 19) and analyzed by single-cell RNAseq (10x Genomics). CNS-infiltrating CD4^{+} T cells were grouped into 7 clusters (defined in Figure 3). A heatmap of the expression of the most characteristic differentially expressed genes belonging to the different clusters is shown.



Supplementary Figure 4, related to Fig. 3: Transcriptome characteristics of CNS Tregs

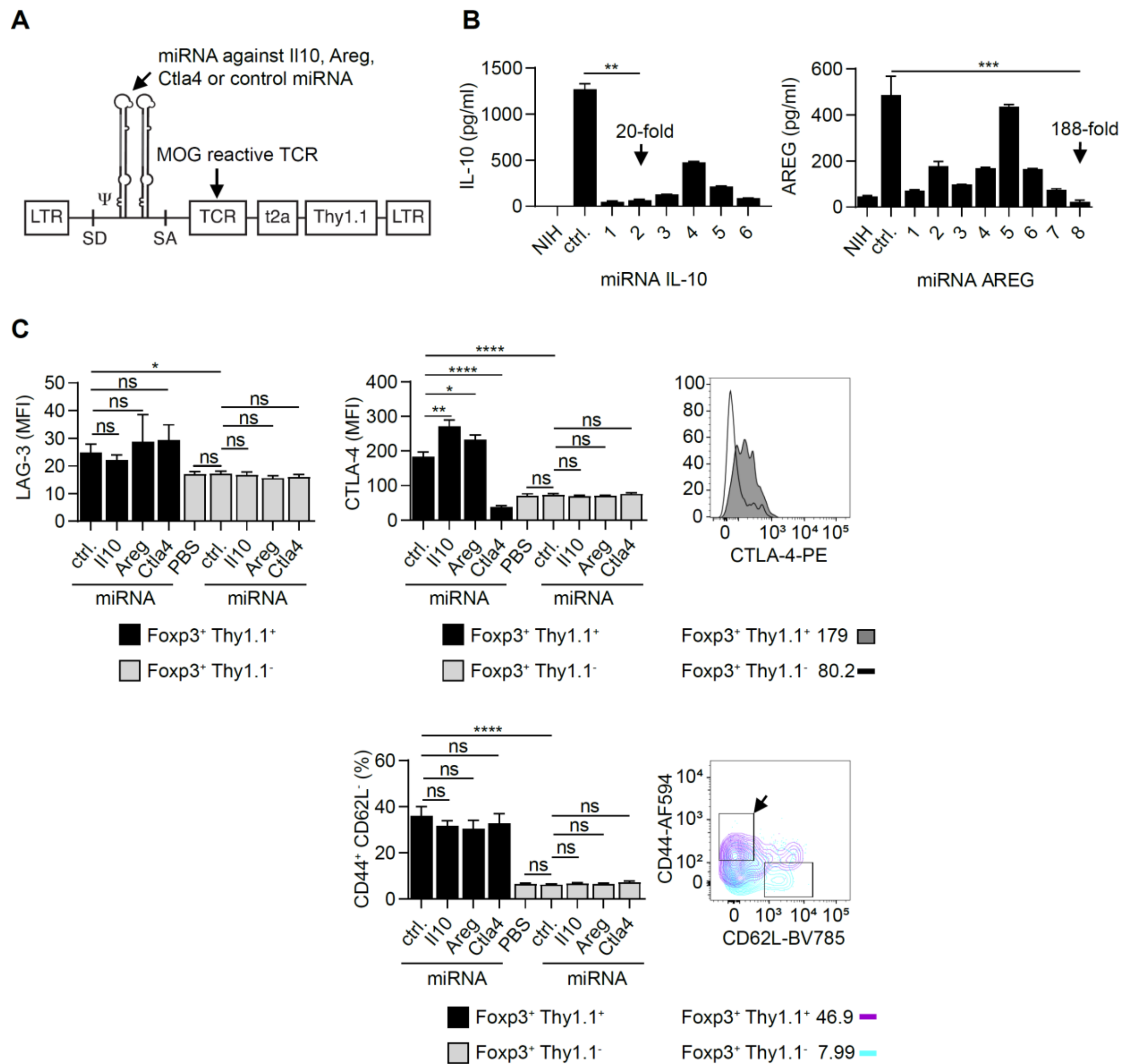
CD45⁺ cells were sorted from the CNS of C57BL/6 mice at the peak of EAE (day 19) and analyzed by single-cell RNAseq (10x Genomics). CNS-infiltrating CD4⁺ T cells were grouped into 7 clusters (defined in Figure 3). Violin plots show normalized expression of selected markers (*Ifng*, *Csf2*, *Il17a*, *Foxp3*, *Il2ra*, *Ctla4*) in each cluster.



Supplementary Figure 5, related to Fig. 3: Transcriptome characteristics of CNS Tregs

(A) CD45+ cells were sorted from the CNS of C57BL/6 mice at the peak of EAE (day 19) and analyzed by single cell RNAseq (10X Genomics). Volcano plot showing differentially expressed genes between Foxp3- (blue) and Foxp3+ (red) cells.

(B) Tregs and Tconvs were sorted from CNS and spleen of Foxp3.eGFP mice at the peak of EAE according to the expression of CD4 and Foxp3 (Treg: CD4+Foxp3.eGFP+ cells; Tconv: CD4+Foxp3.GFP- cells). Samples were hybridized to Affymetrix 430 2.0 whole-genome mouse arrays. Data show a selected Ingenuity network of differentially expressed transcriptional regulators between spleen and CNS Tregs (genes expressed at higher level in CNS or spleen Tregs are shown in red or green, respectively). The brightness of color relates to the fold change of differentially expressed gene (darker color reflects higher fold change).



Supplementary Figure 6, related to Fig. 4: IL-10 is critically involved in the protective function of autoreactive Tregs

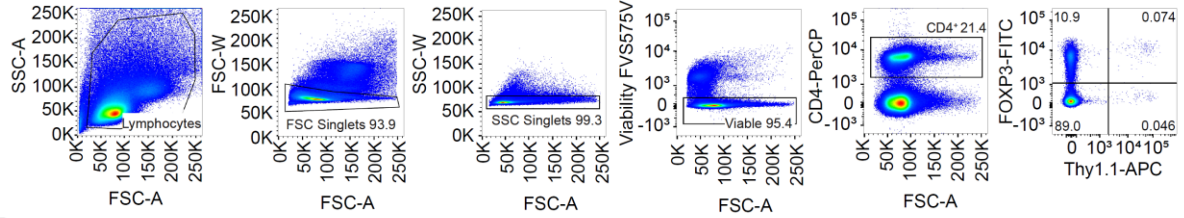
(A) Design of retroviral vector coding for high avidity TCR against MOG peptide and one (*Il10, Areg*) or two (*Ctla4*, ctrl.) intronic miRNA sequences (adopted from Kieback et al., 2016).

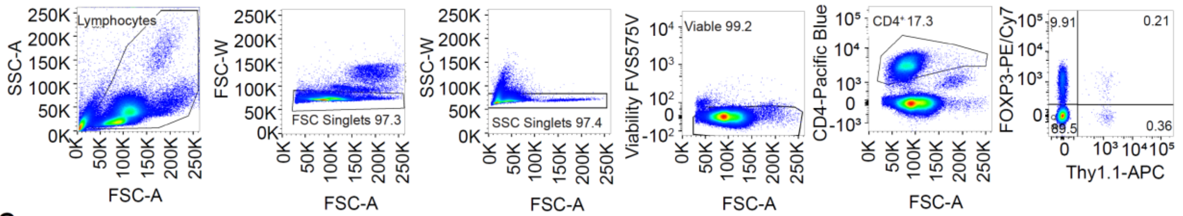
(B) NIH/3T3 cells were left untouched (NIH) or co-transduced with retroviral particles to express mouse *Il10* or *Areg*, and additionally retroviral particles containing the MOG-reactive TCR with the addition of miRNA against *Il10*, *Areg,* and control miRNA (ctrl.). 6 and 8 miRNA were tested for *Il10* and *Areg*, respectively. Supernatants were harvested 72h later to measure IL-10 and AREG concentrations in supernatants by Bioplex or ELISA, respectively. Arrows indicate the miRNA sequences later used in experiments and the fold inhibition of expression. Data show mean + SEM of one out of 2 experiments. Two (IL-10) or three (AREG) technical repetitions were made for each condition in each experiment.

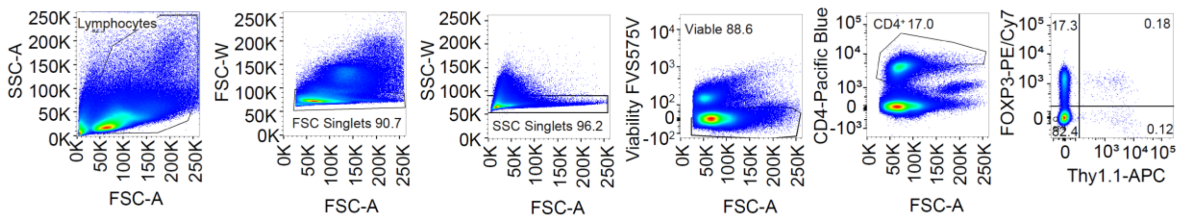
(C) 2 x 10^5 Tregs engineered to express MOG-reactive TCR and either control miRNA, miRNA to silence *Il10*, *Areg,* or *Ctla4* expression were adoptively transferred into C57BL/6 recipient mice on day -1. Control groups were treated with PBS. On day 0, mice were immunized with MOG(35-55) to induce EAE. Peripheral blood was collected 9 days p.i. and cells were stained for LAG-3, CTLA-4 (intracellularly) and CD44 as well as CD62L (cell surface). Expression of LAG-3 (MFI), CTLA-4 (MFI) and percentages of $CD44^+CD62L^-$ cells were compared between engineered Tregs ($Foxp3^+Thy1.1^+$) and endogenous Tregs ($Foxp3^+Thy1.1^-$). Representative flow cytometry plots show the expression of selected markers.

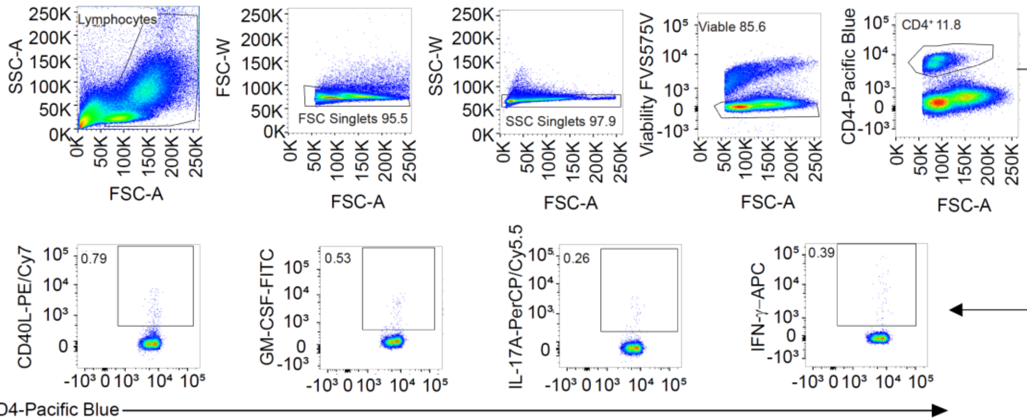
Data are pooled from four experiments (n = 22 per group). Graphs show mean + SEM.

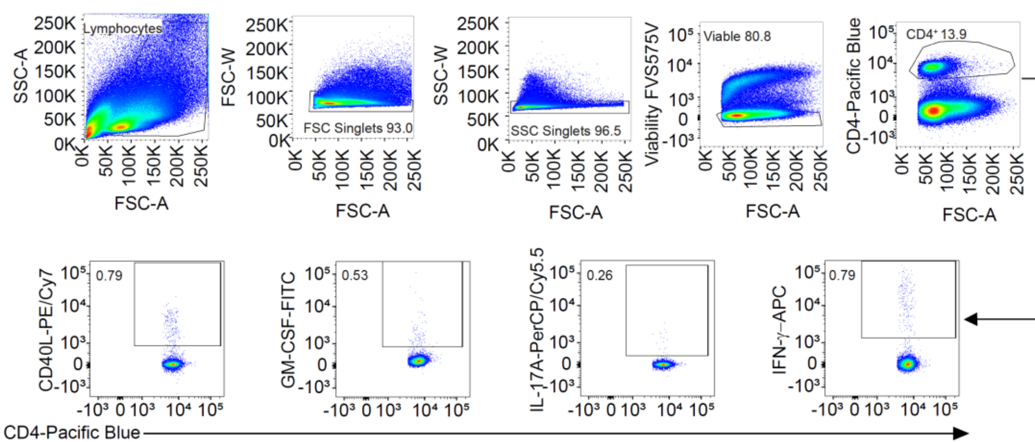
****p<0.0001, ***p<0.001, **p<0.01, *p<0.05, ns – not significant


A Lymph nodes - 3 days p.i.
SSC-A
FSC-A
Lymphocytes
FSC-W
FSC Singlets 93.9
SSC-W
SSC Singlets 99.3
Viability FVS575V
Viable 95.4
CD4-PerCP
CD4+ 21.4
FOXP3-FITC
Thy1.1-APC
10.9
0.074
89.0
0.046


B Blood - 9 days p.i.
SSC-A
FSC-A
Lymphocytes
FSC-W
FSC Singlets 97.3
SSC-W
SSC Singlets 97.4
Viability FVS575V
Viable 99.2
CD4-Pacific Blue
CD4+ 17.3
FOXP3-PE/Cy7
Thy1.1-APC
9.91
0.21
89.5
0.36


C Spleen - 35 days p.i.
SSC-A
FSC-A
Lymphocytes
FSC-W
FSC Singlets 90.7
SSC-W
SSC Singlets 96.2
Viability FVS575V
Viable 88.6
CD4-Pacific Blue
CD4+ 17.0
FOXP3-PE/Cy7
Thy1.1-APC
17.3
0.18
82.4
0.12


D Blood - 9 days p.i.
SSC-A
FSC-A
Lymphocytes
FSC-W
FSC Singlets 95.5
SSC-W
SSC Singlets 97.9
Viability FVS575V
Viable 85.6
CD4-Pacific Blue
CD4+ 11.8
CD40L-PE/Cy7
0.79
GM-CSF-FITC
0.53
IL-17A-PerCP/Cy5.5
0.26
IFN-γ-APC
0.39
CD4-Pacific Blue


E Spleen - 35 days p.i.
SSC-A
FSC-A
Lymphocytes
FSC-W
FSC Singlets 93.0
SSC-W
SSC Singlets 96.5
Viability FVS575V
Viable 80.8
CD4-Pacific Blue
CD4+ 13.9
CD40L-PE/Cy7
0.79
GM-CSF-FITC
0.53
IL-17A-PerCP/Cy5.5
0.26
IFN-γ-APC
0.79
CD4-Pacific Blue

Supplementary Figure 7: Gating strategy

(A) Gating strategy to analyse expression of surface markers on $Foxp3^+Thy1.1^+$ and $Foxp3^+Thy1.1^-$ cells in LNs. Representative gating of LNs cells 3 days post infection (p.i.). Related to Fig. 1 and Fig. S1.

(B) Gating strategy to analyse the number, percentage, and expression of surface markers on $Foxp3^+Thy1.1^+$ and $Foxp3^+Thy1.1^-$ cells in blood 9 days p.i. Related to Fig. 2C and E, Fig. 4C, Fig. 5B and Fig. S6C.

(C) Gating strategy to analyse the number, percentage, and expression of surface markers on $Foxp3^+Thy1.1^+$ and $Foxp3^+Thy1.1^-$ cells in spleens 35 days p.i. Related to Fig. 2D, Fig. 4D, Fig. 5C-D and Fig. S2B.

(D) Gating strategy to analyse the expression of CD40L, GM-CSF, IL-17A, and IFN-γ after restimulation with MOG(35-55) on $CD4^+$ cells in blood 9 days p.i. Related to Fig. 2B and Fig. 4B.

(E) Gating strategy to analyse the expression of CD40L, GM-CSF, IL-17A, and IFN-γ after restimulation with MOG(35-55) on $CD4^+$ cells in spleens 35 days p.i. Related to Fig. S2A.