

Immunity

Thymus-Derived Regulatory T Cells Are Positively Selected on Natural Self-Antigen through Cognate Interactions of High Functional Avidity

Highlights

- Autoreactive Treg cells express TCR of higher functional avidity than Tconv cells
- Expression of endogenous MOG controls the development of MOG-reactive Treg cells
- The avidity of their TCR controls the protective value of autoreactive Treg cells
- Treg cells use CTLA-4 to limit autoimmune disease of the central nervous system

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In Brief

Autoreactive Treg cells are essential for limiting immunopathology. Fillatreau and colleagues demonstrate that the thymic development of these cells depends on the expression of endogenous self-antigens and TCR interactions of high functional avidity. TCR of high functional avidity are essential for the capacity of Treg cells to suppress autoimmunity.



Thymus-Derived Regulatory T Cells Are Positively Selected on Natural Self-Antigen through Cognate Interactions of High Functional Avidity

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SUMMARY

Regulatory T (Treg) cells expressing Foxp3 transcription factor are essential for immune homeostasis. They arise in the thymus as a separate lineage from conventional CD4⁺Foxp3⁻ T (Tconv) cells. Here, we show that the thymic development of Treg cells depends on the expression of their endogenous cognate self-antigen. The formation of these cells was impaired in mice lacking this self-antigen, while Tconv cell development was not negatively affected. Thymus-derived Treg cells were selected by self-antigens in a specific manner, while autoreactive Tconv cells were produced through degenerate recognition of distinct antigens. These distinct modes of development were associated with the expression of T cell receptor of higher functional avidity for self-antigen by Treg cells than Tconv cells, a difference subsequently essential for the control of autoimmunity. Our study documents how self-antigens define the repertoire of thymus-derived Treg cells to subsequently endow this cell type with the capacity to undermine autoimmune attack.

INTRODUCTION

CD4⁺ T regulatory (Treg) cells expressing the transcription factor Foxp3 play an essential role in immune homeostasis. Humans bearing null mutations in the *FOXP3* gene display a deficit in Treg cells and develop the fatal immunodysregulation polyen-

docrinopathy enteropathy X-linked syndrome (IPEX) early in childhood (Bennett et al., 2001). Similarly, deficiency in the *Foxp3* gene in mice results in a lack of Treg cells and lethal immunopathology (Fontenot et al., 2003).

The inhibition of immunity by Treg cells necessitates their activation via the T cell receptor (TCR) (Levine et al., 2014). The antigens stimulating Treg cell activation at steady state must be self-antigens because Treg cells restrain immune-mediated pathogenesis also in germ-free mice (Chinen et al., 2010).

The antigen recognition properties of Treg cells are largely defined during their development in the thymus (Hsieh et al., 2006; Pacholczyk et al., 2006). Experiments performed with *Nur77*-eGFP reporter mice indicate that Treg cells received stronger TCR signals than CD4⁺Foxp3⁻ conventional T cells (Tconv) during their thymic development (Moran et al., 2011). Accordingly, a study using “TCR-cognate antigen” double-transgenic mice has shown that a TCR with high affinity for the selecting antigen drives Treg cell development, whereas TCRs of low affinity do not (Jordan et al., 2001). A role for strong TCR signaling during early Treg cell specification might be to promote the expression of TNF receptor super-family members GITR, OX40, and TNFR2, which facilitate Treg cell formation (Mahmud et al., 2014), and to stimulate the epigenetic changes of the *Foxp3*, *Ctla4*, *Ii2ra*, and *Itgal* loci associated with Treg cells (Morikawa and Sakaguchi, 2014).

The nature of the cognate antigens governing Treg cell selection in the thymus remains controversial. None of the studies mentioned above has addressed this issue because they were either performed using artificial transgenic antigens (Jordan et al., 2001) or only have provided indication on the intensity of the TCR signaling associated with Treg cell formation in response to non-defined epitopes (Moran et al., 2011). Although self-antigens are regularly evoked as mediators of this process,

no self-antigen that selects Treg cells has been identified. Moreover, this concept has been repeatedly questioned, and some investigators claim that microbiota has been the primary source of antigens mediating Treg cell selection (van Santen et al., 2004; Pennington et al., 2006; Pacholczyk et al., 2007).

Here, we sought to investigate the identity of the antigens implicated in thymic Treg cell selection. We focused on Treg cells specific for myelin oligodendrocyte glycoprotein (MOG), a minor component of the myelin sheath that is an important target in autoimmune diseases of the central nervous system (CNS). Upon activation, CD4⁺ T cells reacting against MOG can provoke an inflammatory demyelinating disease of the CNS in mice, called experimental autoimmune encephalomyelitis (EAE), which is the primary pre-clinical model for multiple sclerosis (Fillatreau et al., 2002). In this disease, MOG-reactive Treg cells provide an essential mechanism of protection from immune-mediated pathogenesis (McGeachy et al., 2005). We demonstrate that endogenous MOG expression contributed significantly to the thymic development of MOG-reactive Treg cells. We also have shown that these Treg cells expressed TCRs of higher functional avidity for MOG than Tconv cells and reveal the importance of this difference for the regulation of autoimmunity.

RESULTS

A TCR- β Transgenic Mouse to Study MOG-Reactive Tconv and Treg Cells

Autoreactive Tconv cells differ in their capacity to cause autoimmune disease. Those expressing public TCR clonotypes are prominent drivers of pathogenesis (van den Elzen et al., 2004). In order to analyze such cells, we generated a transgenic mouse (named Kaa mouse) expressing the public TCR- β clonotype of the MOG-reactive CD4⁺ T cell response during EAE in C57BL/6 mice (Fazilleau et al., 2006). This public TCR- β chain contains a V β 8.2 segment (Figure S1A), which is expressed in both Tconv and Treg cells in the central nervous system (CNS) of C57BL/6 mice with EAE (Figure S1B).

Naive Kaa mice contained an increased frequency of MOG-reactive Tconv cells, estimated at around 1.8% in spleen using CD40L expression as an indicator of TCR stimulation (Figure S1C) (Frentsch et al., 2005). Kaa Tconv cells were encephalitogenic because cells isolated from lymph nodes (LN) of Kaa mice immunized with MOG(35-55) expressed interferon- γ (IFN- γ) as well as tumor necrosis factor- α (TNF- α) upon ex vivo re-stimulation and induced EAE in recipient mice upon adoptive transfer (Figure 1A). Tconv cells from naive Kaa mice stimulated under the same condition also induced EAE in recipient mice, while non-transgenic Tconv cells had no effect (Figure 1B).

Despite harboring encephalitogenic Tconv cells, Kaa mice developed a milder EAE than wild-type mice upon immunization with MOG(35-55) (Figure 1C). We hypothesized that the Kaa mouse contained elevated numbers of MOG-reactive Treg cells. Using MOG-I-A^b tetramers, we found that 7.2% of Treg cells were MOG-reactive in the LN of Kaa.Foxp3.IRES.eGFP mice (Figure 1D). These Treg cells were functional since the proliferation of Kaa T cells stimulated with MOG(35-55) was increased after Treg cell depletion (Figure 1E) and adoptive transfer of Kaa Treg cells improved the course of EAE in recip-

ient C57BL/6 mice, while non-transgenic Treg cells had no effect (Figure 1F). This protection was associated with an accumulation and proliferation of adoptively transferred Kaa Treg cells in the draining LN of recipient mice immunized with MOG(35-55), which was not observed in control groups (Figure 1G and S1D). Moreover, interfering with Treg cell function in Kaa mice exacerbated EAE (Figure 1H). Collectively, these data demonstrate that Kaa mice contain protective MOG-reactive Treg cells that efficiently counteract encephalitogenic MOG-reactive Tconv cells.

Treg and Tconv Cells Differently Recognize MOG in the Kaa Mouse

Treg and Tconv cells express distinct TCR repertoires in wild-type mice (Hsieh et al., 2006; Pacholczyk et al., 2006), suggesting that they differ in their antigen recognition properties. We sought to address whether this was the case for cells specific for a disease-relevant autoantigen.

To characterize the TCR- α chains expressed by disease-relevant MOG-reactive Treg and Tconv cells, we induced EAE in Kaa mice on a *Foxp3.IRES.eGFP.Tcr α ^{+/-}Tcr β ^{+/-}* background, and isolated CD4⁺GFP⁺ cells (Treg cells) as well as CD4⁺GFP⁻ cells (Tconv cells) from their CNS at the peak of disease. TCR- α sequences were amplified from these cells using a 5' RACE PCR to allow unbiased repertoire analysis. Three independent experiments were performed, yielding a total of 3,592 and 3,928 TCR- α sequences, which corresponded to 1,011 and 1,086 unique TCR- α sequences for Treg and Tconv cells, respectively. We focused our analyses on sequences found in each of the three samplings for Treg or Tconv cells, assuming that these would derive from disease-relevant MOG-reactive cells. This yielded 21 and 22 TCR- α for Tconv and Treg cells, respectively (Figure S2A), corresponding to 40 distinct TCR α (three sequences were present in both subsets). Importantly, the frequency of these TCR- α among the total number of sequences analyzed for each experiment confirmed their biased representation in the Treg or Tconv cell compartments (Figure 2A).

We could re-express 30 of these 40 TCR- α (Figure S2B and Table S1) together with the Kaa TCR- β in a TCR-deficient cell line that produced IL-2 proportionally to the strength of stimulation of the ectopic TCR (Letourneur and Malissen, 1989). The majority of the TCRs tested (28/30) were MOG-reactive (Figure S2B). The TCRs from Treg cells ($n = 17$) conferred the cell line a markedly stronger reactivity toward MOG than did Tconv cell-derived TCRs ($n = 11$) (Figure 2B). The bi-modal distribution of this response showed that TCRs of Treg and Tconv cells belonged to two distinct classes differing by their functional avidity for MOG.

To assess whether this difference in functional avidity was associated with qualitative differences in the recognition of the antigen, we evaluated whether distinct residues within MOG(35-55) were important for stimulating Treg versus Tconv cell TCRs. First, we activated a sample of the cell lines expressing TCRs from Treg ($n = 7$) or Tconv ($n = 6$) cells with modified MOG(35-55) peptides containing alanine substitutions at each of the 21 amino acid positions, and quantified their IL-2 production relative to the response elicited by the original MOG(35-55) peptide (Figure S2C). On the basis of the obtained results, we then focused on six altered peptides that affected the cell lines

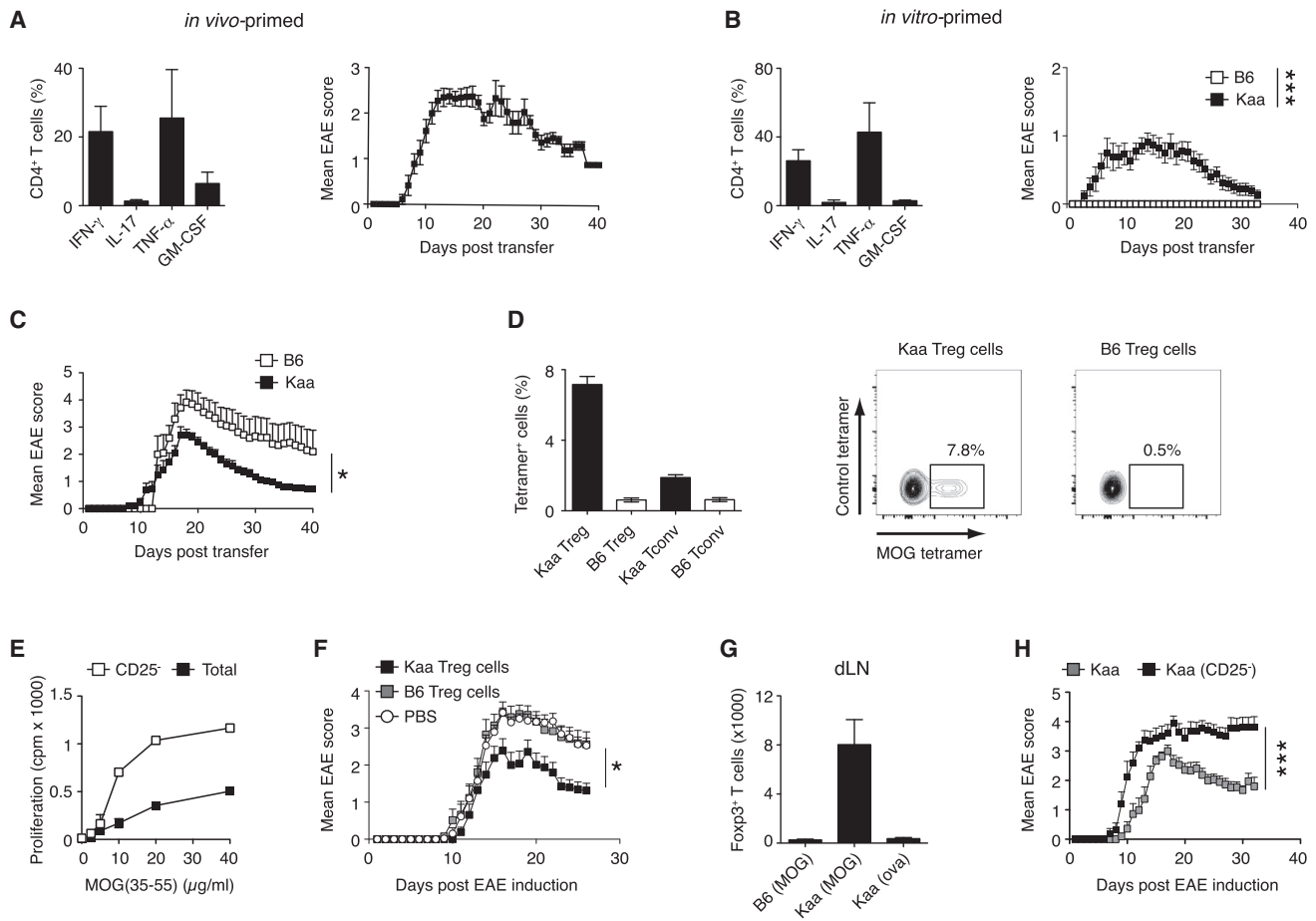


Figure 1. The Kaa Mouse

(A) LN cells isolated from Kaa mice on day 8 post-immunization with MOG(35-55) were re-stimulated with MOG(35-55) for 3 days, and (left panel) analyzed by flow cytometry to quantify cytokine-expressing CD4⁺ T cells, or (right panel) adoptively transferred in recipient mice. Data show mean EAE score \pm SEM from a pool of two experiments (n = 12).

(B) Cells from LN and spleens from naive Kaa or C57BL/6 mice were depleted from CD25⁺ cells and stimulated with MOG(35-55) for 3 days *in vitro*, prior to analysis by flow cytometry (left, only Kaa) or adoptive transfer (right). Pooled data (mean EAE score \pm SEM) from three experiments (C57BL/6: n = 10, Kaa: n = 13).

(C) EAE was induced in Kaa (n = 12) and C57BL/6 (n = 6) mice by immunization with MOG(35-55). Data show mean EAE score \pm SEM.

(D) LN cells of Kaa.*Foxp3*.eGFP (Kaa, n = 4) or *Foxp3*.eGFP (B6, n = 2) mice were stained with MOG-I-A^b-PE and control-I-A^b-APC tetramers. Data show frequency of MOG-I-A^b-binding cells in Treg and Tconv cells (left, pooled results from all mice) and representative stainings (right) gated on CD4⁺GFP⁺ cells for Kaa.*Foxp3*.eGFP (Kaa Treg cells) and *Foxp3*.eGFP (B6 Treg cells) mice.

(E) Splenocytes from naive Kaa mice were depleted or not of CD25-expressing cells, and stimulated for 72 hr with MOG(35-55) to measure their proliferation by ³H-thymidine incorporation. Data show mean values of triplicates. Representative results from one of two experiments.

(F) EAE course of C57BL/6 mice that received Treg cells from naive Kaa (n = 12) or C57BL/6 (n = 13) mice or PBS (n = 13) on day 1 before EAE induction by immunization with MOG(35-55). Data show results (mean EAE score \pm SEM) pooled from three experiments. Statistic was calculated comparing B6 Treg versus Kaa Treg cells.

(G) Treg cells from Kaa or C57BL/6 mice were labeled with Cell Proliferation Dye eFluor450 and injected into CD45.1⁺ C57BL/6 mice. Mice were immunized with either MOG(35-55) or OVA(323-369) and the number of CD45.2⁺CD4⁺Foxp3⁺ cells in the draining LN was determined 6 days later. Data are pooled from two experiments (n = 9 per group).

(H) Kaa mice were treated with anti-CD25 (PC61) (n = 11) or left untreated (n = 10) and immunized with MOG(35-55) to induce EAE. Data show results (mean EAE score \pm SEM) pooled from two experiments. See also Figure S1.

expressing TCRs from Treg and Tconv cells in a distinct manner (Figure S2C), and re-tested them on the 28 cell lines available (Figure 2C). Five of these peptides elicited distinct IL-2 responses depending on the origin of the TCR expressed by the cell lines. In particular, the 49(H→A) peptide increased the amount of IL-2 produced by cell lines expressing Tconv cell TCRs by about 10-fold while having little effect on the response

of cells bearing Treg cell TCRs. From these results, we conclude that the TCRs from Treg and Tconv cells characterized in this study differently recognize the MOG antigen with conserved properties within each class.

A limitation of the clonal analyses described above is that they are only feasible for a limited number of TCRs. In an attempt to generalize these findings, we tested the prediction that the

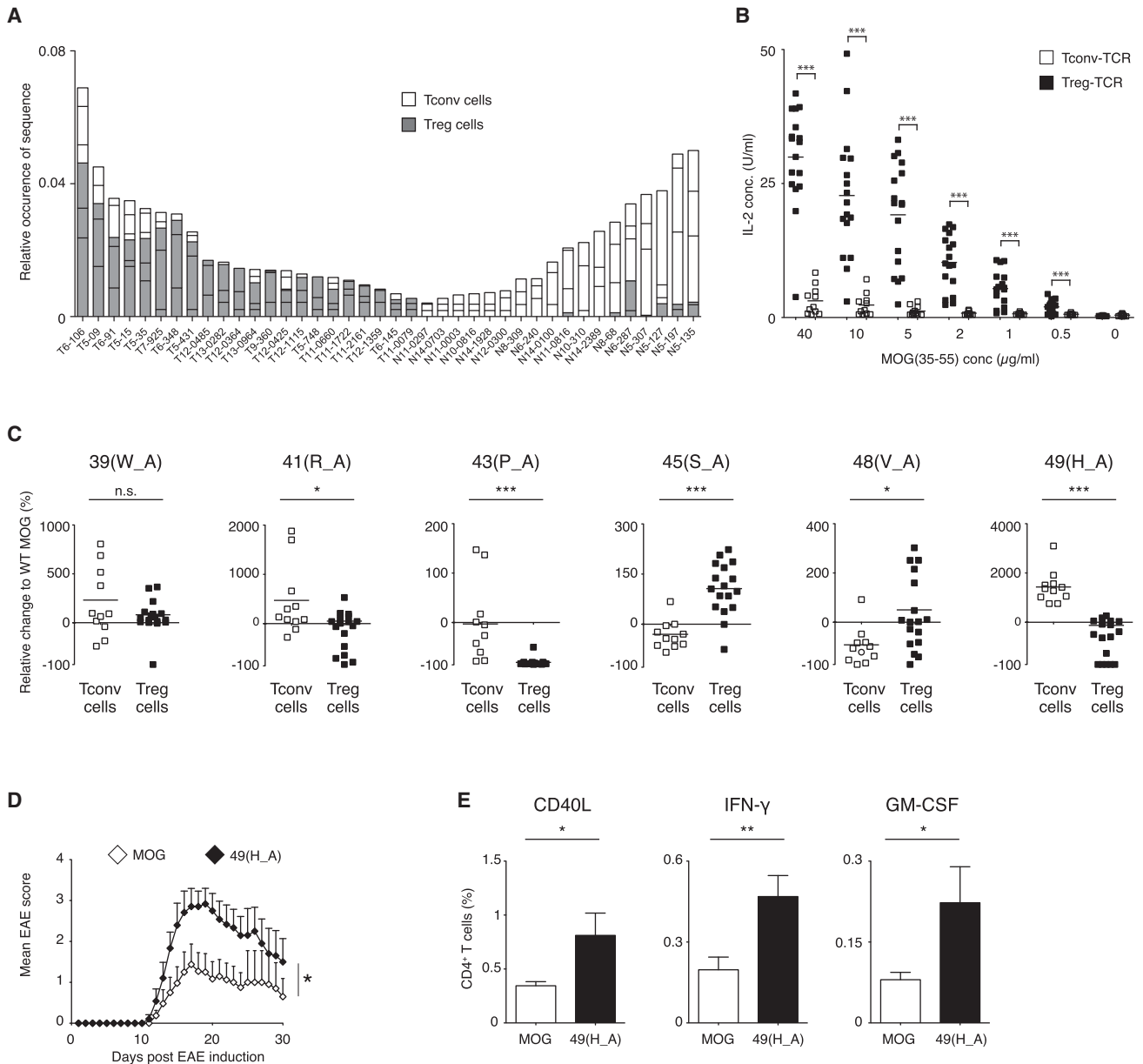


Figure 2. Antigen Recognition Properties of TCR from MOG-Reactive Treg and Tconv Cells

(A) Relative frequencies of the selected TCR- α among the total number of sequences analyzed for Treg (gray) and Tconv (white) cells. Each segment within a bar corresponds to one of the three independent experiments. Relative frequencies were calculated by dividing the counts of each selected sequence per experiment by the total number of sequences analyzed in this experiment. The indexes “T” and “N” indicate sequences found in the three Treg and Tconv cells samplings, respectively.

(B) The T.54 ζ 17 cell lines expressing MOG-reactive TCRs from Treg ($n = 17$) or Tconv ($n = 11$) cells were stimulated with MOG(35-55) in indicated concentrations, and IL-2 production was quantified by ELISA. Data show one representative of three experiments.

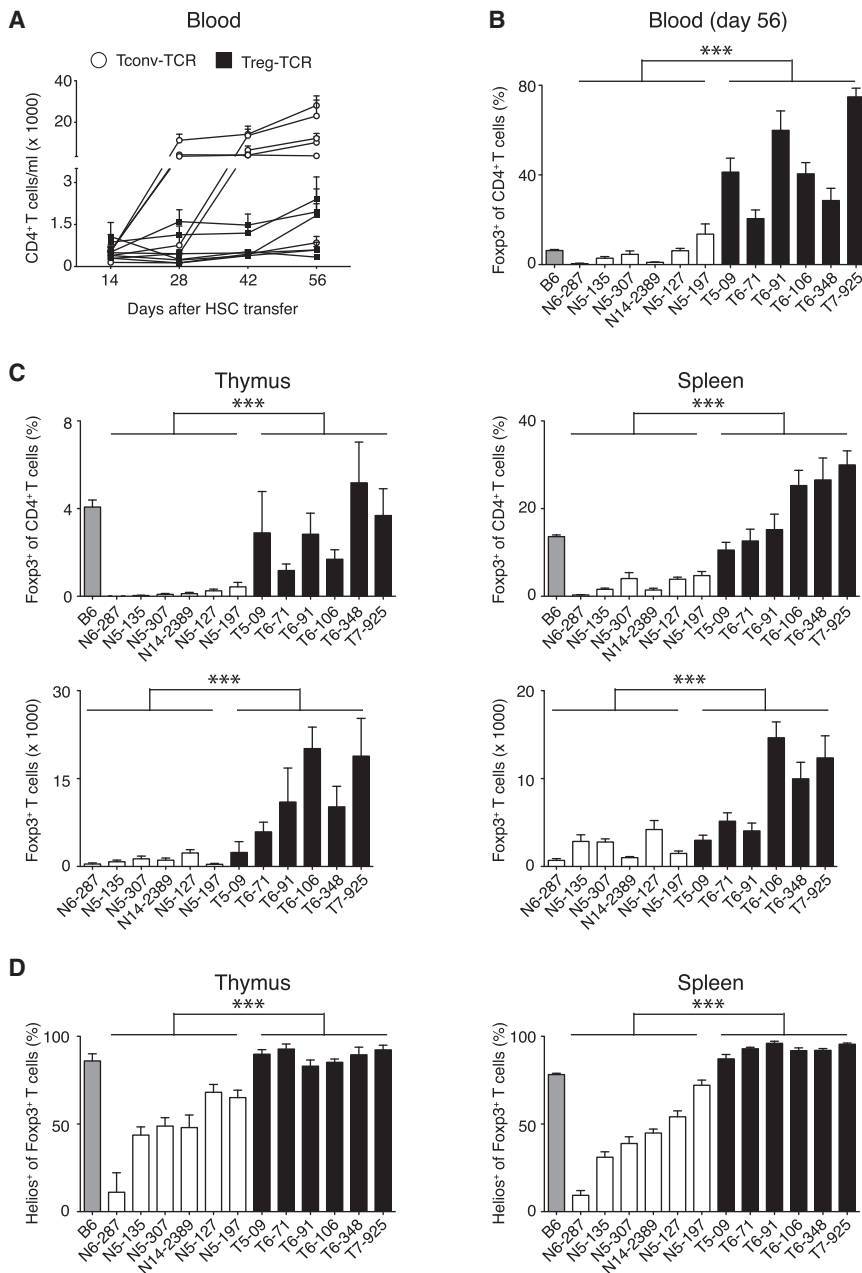
(C) The cell lines used in B (Treg cells: $n = 17$, Tconv cells: $n = 11$) were stimulated with MOG(35-55) peptide variants or WT MOG(35-55). Data show the change in IL-2 produced after stimulation with each variant peptide relatively to WT MOG(35-55). One representative of two experiments.

(D) Kaa mice were immunized with 49(H \rightarrow A) or MOG(35-55). Data show mean EAE score + SEM, and are pooled from two experiments ($n = 12$).

(E) Blood was collected from mice shown in D on day 10 after immunization, and re-stimulated for 5 hr with MOG(35-55) ex vivo to quantify CD40L- and cytokine-expressing CD4 $^{+}$ T cells. Shown are mean + SEM. See also [Figure S2](#) and [Table S1](#).

49(H \rightarrow A) peptide should be more encephalitogenic than the original self-antigen because it was a stronger Tconv cell stimulant. Indeed, the variant 49(H \rightarrow A) induced a more severe EAE than the original MOG(35-55) peptide ([Figure 2D](#)), correlating

with the induction of stronger encephalitogenic CD4 $^{+}$ T cell responses ([Figure 2E](#)). These results corroborate the notion that Tconv and Treg cells carry TCRs with different antigen recognition properties.



The TCR Instructs the Development of MOG-Reactive Treg Cells

We evaluated the capacities of TCRs cloned from MOG-reactive Treg and Tconv cells to drive Treg cell development in vivo generating TCR retrogenic mice using six TCRs from Treg cells and six TCRs from Tconv cells.

Donor-derived CD4⁺ T cells were detected in the blood of retrogenic mice starting 2 weeks after reconstitution and followed distinct patterns of repopulation depending on the origin of the TCR. Donor-derived CD4⁺ T cell numbers were lower (Figure 3A), while Treg cell frequencies were higher (Figure 3B) in mice prepared with TCRs from Treg cells compared to those produced with TCRs from Tconv cells. Similar differences were observed in thymus and spleen (Figure 3C), as

Figure 3. Role of TCR for Treg Cell Development

HSC from CD45.2⁺Rag1^{-/-} mice were transduced with one of 12 MOG-reactive TCR and used to reconstitute CD45.1⁺ C57BL/6 mice.

(A) Donor-derived CD45.2⁺CD4⁺ T cells were quantified in blood.

(B) Blood lymphocytes were analyzed for the percentage of Fopxp3⁺ cells among donor-derived CD45.2⁺CD4⁺ cells. C57BL/6 mice were included as control.

(C) Thymus and spleen of retrogenic mice were analyzed for percentages of Fopxp3⁺ cells among donor-derived CD45.2⁺CD4⁺CD8⁻ T cells (upper panels) and absolute numbers (lower panels) of CD45.2⁺CD4⁺CD8⁻Fopxp3⁺ cells 9 weeks after reconstitution.

(D) Expression of Helios in donor-derived CD45.2⁺CD4⁺CD8⁻ Fopxp3⁺ Treg cells in thymus and spleen.

(A–D) Data (mean + SEM) show pool from two experiments (n = 9/10 mice per TCR) and are analyzed comparing all mice prepared with Tconv cell TCRs versus all mice made with Treg cell TCRs (unpaired t test). See also Figure S3.

well as LN (Figure S3A), both in frequency and absolute number of Treg cells. Thus, TCRs from MOG-reactive Treg cells generated Treg cells more efficiently than TCRs from Tconv cells. In line with previous studies, however, none of the TCRs induced the formation of only Treg cells (Bautista et al., 2009; Leung et al., 2009).

We then measured the expression of Helios, a transcription factor of the Ikaros family described as a marker of thymus-derived Treg cells (Thornton et al., 2010). Nearly all donor-derived Treg cells expressed Helios in mice made with Treg cell TCR, while fewer Treg cells expressed Helios in mice generated with TCRs from Tconv cells (Figure 3D). As expected, Tconv cells did not express Helios (Figure S3B).

Further cytometric analyses indicated that donor-derived Treg cells were mostly CD25⁺CD62L⁺GITR^{high} irrespective of the origin of their TCRs, while Tconv cells displayed different phenotypes (Figure S3C).

Taken together, our results show that TCRs of high functional avidity for MOG have a superior capacity to generate Helios-expressing Treg cells.

Endogenously Expressed MOG Controls the Development of MOG-Reactive Treg Cells

The role of self-antigens in Treg cell development remains controversial. Here, we examined whether MOG was required for the formation of MOG-reactive Treg cells using *Mog*^{-/-} mice (Hövelmeyer et al., 2005).

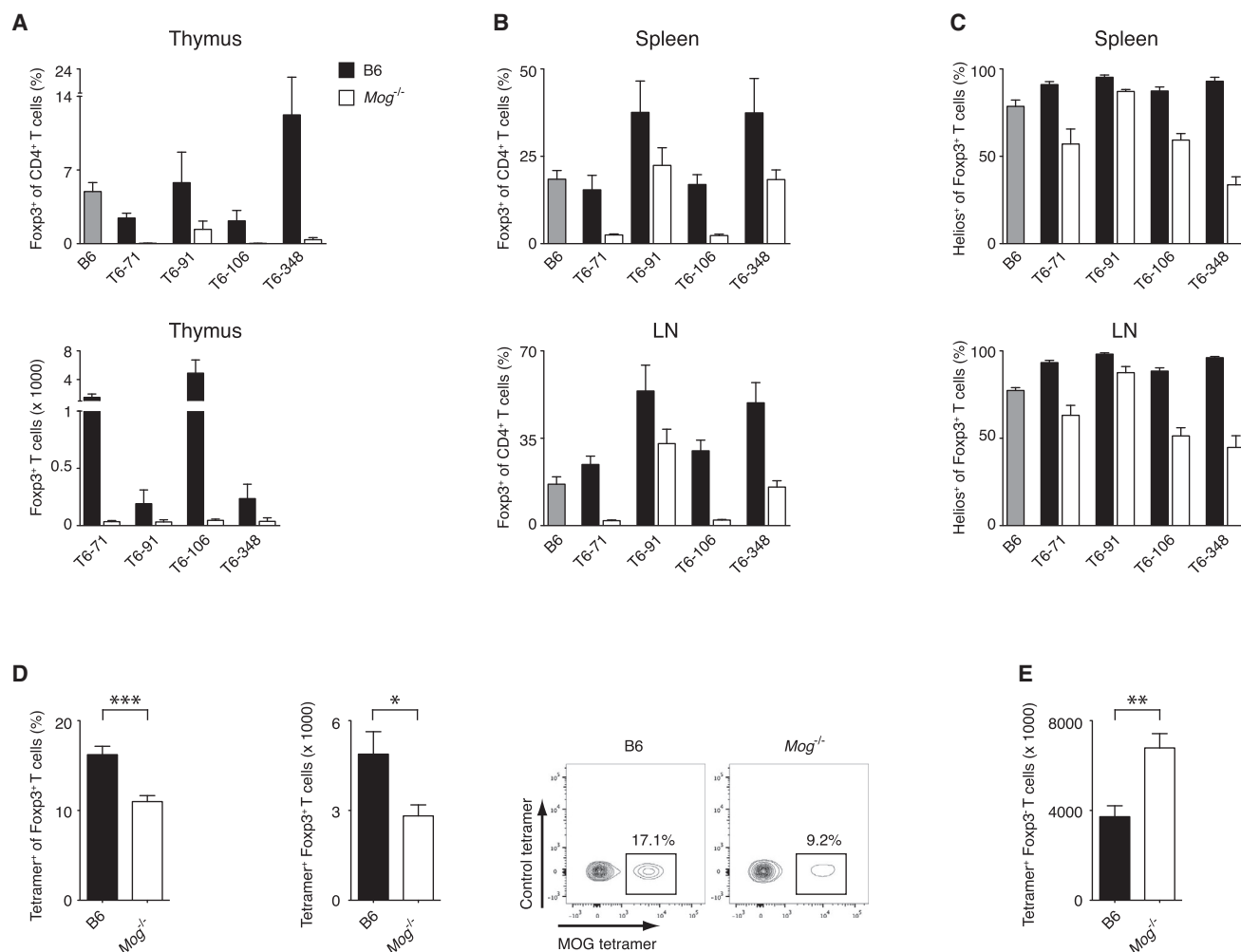


Figure 4. Role of MOG for the Development of MOG-Reactive Treg Cells

(A–C) HSC of CD45.1⁺Rag1^{-/-} mice were transduced with one of four MOG-reactive TCR from Treg cells, and used to reconstitute C57BL/6 (black bars) or *Mog*^{-/-} (white bars) mice. Nine weeks later, thymus, spleen, and LN were analyzed. WT C57BL/6 (gray bars) were included as control. (A and B) Percentage of Fopx3⁺ cells among donor-derived CD45.1⁺CD4⁺ cells, and absolute numbers of CD45.1⁺CD4⁺Fopx3⁺ Treg cells. (C) Frequency of Helios-expressing cells in CD45.1⁺CD4⁺Fopx3⁺ Treg cells. (A–C) Data pooled from two experiments (n = 9/10 mice per TCR).

(D and E) C57BL/6 (black bars, n = 8) and *Mog*^{-/-} (white bars, n = 7) mice were reconstituted with CD90-depleted bone marrow cells from Kaa.Foxp3.eGFP mice. Thymocytes were analyzed 5 weeks later by flow cytometry after staining with MOG-I-A^b tetramer and α CD4. Data show percentage of MOG-I-A^b-binding cells among CD4⁺CD8⁻GFP⁺ Treg cells and absolute numbers of MOG-I-A^b-binding CD4⁺CD8⁻GFP⁺ Treg cells. Representative stainings show MOG-I-A^b and control-I-A^b tetramer staining gated on live thymic donor-derived CD4⁺CD8⁻GFP⁺ Treg cells in C57BL/6 and *Mog*^{-/-} recipients. (E) Absolute numbers of MOG-I-A^bCD4⁺CD8⁻GFP⁺ T cells in thymus of reconstituted C57BL/6 and *Mog*^{-/-} mice were analyzed in (D). Data show mean + SEM and are pooled from two experiments. See also Figure S4.

We selected four Treg cell-derived TCRs to prepare retrogenic mice using wild-type and *Mog*^{-/-} mice as recipients. Absolute numbers and frequencies of Treg cells among donor-derived CD4⁺ T cells were reduced in the thymus of *Mog*^{-/-} mice compared to controls (Figure 4A). Similar differences were observed in spleens and LN, although they were less striking for some TCRs (Figure 4B and S4A). Fewer donor-derived Treg cells expressed Helios in *Mog*^{-/-} mice, compared to controls, illustrating the contribution of MOG to the normal development of MOG-reactive Treg cells (Figure 4C and S4B). Expression of CD25, CD62L, or GITR was in contrast not influenced by MOG (Figures S4C–S4E).

To address how endogenous MOG expression influenced the development of MOG-reactive Treg cells in a polyclonal setting, we reconstituted wild-type and *Mog*^{-/-} mice with bone marrow cells from Kaa.Foxp3.IRES.eGFP mice and used MOG-I-A^b tetramer to quantify MOG-reactive Treg and Tconv cells (Figure 4D). The frequency of MOG-I-A^b-binding cells among CD4⁺GFP⁺ Treg cells, and the absolute number of MOG-I-A^b-binding CD4⁺GFP⁺ Treg cells were markedly reduced (absolute number reduced by 42%) in the thymus of *Mog*^{-/-} mice compared to controls (Figure 4D). These results show that the absence of a single self-antigen, here MOG, is sufficient to reduce the formation of MOG-reactive Treg cells.

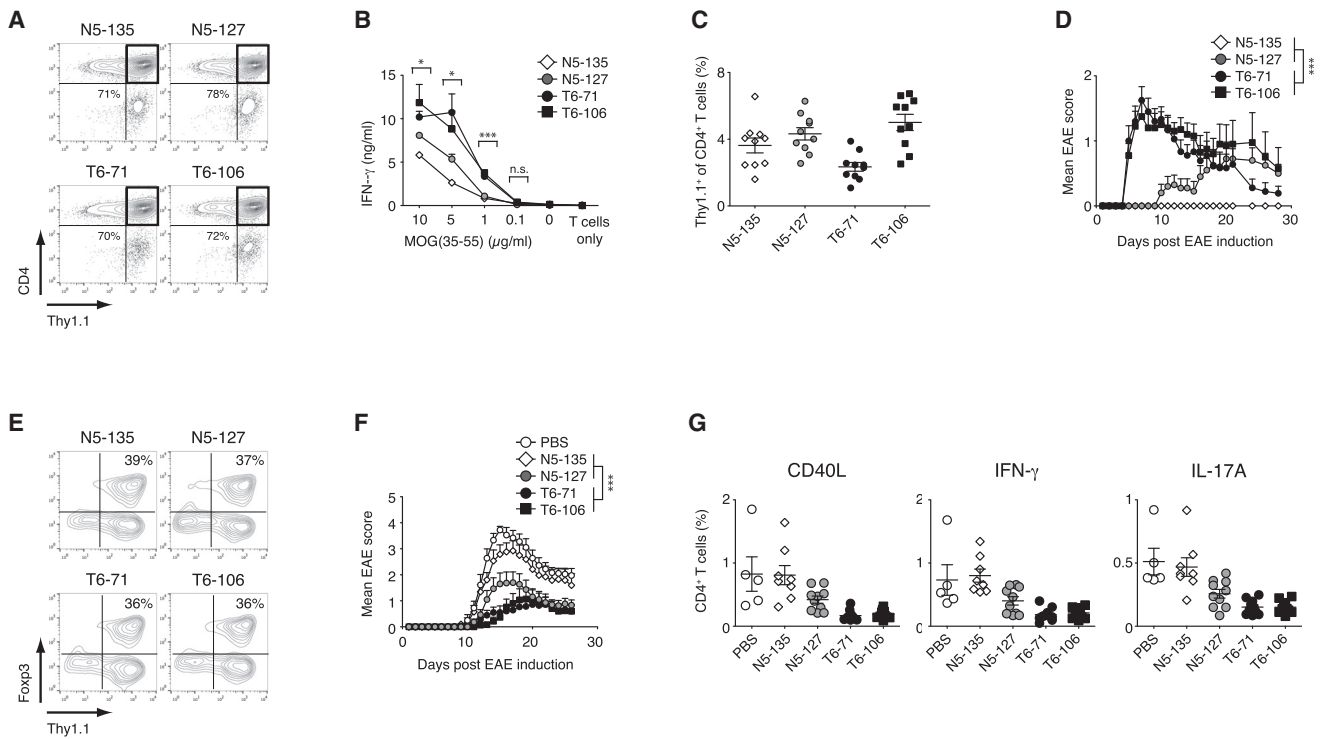


Figure 5. TCR Functional Avidity Determines the Function of MOG-Reactive Treg and Tconv Cells

(A) Polyclonal Tconv cells were activated and transduced with retroviral vectors encoding for one of four MOG-reactive TCRs and the marker Thy1.1. Flow cytometry plots show expression of Thy1.1 by T cells two days after transduction. Numbers indicate the percentage of Thy1.1⁺CD4⁺ cells among lymphocytes in the culture.

(B) TCR-modified Tconv cells obtained as in (A) were stimulated for 24 hr with MOG(35-55) in the presence of irradiated splenocytes. IFN- γ concentration in supernatant was measured by ELISA. Data show means + SEM of duplicates. One representative of two experiments. Statistics were calculated comparing all values from Tconv cell TCR versus all values from Treg cell TCRs for each peptide concentration.

(C and D) TCR-modified Tconv cells obtained as in (A) were used to induce EAE in recipient C57BL/6 mice via adoptive transfer. (C) Seven days after transfer, the percentage of Thy1.1⁺ cells among peripheral blood CD4⁺ T cells was determined. (D) Data show mean EAE score + SEM. Data in (C) and (D) are pooled from two experiments (n = 10 per group). Statistics were calculated comparing the EAE curves from all mice receiving Tconv cell TCRs versus those from mice receiving Treg cell TCRs.

(E) Polyclonal Treg cells from C57BL/6 mice were activated and transduced with retroviral vectors encoding for one of the four different MOG-reactive TCRs and Thy1.1. Flow cytometry plots show expression of Foxp3 and Thy1.1 by CD4⁺ T cells on day two after transduction. Numbers indicate the percentage of Thy1.1⁺Foxp3⁺ cells among CD4⁺ T cells.

(F) TCR-engineered Treg cells produced as in (E), or PBS, were administered to C57BL/6 mice one day before EAE induction by immunization with MOG(35-55). Data show mean EAE score + SEM, and are pooled from three experiments (n = 9 to 15 per group). Statistics were calculated comparing the EAE curves from all mice receiving Tconv cell TCRs versus those from mice receiving Treg cell TCRs.

(G) Blood samples of mice from (F) were collected on day 10 after immunization, re-stimulated with MOG(35-55) for 5 hr, and analyzed by flow cytometry to quantify the frequency of CD4⁺ T cells expressing CD40L, IFN- γ , or IL-17A. Graphs show mean \pm SEM, and are pooled from two experiments (n = 5 to 10 per group). See also Figure S5.

In contrast, MOG-reactive Tconv cells were efficiently selected in the absence of MOG. They were present in similar numbers in wild-type and *Mog*^{-/-} retrogenic mice (Figure S4F), and were more abundant in the thymus of the chimera mice bearing a polyclonal repertoire that lacked MOG expression compared to controls (Figure 4E). Thus, distinct cognate antigens control the formation of MOG-reactive Treg and Tconv cells in the thymus.

TCR Functional Avidity Controls the Activity of Treg and Tconv Cells during Autoimmune Disease

We next addressed whether the function of Treg and Tconv cells in the periphery was influenced by the functional avidity of their TCRs for the disease-relevant self-antigen.

First, we ectopically expressed two MOG-reactive TCRs from Treg cells and two MOG-reactive TCRs from Tconv cells in polyclonal Tconv cells through TCR gene transfer (Figure 5A). Although the four T cell populations displayed similar IFN- γ production upon stimulation with PMA and ionomycin (Figure S5A), those expressing a TCR from Treg cells produced more IFN- γ upon stimulation with MOG(35-55) (Figure 5B). Upon adoptive transfer, these four Tconv cell populations expanded comparably in recipient mice (Figure 5C). However, Tconv cells expressing a TCR from Treg cells caused a more severe EAE in recipient mice with an earlier disease onset (Figure 5D) and a higher cumulative disease burden (Figure S5B). Thus, the functional avidity of their TCR for MOG determines the encephalitogenic potential of Tconv cells.

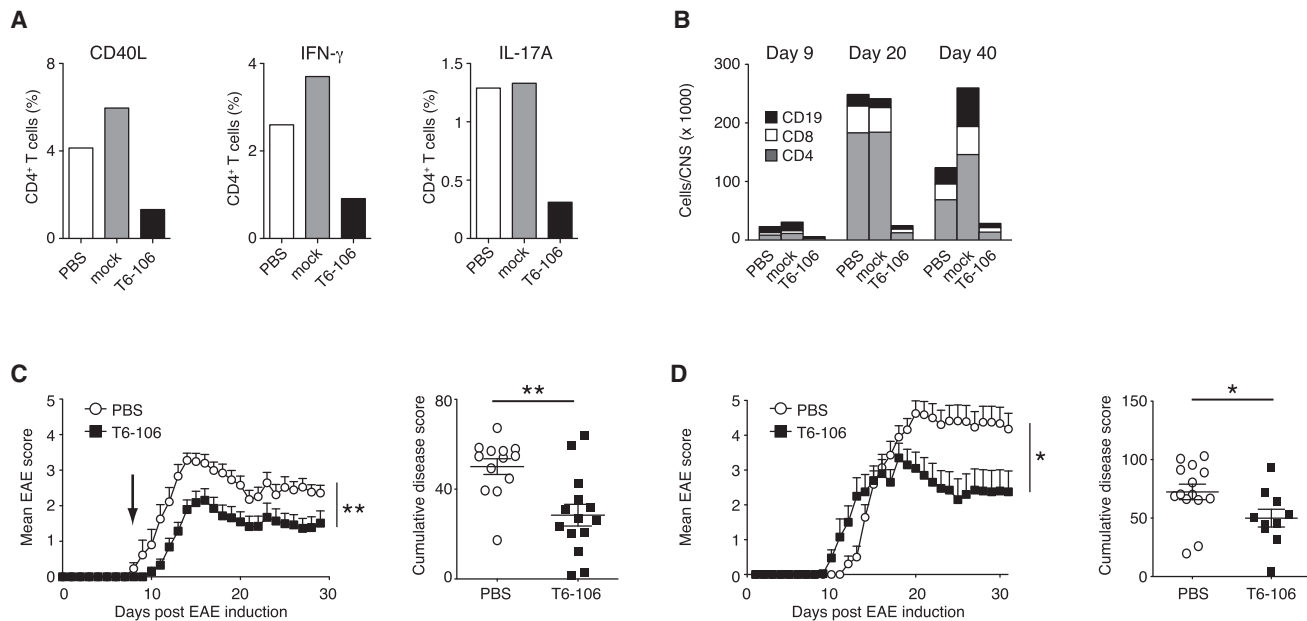


Figure 6. Protective Function of Engineered MOG-Reactive Treg Cells

(A and B) C57BL/6 mice were treated with PBS, engineered Treg cells expressing the TCR T6-106 and Thy1.1, or a mock vector coding only for Thy1.1, and immunized with MOG(35-55) 1 day post-transfer to induce EAE. (A) On day 9 post-immunization, draining LN (popliteal and inguinal) cells from five mice were pooled and re-stimulated *in vitro* for 6 hr with MOG(35-55), and analyzed for intracellular cytokines and CD40L expression by flow cytometry. One representative of two experiments. (B) CNS (brain plus spinal cord) from five mice were pooled and analyzed at indicated days after EAE induction by flow cytometry. One representative of two experiments.

(C) C57BL/6 mice were immunized with MOG(35-55) and treated 8 days later with 2×10^6 T6-106 TCR-transduced Treg cells or PBS. Data show mean EAE score + SEM, and cumulative disease scores (pooled from three experiments with $n = 14/15$ per group).

(D) C57BL/6 mice received 2×10^6 T6-106 TCR-transduced Treg cells or PBS, and were immunized 6 weeks later with MOG(35-55) to induce EAE. Data show mean EAE score + SEM, and cumulative disease scores (pool from two experiments with $n = 10/14$ per group). See also Figure S6.

We next ectopically expressed the same four TCRs in polyclonal Treg cells (Figure 5E). Upon activation with MOG(35-55), such engineered Treg cells suppressed responder T cell proliferation in a dose-dependent manner *in vitro* (Figure S5C). Upon adoptive transfer, Treg cells expressing a TCR of high avidity for MOG afforded better protection from EAE than Treg cells carrying a low avidity TCR (Figure 5F and S5D), correlating with a stronger reduction of the autoreactive T cell response in recipient mice (Figure 5G). We conclude that the functional avidity of their TCRs for MOG determines the protective function of Treg cells in EAE.

Engineered Treg Cells Have Therapeutic Function when Administered after EAE Induction

We next characterized how polyclonal Treg cells expressing a TCR of high functional avidity for MOG protected recipient mice from EAE in a prophylactic setting and determined whether these Treg cells were also beneficial when adoptively transferred after, or long before, EAE induction.

In a prophylactic setting, the protection afforded by engineered Treg cells was associated with their accumulation (Figure S6A) and a reduced encephalitogenic T cell response (Figure 6A) in the draining LN of recipient mice. The transferred Treg cells also migrated to the CNS of recipient mice (Figure S6B), correlating with a decreased inflammatory immune cell infiltrate (Figures 6B and S6C). Thus, the engineered Treg

cells suppressed the pathogenic reaction in both secondary lymphoid organs and the target tissue. Transduced Treg cells still reduced disease severity when administered in recipient mice at EAE onset (Figure 6C). They also persisted long-term after transfer in naive recipient mice (Figure S6D), and when these mice were immunized 6 weeks after Treg cell transfer, the latter cells reduced the activation of the pathogenic T cell response (Figure S6E), the progression of EAE course (Figure 6D), and disease-associated mortality (Figure S6F). We conclude that polyclonal Treg cells engineered to express a TCR of high functional avidity for a disease-relevant antigen might provide a cellular therapy to treat diseases caused by unwanted T cell responses.

CTLA-4 Expression Is Essential for the Suppressive Function of Treg Cells Engineered with TCRs of High Functional Avidity

We next asked whether polyclonal Treg cells activated *ex vivo* and genetically engineered to express a TCR of high functional avidity for MOG remained reliant on mechanisms commonly used by Treg cells to suppress immunity.

CTLA-4 is pivotal for Treg cell-mediated suppression (Wing et al., 2008), even though one study found that it was dispensable for the limitation of CNS autoimmunity (Verhagen et al., 2009). To produce MOG-reactive Treg cells having reduced amounts of CTLA-4, we developed a retroviral vector containing the MOG-reactive TCR cassette and two miRNAs to silence

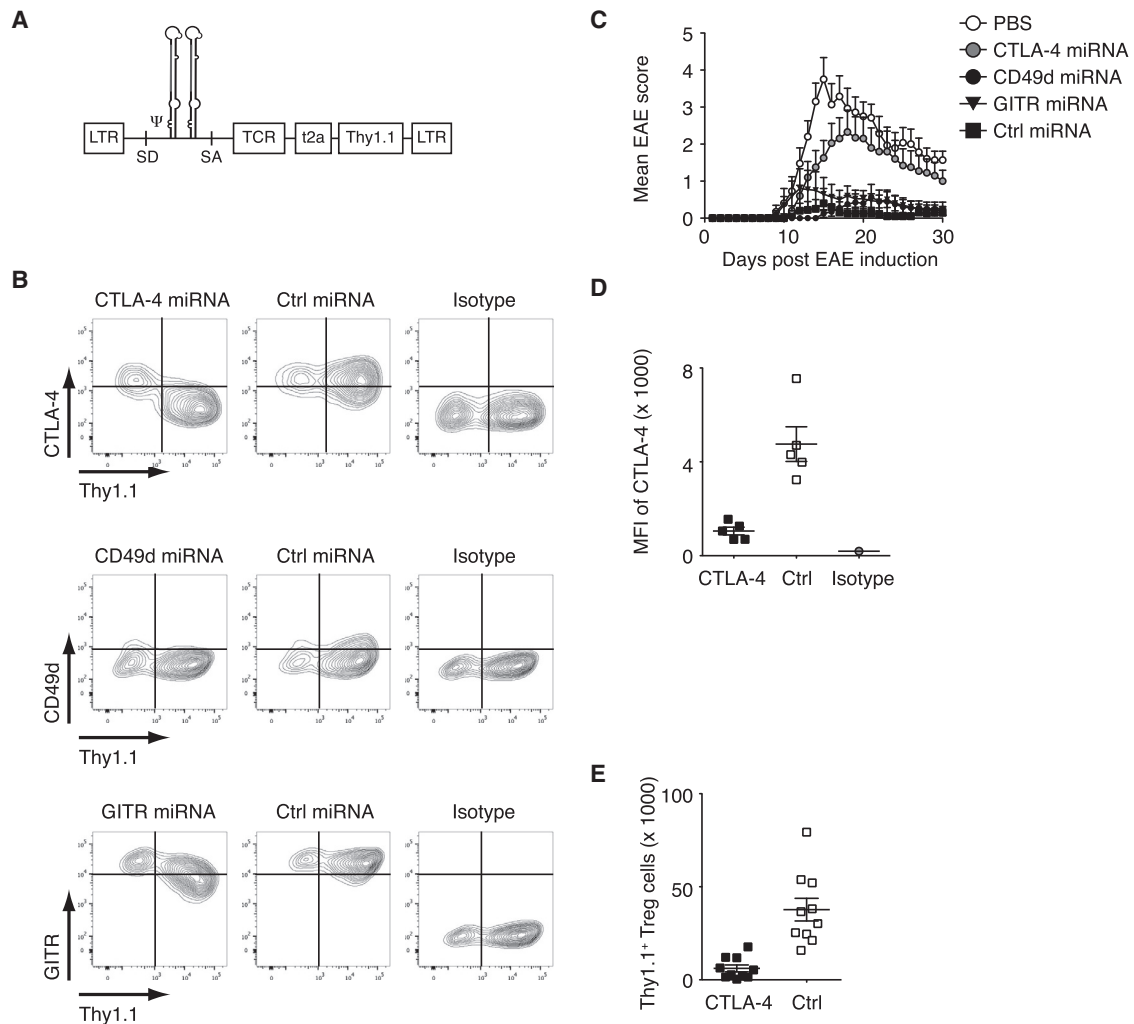


Figure 7. CTLA-4 Controls Protection from EAE by Engineered Treg Cells

(A) Design of the retroviral vector coding for T6-106 TCR, Thy1.1 and two intronic miRNAs.

(B) Polyclonal Treg cells were activated and transduced with a vector as in A harboring *Ctla-4*-, *Cd49d*-, or *Tnfrsf18*- (GITR) specific miRNAs, or control miRNAs, and analyzed 2 days post-transduction for CTLA-4, CD49d, or GITR expression, respectively. Plots are gated on Foxp3⁺ cells. One representative of three experiments.

(C) C57BL/6 mice were treated with 2×10^5 Treg cells transduced as in (B), and immunized 1 day later with MOG(35-55). Data show mean EAE score + SEM (pool from two experiments, $n = 10$ per group).

(D) Mice treated with transduced CTLA-4-silenced Treg cells and immunized with MOG(35-55) as in C were analyzed on day 13 post-immunization by flow cytometry to measure expressed amounts of CTLA-4 in the administered transduced CD45.2⁺Thy1.1⁺CD4⁺Foxp3⁺ Treg cells ($n = 5$) in spleen.

(E) Absolute numbers of administered transduced Thy1.1⁺Foxp3⁺ Treg cells in spleen of mice from (D) (pool of two experiments, $n = 10$ per group).

CTLA-4 (Figure 7A). The silencing of CTLA-4 (Figure 7B) abolished the protection afforded by MOG-reactive Treg cells (Figure 7C). These Treg cells retained low CTLA-4 amounts in recipient mice (Figure 7D) and expanded less than control MOG-reactive Treg cells (Figure 7E). Thus, CTLA-4 is essential for the therapeutic effect of engineered Treg cells against EAE. In contrast, silencing of CD49d or GITR (Figure 7B) did not impair the protective effect of MOG-reactive Treg cells (Figure 7C). It is remarkable that CD49d seemed dispensable for the protective function of adoptively transferred Treg cells, because this integrin is essential for the infiltration of pathogenic T cells into the CNS (Yednock et al., 1992).

DISCUSSION

The antigens selecting thymus-derived Treg cells remain poorly defined. Here, we reported the identification of a natural self-antigen controlling the generation of thymus-derived Treg cells. Moreover, we have documented how differences in the properties of the TCR instructing Treg cells' development, compared to those producing Tconv cells, contribute to limit the risk of autoimmunity.

It is noteworthy that the absence of a single antigen, MOG, resulted in a marked loss of MOG-reactive Treg cells in the thymus because it is generally admitted that TCR recognition of antigen is

degenerated (Mason, 1998; Vrisekoop et al., 2014). Degenerate antigen recognition by TCRs is an essential feature of T cell development since T cell progenitors are first positively selected on self-antigens in the thymus before they give rise to mature Tconv cells that can then during infection react toward unrelated non-self-antigens in the periphery. The degeneracy of T cell recognition is also important during heterologous immunity (Welsh and Selin, 2002). Such a high amount of degeneracy has been proposed to be essential for limiting possible holes in the TCR repertoire and to ensure the robustness of T cell immunity (Anderton and Wraith, 2002; Mason, 1998; Sewell, 2012; Vrisekoop et al., 2014). A drawback of degeneracy is however the potential recognition of autoantigens, and consequently the risk of autoimmunity (Skowera et al., 2008; Wooldridge et al., 2012). In contrast, our data obtained with retrogenic mice highlighted that for some MOG-reactive TCR, the expression of MOG was critical for the development of Treg cells. In this case, the selecting peptide can also act as an agonist to stimulate the activation of MOG-reactive Treg cells in the periphery. Thus, some Treg cells might follow the central dogma of the clonal selection theory, which proposes that an individual lymphocyte is specific for a single antigen (Jerne, 1955, 1971). It is possible that antigen recognition by Treg cells is less degenerate than for Tconv cells, and that the resulting holes in the regulatory repertoire are required to allow efficient immunity against non-self-antigens. Our data indicate however that this mode of Treg cell development is not absolute. First, Tconv cell TCRs allowed the development of low numbers of Treg cells. Although these cells might provide little protection from CNS autoimmunity due to the low functional avidity of their TCRs for MOG, they might recognize some foreign antigens with high affinity, which could explain the detection of Treg cells reacting against microbial antigens in the naive peripheral repertoire (Moon et al., 2011). Second, some of the four TCRs cloned from MOG-reactive Treg cells generated low amounts of Treg cells in the thymus of *Mog*^{-/-} retrogenic mice. Thus, in addition to the pathway of Treg cell development driven by endogenous antigen presentation in the thymus, other processes contribute in parallel to the generation of the peripheral Treg cell pool. This might explain why in a polyclonal repertoire deficiency in MOG expression resulted in an incomplete loss of thymic MOG-reactive Treg cells compared to controls. It will be important to assess whether these different pathways of Treg cell development give rise to functionally distinct subsets. Our observation that in retrogenic mice the frequencies of cells expressing Helios vary in these distinct conditions suggests that these various Treg cells are not identical.

The capacity of antigen(s) other than MOG to drive thymic selection of Tconv cells more efficiently than Treg cells suggests that these cells express TCRs with different antigen-recognition properties. Our data obtained using a panel of altered peptides support the notion that the TCRs from Treg and Tconv cells differentially interact with MOG. Some altered peptide ligands increased the amounts of IL-2 produced by the cell lines expressing Tconv cell-derived TCR by 2.7 to 10.1-fold compared to the original peptide, while the maximum increases observed for the Treg cell-derived TCRs were lower (increases of only 0.5- to 1.6-fold). This suggests that wild-type MOG is an optimal agonist for MOG-reactive Treg but not for Tconv cells. This might explain why MOG is so important for the development of MOG-reactive Treg cells. Structural investigations will be necessary to

precise how the TCRs from Treg and Tconv cells recognize MOG. Structural characterization of two TCRs carried by induced human regulatory T cells recently indicate that these TCRs interact differently with their antigen-MHC complex compared to previously characterized TCR-peptide-MHC complexes structures for Tconv cell TCRs (Beringer et al., 2015).

Indirect evidence suggested that Treg cell development was favored by TCR interactions of high functional avidity for the selecting antigens. However, this notion is complicated to evaluate because antigen sensitivity is influenced by numerous parameters beyond the TCR identity, as shown by the fact that individual T cell clones can generate progeny with both high and low antigen sensitivity (Kroger and Alexander-Miller, 2007). Here, the identification of MOG as the cue controlling the differentiation of MOG-reactive Treg cells enabled us to compare the characteristics distinguishing the TCR implicated in the commitment of T cell progenitors into either Treg or Tconv cells. Our data confirmed that a major difference between these two types of TCRs was their functional avidity for MOG, which was markedly higher for Treg cells. Of note, we have compared here the properties of the TCRs expressed by Treg and Tconv cells in a similar context: we isolated these TCRs and then re-expressed them in the same reporter cell line. The difference between our approach and the methodology used by others possibly explains our different findings (Hood et al., 2015).

The expression by Treg and Tconv cells of TCRs of different functional avidity for MOG led us to examine the relevance of this dichotomy for the function of these cells in the periphery. Polyclonal Treg cells engineered to express a MOG-reactive Treg cell TCR almost completely prevented disease induction, whereas Treg cells carrying Tconv cell TCRs had only moderate to no protective value in EAE. On the basis of these observations, we propose that the expression of high-performance TCRs by Treg cells is important for these cells to have a dominant effect over Tconv cells for suppressing anti-self immunity.

There is currently intense interest in the possibility of using Treg cells for the suppression of unwanted immunity in adoptive cell therapy. Several clinical trials have already been initiated to examine the safety and therapeutic value of polyclonal Treg cells in the clinic (Brunstein et al., 2011; Di Ianni et al., 2011; Marek-Trzonkowska et al., 2012; Theil et al., 2015). Data obtained in pre-clinical models demonstrated that in autoimmune diseases, autoantigen-reactive Treg cells were markedly superior to polyclonal Treg cells to suppress pathology (Stephens et al., 2009). Thus, an attractive Treg cell-based strategy for treating autoimmune diseases would be to isolate a patient's own Treg cells reacting against a disease-relevant autoantigen, expand them *ex vivo*, and subsequently use these cells in autologous adoptive cell therapy. However, autoantigen-specific Treg cells are rare and remain difficult to isolate making their application in adoptive cellular therapy challenging. The possibility of engineering Treg cells by TCR gene transfer represents a major advance on this point, yet raises the new question about the choice of the type of TCR that should be used. Our data demonstrate that TCRs from Treg cells are clearly a preferable choice since they confer markedly superior protective functions to engineered Treg cells than TCRs from Tconv cells.

In conclusion, we have demonstrated an unexpected role for endogenous MOG expression as a major cue for the selection

of thymus-derived MOG-reactive Treg cells, providing the first identification of a natural antigen that controls Treg cell differentiation. Although we analyzed specifically Treg cells that express the public TCR- β chain of the MOG-reactive CD4⁺ T cell response during EAE, which we selected because such TCR usually play prominent roles during immune responses (van den Elzen et al., 2004), we anticipate that our findings are also relevant for private TCRs. These results might help to understand how thymus-derived Treg cells have a key role in preventing autoimmune-mediated attack of self-tissues, while allowing efficient defense against infections.

EXPERIMENTAL PROCEDURES

Detailed experimental procedures can be found in the [Supplemental Information](#) section.

Mice

Mouse strains are described in Supplemental Experimental Procedures. Experiments were performed in accordance with German, French, and UK authorities.

EAE Induction through Immunization and Adoptive Transfer

EAE was induced and assessed as described (Fillatreau et al., 2002). Some mice received 200 μ g anti-CD25 (PC61) intravenously 3 days before EAE induction (Figure 1H). The protocol was in cases adjusted to use 5 μ g of MOG(35-55) or 49(H_A) peptides (Figure 2D).

For induction of passive EAE with in vitro-primed T cells, splenic and LN lymphocytes of naive Kaa or C57BL/6 mice were depleted of CD25⁺ cells by separation on autoMACS (Miltenyi Biotech) and stimulated in vitro for 3 days with 10 μ g/ml MOG(35-55) peptide, recombinant mouse IL-2 (10 U/ml), IL-12, and IL-18 (each at 25 ng/ml). 10×10^6 in vitro-primed cells were then injected into naive C57BL/6 mice intravenously to induce EAE. These mice additionally received 240 ng Pertussis toxin intravenously on days 0 and 2 after cell transfer.

For induction of passive EAE with in vivo-primed Kaa cells, Kaa mice were immunized with MOG(35-55), as previously described (Fillatreau et al., 2002). Draining LN were harvested 8 days after immunization, and cells were re-stimulated in vitro for 3 days with 10 μ g/ml MOG(35-55) peptide, recombinant mouse IL-2 (10 U/ml), IL-12, and IL-18 (each at 25 ng/ml). 6×10^6 cells were then injected into naive C57BL/6 mice intravenously to induce EAE. These mice additionally received 240 ng Pertussis toxin intravenously on days 0 and 2 after cell transfer.

For production of transduced MOG-reactive Tconv cells by TCR gene transfer, LN and spleen were collected from naive C57BL/6 mice, subjected to a mouse CD4⁺ T cell pre-enrichment EasySep kit (STEMCELL technologies), and CD25⁺ cells were subsequently eliminated using a mouse CD25 positive selection EasySep kit (STEMCELL technologies). The obtained cells were then activated with anti-CD3 (1 μ g/ml), anti-CD28 (0.1 μ g/ml), IL-2 (40U/ml), IL-12 (25 ng/ml), and IL-18 (25 ng/ml), and transduced at 48 hr and 72 hr with MP71 retroviral particles, and used in vitro or in adoptive transfer at 96 hr ($1-2 \times 10^6$ cells/mouse). Recipient mice additionally received 240 ng Pertussis toxin intravenously on days 0 and 2 after cell transfer.

Identification and Cloning of Kaa TCR α Chains

Kaa.Foxp3.IRES.eGFP⁺Tcr α ^{+/+}Tcr β ^{+/+} mice were immunized with MOG(35-55) to induce EAE, and CD4⁺GFP⁺ and CD4⁺GFP⁻ cells were isolated from their CNS (brain and spinal cord) using a combination of magnetic and cytometric procedures. RNA was isolated from these cells using RNeasy mini Kit (QIAGEN). cDNA was generated using a SMARTer 5' RACE cDNA Amplification Kit, and an Advantage 2 PCR Kit (both Clontech), using the primer 5'-ACT GGA CCA CAG CCT CAG CGT CA-3'. PCR products were cloned into the pcDNA3.1/V5-His-TOPO vector (Life Technologies). After transformation into *E. coli*, positive colonies were sequenced (GATC Biotech). TCR- α sequences were identified by IMG T HighV-quest software (<http://www.imgt.org/HighV-QUEST>) (Alamyar et al., 2012).

Expression of TCR in T.54 ζ 17 Cells

T.54 ζ 17 cells (gift of B. Malissen, France) (Letourneur and Malissen, 1989), which express mouse CD4, were modified to stably express the Kaa TCR- β chain cloned into a pMig retroviral vector (Van Parijs et al., 1999). TCR- α chains were cloned into the pMY-IRES-GFP retroviral vector (Cell Biolabs). T.54 ζ 17 cells expressing the Kaa TCR- β chain were transduced with pMY-IRES-GFP encoding the TCR- α , and GFP⁺V β 8.2⁺ cells were sorted on a FACSAria II several times until cultures stably containing > 95% TCR-expressing cells were obtained. For each TCR- α chain, two TCR-expressing cell lines were independently generated.

Expression of TCR in HSC, Tconv Cells, and Treg Cells

The TCR- α and Kaa TCR- β sequences were linked via a 2A element of porcine teschovirus (p2a) by PCR and cloned into the MP71 vector (Engels et al., 2003) via NotI and EcoRI restriction sites to obtain MP71-TCR- β -p2a-TCR- α . The *Thy1.1* gene was additionally linked to the TCR chains using a 2A linker element of *Thosea asigna* virus (t2a) to yield MP71-TCR- β -p2a-TCR- α -t2a-Thy1.1. To simultaneously express miRNAs in the MP71 vector, we predicted *Ctla4*-specific miRNA target sites GGT TCC AAA GGT TGT AGT GTT and AAC TGA AAG GCC GTT TAT GAA by BLOCK-iT RNAi Designer (<http://rnaidesigner.lifetechnologies.com/maixpress>) and integrated the respective antisense sequences into two different miRNA environments (murine miR-155 [Chung et al., 2006] and an artificial miRNA [Saetrom et al., 2006]). Then, the miRNAs were inserted into the MP71 intron (Bunse et al., 2014).

Retroviral Transduction

Viral particles for transduction of HSC and primary Treg and Tconv cells were produced using Plat-E cells through transient transfection with the MP71 retrovirus vector using CaPO₄ precipitation. Supernatants containing retroviral particles were harvested at 48 hr and 72 hr after transfection and subsequently used for transduction of HSC, Tconv, or Treg cells.

CD4⁺CD25⁺ Treg and CD4⁺CD25⁻ Tconv cells were isolated from spleen and LN of naive C57BL/6 mice and stimulated in non-tissue culture plates coated with 1 μ g/ml anti-CD3 and anti-CD28 (Biolegend). Cells were cultured with 750 or 40 U/ml recombinant human IL-2 (Chiron), respectively, and transduced on day 2 and 3 after isolation by spinoculation in plates coated with RetroNectin (TaKaRa Biomedicals). Four to six days after isolation cells were used for adoptive transfer.

Retroviral supernatant for T.54 ζ 17 cells was produced with HEK293T cells by transient CaPO₄ transfection with pCgp (Soneoka et al., 1995), MLV-eco, and pMY-TCR- α -IRES-eGFP or pMig-Kaa-TCR- β -Tomato, respectively. Cells were sequentially transduced to express TCR- β and TCR- α and Tomato⁺eGFP⁺ were subsequently sorted.

TCR-retrogenic mice were prepared as described (Kieback et al., 2014).

Statistical Analyses

Cumulative disease scores and data from peptide stimulations and TCR retrogenic mice were analyzed with an unpaired or paired two-tailed Student's *t* test. EAE disease courses were compared by repeated-measures two-way ANOVA. Statistical analysis was performed with GraphPad Prism (GraphPad Software). **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2016.04.018>.

AUTHOR CONTRIBUTIONS

E.K., E.H., U.S., V.L., P.S., M.B., Y.J., P.B., A.R., U.K., A.A.K., R.L., N.H., S.M.A., W.U., and S.F. performed experiments and contributed to the development of the project and the writing of the manuscript.

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