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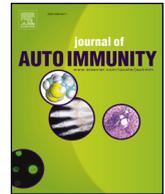
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# Treatment of experimental autoimmune encephalomyelitis with engineered bi-specific Foxp3<sup>+</sup> regulatory CD4<sup>+</sup> T cells

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## ABSTRACT

The use of autoantigen-specific regulatory T cells (Tregs) as a cellular therapy for autoimmune diseases is appealing. However, it is challenging to isolate and expand large quantity of Tregs expressing disease-relevant T-cell receptors (TCR). To overcome this problem, we used an approach aiming at redirecting the specificity of polyclonal Tregs through autoreactive TCR gene transfer technology. In this study, we examined whether Tregs engineered through retroviral transduction to express a TCR cross-reactive to two CNS autoantigens, myelin oligodendrocyte glycoprotein (MOG) and neurofilament-medium (NF-M), had a superior protective efficacy compared with Tregs expressing a MOG mono-specific TCR. We observed that engineered Tregs (engTregs) exhibited *in vitro* regulatory effects related to the antigenic specificity of the introduced TCR, and commensurate in potency with the avidity of the transduced TCR. In experimental autoimmune encephalomyelitis (EAE), adoptively transferred engTregs proliferated, and migrated to the CNS, while retaining FoxP3 expression. EngTregs expressing MOG/NF-M cross-reactive TCR had superior protective properties over engTregs expressing MOG-specific TCR in MOG-induced EAE. Remarkably, MOG/NF-M bi-specific TCR-engTregs also improved recovery from EAE induced by an unrelated CNS autoantigen, proteolipid protein (PLP). This study underlines the benefit of using TCRs cross-reacting towards multiple autoantigens, compared with mono-reactive TCR, for the generation of engTregs affording protection from autoimmune disease in adoptive cell therapy.

## 1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) likely caused by an autoimmune response against self-antigens expressed in the CNS [1–3]. Prevention of autoimmunity relies, at least in part, on a subset of regulatory CD4 T cells (Tregs) characterized by the expression of the transcription factor forkhead-box protein P3 (Foxp3), which is essential for their development and suppressive functions [4,5]. This population limits tissue damage and inflammation by inhibiting the activation and effector functions of several immune cells, including conventional CD4 and CD8 T cells, B cells, NK cells, NKT cells, and monocytes as well as dendritic cells [6,7].

Autoreactive Tregs express self-reactive T-cell receptor (TCR) with higher affinity than conventional T cells (Tconvs) for the corresponding self-antigen [8]. An imbalance in Treg/Tconv immune homeostasis contributes to autoimmune pathogenesis [9]. In multiple sclerosis (MS) patients [10] and other autoimmune diseases, such as rheumatoid arthritis [11], type 1 diabetes [12] and myasthenia gravis, either the frequency of Tregs is reduced or their functional properties are altered [13–15]. One possibility to restore the Treg/Tconv balance would be to

adoptively transfer polyclonal Tregs. However, the adoptive transfer of polyclonal Tregs could have beneficial effects on autoimmunity while bearing deleterious consequences on protective systemic immune responses [16,17]. Thus, the adoptive transfer of disease-relevant antigen-redirected functional Tregs might be a possible therapeutic strategy in MS and other autoimmune diseases [18–21].

Importantly, whereas the adoptive transfer of polyclonal Tregs had little effect, myelin-reactive Tregs cured recipient mice from experimental autoimmune encephalomyelitis (EAE), an animal model of MS [8,22]. However, autoantigen-specific Tregs are rare and remain difficult to isolate. Furthermore, to obtain the desired number of antigen-specific Tregs for adoptive transfer studies, multiple rounds of *ex vivo* expansion using antigen-loaded antigen-presenting cells (APC) are needed, which can lead to decreased suppressive function of Tregs and promote their plasticity into Th17 cells [23,24]. Several alternate methods for *in vivo* expansion of antigen-specific Tregs have been reported, such as injection of nanoparticle attached self-peptides [25], and oral or intravenous injection of self-peptides [26,27] or low dose IL-2 [21]. However, the injected self-peptides can also be taken-up, processed, and presented by APC to Tconvs, which can further enhance pathogenic immune response to self-antigens.

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A promising approach for generating self-antigen-specific Tregs is the introduction of chosen TCR genes by retroviral or lentiviral gene transfer in polyclonal Tregs. Several studies have demonstrated the feasibility of this method both in mice and humans [28–30]. For example, the adoptive transfer of myelin oligodendrocyte glycoprotein (MOG)-specific Tregs generated by TCR gene transfer was more potent at preventing mice from EAE than that of polyclonal Tregs [8]. Similarly, auto-antigens redirected engTregs generated with chimeric antigen receptors were superior to polyclonal Tregs at preventing experimental models of inflammatory bowel disease, autoimmune disease, and graft-versus host disease [31–35].

It is now well documented that the TCR cross-react with multiple antigens, though with different avidity, so that the limited number of T cells can cope with the vast number of peptide ligands. Autoreactive T cells recognizing multiple self-antigens have been reported in several immune-mediated diseases, including MS [36,37]. Interestingly, effector CD4 T cells targeting both MOG and neurofilament-medium (NF-M) have emerged as highly pathogenic from our studies of EAE in C57BL/6 mice. These dual self-antigens cross-reactive Tconv cells were able to induce EAE when either of the two target auto-antigens were present (MOG<sup>-/-</sup> or NF-M<sup>-/-</sup> mice), and were highly pathogenic when both target antigens were expressed in wild type (WT) mice [38–41]. Harnessing the functional properties of high-avidity dual self-antigens cross-reacting TCR for therapeutic application could represent an interesting option to generate bi-specific protective Tregs. In the present study, we investigated the *in vivo* therapeutic effect of MOG/NF-M bi-specific TCR-engineered Tregs (engTregs) and compared them with MOG-specific TCR-engTregs. To this goal, we engineered polyclonal Tregs to express one of two MOG/NF-M cross-reactive TCRs, and one of two MOG mono-specific TCRs with varying avidity towards their cognate antigen(s), and tested whether cross-reactivity of TCR, its avidity or both on the engTregs correlated with the level of protection against T cell-mediated immunopathology in EAE. We demonstrate that TCR-engTregs bearing TCR cross-reactive to two self-antigens not only strongly suppress an autoimmune response to these self-antigens, but also inhibit EAE induced by an unrelated autoantigen. Hence these data suggest that generating Tregs with cross-reactive TCR may be a valuable therapeutic option in MS, and other autoimmune diseases.

## 2. Experimental procedures

### 2.1. Mice

Male C57BL/6 mice, 10–15 weeks old, were purchased from Charles River Laboratories, France. MOG-specific TCR-transgenic 2D2 mice on a C57BL/6 background [42] have been backcrossed with CD45.1 congenic animals. Mice were kept in specific pathogen-free conditions and used in accordance of the European Union guidelines following approval of the local ethics committee.

### 2.2. Peptides

The peptides MOG (35–55) (MEVGWYRSPFSRVVHLYRNGK), NF-M (15–35) (RRVTETRSSFSRVSGSPSSGF), and (PLP<sub>178–191</sub>) (NTWTTQQS-IAFPSK) were purchased from Polypeptide Laboratories (San Diego, CA) with a purity > 95%.

### 2.3. Cloning of the TCR genes into a retroviral vector

MOG-specific T-cell hybridomas were previously described [40,43,44]. The paired TCR $\alpha$  and TCR $\beta$  cDNA sequences of chosen TCR clones were amplified using gene specific V $\alpha$  and V $\beta$  forward primers and a common C $\alpha$  and C $\beta$  reverse primers as described previously [43], and linked using a 2A element of porcine teschovirus (P2A) by PCR and cloned into the MP71 retrovirus vector [8,45] using NotI and EcoRI restriction sites to obtain TCR $\beta$ -P2A-TCR $\alpha$ . In addition, the Thy1.1 gene

was linked to the TCR chains using a 2 A linker element of teschovirus (T2A) to produce TCR $\beta$ -P2A-TCR $\alpha$ -T2A-Thy1.1 that provided a surface marker for the identification of virus transduced T cells.

### 2.4. Production of retrovirus and T-cell transduction

Retrovirus particles were produced using Plat-E cells by transient transfection with the TCR or control retrovirus vector using Lipofectamine<sup>®</sup>2000 transfection reagent (Invitrogen). Supernatants containing retroviral particles were harvested at 48 h and 72 h after transfection, centrifuged at 350 g for 1 min and carefully filtered using 40  $\mu$ m sterile cell strainer to remove cell debris. Supernatants were mixed with 5  $\mu$ g/ml polybrene infection reagent (Sigma) for 30 min at 37 °C and used for transduction of Tregs or Tconvs.

CD4<sup>+</sup>TCR<sup>+</sup>CD25<sup>hi</sup> Tregs or CD4<sup>+</sup>TCR<sup>+</sup>CD25<sup>-</sup> Tconvs were isolated via cell sorting using a BD FACSAria flow cytometer from pooled spleen and lymph nodes of four to five donor male C57BL/6 mice. These cells were then stimulated in a flat bottom 96-well tissue culture plate (TRP) coated with 1  $\mu$ g/ml anti-CD3 and soluble anti-CD28 (BD) mAbs or with anti-CD3/CD28 dynabeads (Invitrogen). Activated CD4 Tconvs were cultured in complete medium (RPMI 1640 medium with 10% FBS, 100 units/mL penicillin, 100 mg/mL streptomycin, 1 mM non-essential amino acids, 50 mM  $\beta$ -mercaptoethanol, 1 mM sodium pyruvate, and HEPES). The Treg culture medium also contained 1000 U/ml recombinant mouse IL-2 (R&D systems), and, where indicated, 100 nM rapamycin (Sigma). Cultured Tregs or Tconv were transduced on day 2 and 3 after isolation, by centrifugation at 32 °C for 2 h in six-well plates coated with 10  $\mu$ g/ml RetroNectin (TaKaRa Biomedicals). The *in vitro* and *in vivo* experiments were performed 4–6 days after the transduced Treg cells were generated.

### 2.5. Adoptive transfer of Tregs and EAE induction

EAE was induced and assessed as described [8,39,41]. In brief, 10 to 12-week-old C57BL/6 male mice were immunized subcutaneously at the base of the tail either with 100  $\mu$ g of MOG (35–55) or 200  $\mu$ g of PLP (178–191) emulsified in complete Freund's adjuvant (CFA; BD) containing 500  $\mu$ g of *Mycobacterium tuberculosis* (Strain H37 Ra, BD). Pertussis toxin (200 ng; List Biological Laboratories) was injected intravenously at days 0 and 2 post-immunization. Retrovirus transduced TCR-engTregs (10<sup>6</sup> cells), control Tregs (10<sup>6</sup> cells) or PBS were injected intravenously either one day before EAE induction or on day 9 post-immunization. Disease severity was scored daily on a 5 point scale: 0, no neurological sign; 1, tail atony; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb paralysis; and 5, moribund.

### 2.6. Peripheral and CNS mononuclear cell isolation

Mice were anaesthetized with ketamine and transcardially perfused with cold PBS. Spleen, lymph nodes (cervical, axillary & inguinal), brain and spinal cord were collected separately. Blood samples were collected in EDTA-coated tubes before perfusion. Cells from spleen, lymph nodes and blood were treated with ACK (Ammonium-Chloride-Potassium) lysing buffer before CD4 T cells were enriched using the dynabeads untouched Mouse CD4 kit (Invitrogen). Mononuclear cells from brain and spinal cord were isolated by homogenization and digestion using collagenase D (Roche) and DNaseI (Sigma) followed with a triple percoll gradient (30, 37, 70%) and centrifugation for 20 min at 2000 rpm [41,46].

### 2.7. Flow cytometry

Cell surface staining was performed with mAbs specific for CD4, CD8, TCR, Thy1.2, Thy1.1, PD1, CTLA-4, CD25, ICOS, GITR, CD103 as well as a viability dye (eFluor 780). Expression of other cell surface markers CD11b, CD11c, CD19, MHC-II, CD45, ICOS and CCR7 was also

assessed by staining of mononuclear cells isolated from the CNS of mice with EAE, using the respective mAbs.

For intracellular cytokine staining, cells were stimulated in culture medium either with MOG or NF-M peptide loaded APC for 72 h and with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, Sigma), ionomycin (1 mg/ml, Sigma) for the last 5 h in culture medium containing GolgiStop (1  $\mu$ l/ml, BD Biosciences) at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere [46]. After staining for surface markers (CD4, CD8, Thy1.1 and Thy1.2) and viability, cells were fixed and permeabilized for staining of IL-2, IL-10, IL-17, IL-35(IL-12p35), GM-CSF, TNF $\alpha$ , IFN $\gamma$ , TGF $\beta$ (LAP), Granzyme B and FoxP3 using respective cytoplasmic or intra-nuclear fixation/permeabilization buffer kit from eBiosciences according to the manufacturer's instructions.

For the peptide:MHC-II tetramer staining, 10<sup>6</sup> cells were incubated with MOG-PE:I-A<sup>b</sup>, NF-M-APC:I-A<sup>b</sup> or control CLIP-PE/APC:I-A<sup>b</sup> tetramers overnight at 37 °C with 5  $\mu$ l tetramer in 500  $\mu$ l RPMI culture medium.

Data were acquired on cytometer LSRII or Fortessa (BD) and analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA).

### 2.8. Regulatory T cell-mediated suppression of proliferation assays

Treg suppression assays were performed following the protocol of [47] with slight modifications. Briefly, Tconvs from 2D2 mice (CD4<sup>+</sup>TCR<sup>+</sup>CD25<sup>-</sup>) expressing a MOG/NF-M bi-specific transgenic TCR were isolated and labeled with CellTrace Violet (CTV) dye. Hundred thousand labeled 2D2 Tconvs per well were activated with 50  $\mu$ g/ml of MOG or NF-M peptide loaded APC at the ratio of 1:3 in a round bottom 96-well tissue culture plate (TRP) and cultured alone (no Tregs) or in the presence of either TCR-engTregs or control engTregs at different Tregs:Tconv ratios. After 72 h, the suppressive efficiency of engTregs was determined by flow cytometry analysis of CTV dilution in the responder 2D2 Tconv cells.

### 2.9. Statistical analyses

EAE disease courses were compared by repeated-measure two-way ANOVA. Cumulative disease scores and data from peptide stimulations were analyzed with one-way ANOVA test. Reported significant values were obtained after multiple comparisons performed using two-way and one-way ANOVA by comparing mean of each group with the mean of every other group. Statistical analyses were performed with GraphPad Prism Software. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001.

## 3. Results

### 3.1. Validation of recombinant exogenous TCR on Tconv cells

In MS, the paucity of Tregs within inflammatory CNS lesions, as confirmed here in Table 1, likely contributes to the disease process [48,49]. Adoptive cell therapy using Tregs in human autoimmune diseases is a highly investigated option that has the potential to increase the frequency of Tregs in the blood and in inflamed tissues [20,21].

In the present study, we investigated, in an animal model of MS, the potential of adoptive cell therapy using Tregs redirected to recognize neural autoantigens by TCR gene transfer. We selected TCRs from two clones reacting only to MOG and from two MOG/NF-M cross-reactive T cell clones [40]. We reprogrammed the antigen specificity of polyclonal T cells by introducing either MOG-specific or MOG/NF-M bi-specific TCR through retroviral gene transfer. In order to recognize the two self-antigens, MOG/NF-M cross-reactive TCR likely contact the shared amino acid residues within the core epitopes of MOG (38–50) and NF-M (18–30) (Fig. 1A). The TCRs used in this study have either low or high avidity for the cognate peptide(s), use different V(D)J germline gene segments and have distinct CDR3 sequences (Fig. 1B). We used a

**Table 1**

Quantification of FoxP3 Tregs in the CNS of MS patients versus non-neurological controls.

Gender	Age (Years)	CD3 (cells/mm <sup>2</sup> )	% FoxP3+ Treg cells
<b>Multiple sclerosis</b>			
Female	68	52.7	0.0
Female	45	190.4	3.0
Female	45	204.2	0.0
Female	40	39.8	0.0
Female	57	63.7	0.0
Female	20	56.5	1.7
Female	55	126.2	0.9
Female	71	22.6	0.1
Female	83	16.0	0.0
Male	45	190.4	3.0
Male	36	32.5	0.0
Average	<b>52.0</b>	75.3	<b>0.5</b>
<b>Controls</b>			
Female	45	2.8	0.0
Female	42	13.7	0.0
Female	30	26.3	0.0
Female	47	13.0	0.0
Female	36	5.4	0.0
Female	39	12.2	0.0
Female	71	18.6	0.0
Female	70	9.6	0.0
Male	37	13.3	0.0
Male	65	6.0	0.0
Male	83	6.4	0.0
Average	51.4	11.5	0.0

multicistronic retroviral expression vector to produce engineered Tconvs (engTconvs) or Tregs (engTregs) expressing the recombinant TCR together with an identifiable surface marker, Thy1.1. A retroviral vector expressing Thy1.1 but no exogenous TCR was used to generate control engTregs throughout this study (Fig. 1C & D). The engTregs expressing a recombinant TCR are named TCR-engTregs, while those transduced with the control vector are named control-engTregs.

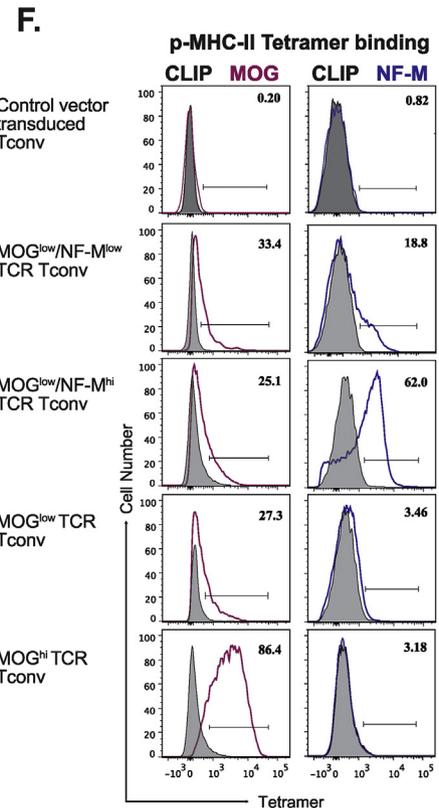
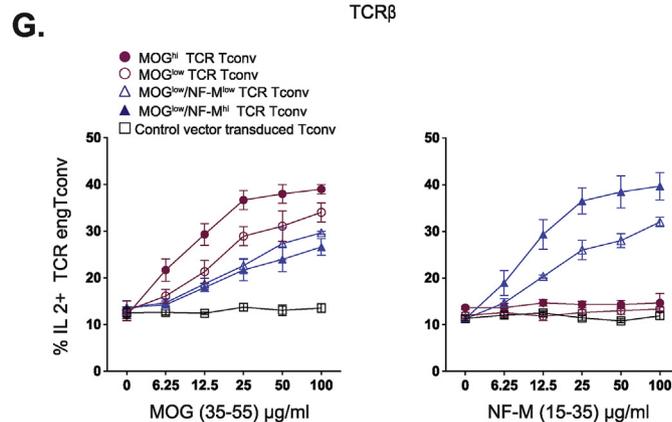
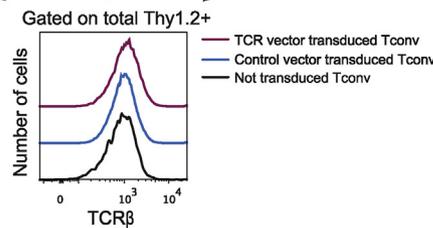
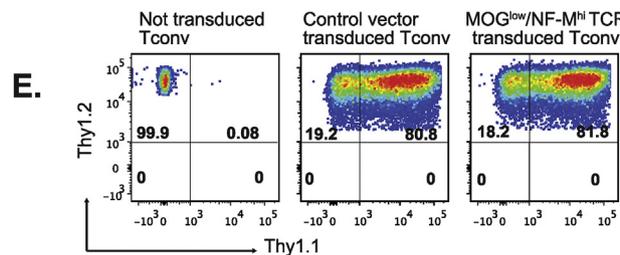
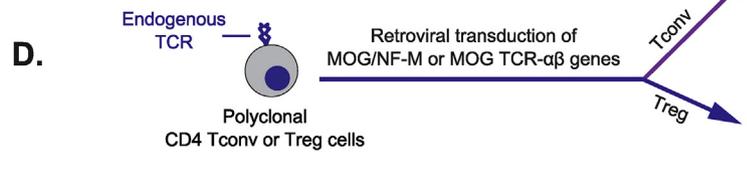
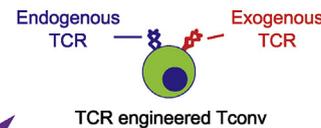
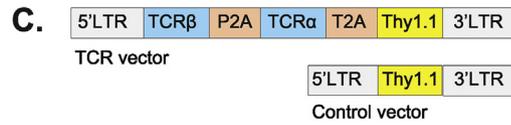
First, we validated this engineering strategy in engTconvs by assessing surface expression of the recombinant TCRs and Thy1.1, and by performing functional analysis of transduced cells. For this purpose, we isolated polyclonal CD4<sup>+</sup>TCR<sup>+</sup>CD25<sup>-</sup> Tconvs and activated them with anti-CD3 $\epsilon$  and anti-CD28 antibodies 48 h before transducing them with TCR-encoding or control retroviruses. Due to the unavailability of clonotype-specific antibodies to specifically detect the expression of the recombinant TCR on the transduced cells, we relied on Thy1.1 expression to evaluate transduction efficiency. Transduction efficiency was about 80% (Fig. 1E). The surface expression level of TCR $\beta$  on TCR-engTconvs and on non-transduced T cells was similar (Fig. 1E).

We then assessed the pairing of recombinant TCR $\alpha$  and  $\beta$  chains at the cell surface of TCR-engTconvs, using fluorescently-labeled peptide:I-A<sup>b</sup> tetramers and functional response to cognate antigen(s). Transduced CD4 Tconvs were incubated with MOG:I-A<sup>b</sup>, NF:M-I-A<sup>b</sup> and the non-specific CLIP:I-A<sup>b</sup> tetramers, and the tetramer staining among the Thy1.1<sup>+</sup> population was analyzed by flow cytometry. As expected, engTconvs expressing a MOG mono-specific TCR bound to MOG:I-A<sup>b</sup> only (Fig. 1F), whereas Tconvs expressing a MOG/NF-M cross-reactive TCR bound to both MOG:I-A<sup>b</sup> and NF-M:I-A<sup>b</sup> tetramers (Fig. 1F). The two MOG/NF-M bi-specific TCR have low avidity towards MOG (Fig. 1F). However, one of them exhibited a strong binding to the NF-M:I-A<sup>b</sup> tetramers, as assessed by frequency and MFI of staining, and was therefore named MOG<sup>low</sup>/NF-M<sup>hi</sup> TCR, while the other was named MOG<sup>low</sup>/NF-M<sup>low</sup> TCR (Fig. 1F). Similarly, among the two MOG mono-specific TCRs, MOG:I-A<sup>b</sup> tetramer binding confirmed that one has high avidity for MOG, thus named MOG<sup>hi</sup> TCR, and the other one has low avidity, hence named MOG<sup>low</sup> TCR (Fig. 1F). These data indicate the efficient pairing of the recombinant TCR $\alpha$  and  $\beta$  chains on the surface of transduced cells.

To test the functionality of the recombinant TCRs, engTconvs were

**A. MOG(38-50) G W Y R S P F S R V V H L**  
**NF-M(18-30) T E T R S S F S R V S G S**

B. TCR	Specificity	TRAV	TRAJ	CDR3 $\alpha$	TRBV	TRBD	TRBJ	CDR3 $\beta$	Relative avidity		
									MOG	NF-M	Short Name
2MOG60	MOG/NF-M	9D-3	23	CALSGYNQKLI	14	1	1-2	CASRDSANSDYTF	++	++	MOG <sup>low</sup> /NF-M <sup>low</sup>
3MOG204	MOG/NF-M	3-1	23	CAVSGYNQKLI	13-2	2	2-3	CASGGGLGTSAETLYF	++	+++	MOG <sup>low</sup> /NF-M <sup>hi</sup>
1MOG26	MOG	9D-3	17	CALSIGANSAGNKLT	12-2	2	2-7	CASSLETAGYEQYF	++	-	MOG <sup>low</sup>
1MOG210	MOG	12D-2	18	CALSDRGSALGRLHF	19	1	2-3	CASSIADSSAETLYF	++++	-	MOG <sup>hi</sup>



(caption on next page)

co-cultured with MOG or NF-M peptide-loaded APC and production of IL-2 was assessed by flow cytometry. MOG (35–55) peptide induced significant IL-2 expression in both MOG/NF-M cross-reactive and MOG mono-specific TCR-engTconvs, confirming that all recombinant TCRs are MOG reactive (Fig. 1G). The proportion of IL-2-producing cells in response to the MOG (35–55) peptide correlated well with the binding of MOG:I-A<sup>b</sup> tetramers to the exogenous TCR (Fig. 1G, left panel). On the other hand, stimulation with NF-M(15–35) peptide elicited IL-2

production only in MOG/NF-M cross-reactive TCR-engTconv cells, confirming the cross-reactivity of these TCR towards MOG and NF-M (Fig. 1G, right panel). Here again the functional avidity of the TCR-engTconvs correlated with the binding of NF-M:I-A<sup>b</sup> tetramers to the exogenous TCR. These data indicate that the recombinant TCR induce efficient signaling in the transduced cells, and show that the functional avidity of these TCR correlated with the magnitude of tetramer staining.

**Fig. 1.** Transfer of recombinant TCR from autoreactive CD4 T-cell hybridomas into polyclonal T cells via a retroviral expression system. **(A)** Shared amino acid residues between the MOG and NF-M peptides in the context of I-A<sup>b</sup> are shown in red. **(B)** CDR3 regions of the two chosen MOG/NF-M cross-reactive TCR (2MOG60 & 3MOGN204) and the two MOG-specific TCR (1MOG26 & 1MOG210) as reported in Lucca et al., 2014. The short names attributed to each TCR for easy reading are shown in the right column. **(C)** Retroviral vector genome: the paired TCR $\alpha$  and  $\beta$  chain genes of each MOG-reactive T-cell hybridoma were inserted in frame into a MP71 retroviral vector together with a Thy1.1 gene as a congenic surface marker. The P2A and T2A peptide sequences were used to link the TCR $\beta$ ,  $\alpha$ , and Thy1.1 genes. The control retroviral vector carries only the Thy1.1 gene. LTR - long terminal repeat. **(D)** Experimental scheme: autoreactive TCRs were transferred into polyclonal CD4 Tregs or Tconvs via retroviral transduction to redirect their specificity resembling a monoclonal population. **(E)** Representative flow cytometry plots showing Thy1.1 staining (upper panels) and TCR $\beta$  staining (endogenous + exogenous; lower panel) among not transduced versus control vector or TCR vector (MOG<sup>low</sup>/NF-M<sup>hi</sup> TCR) engTconvs on day 6 after transduction. The lower panel is an overlay histogram showing TCR $\beta$  expression on the X-axis and the count of events on the Y-axis. **(F)** Representative histograms showing frequency of peptide:MHC-II tetramer binding cells among Thy1.1<sup>+</sup> engTconvs. Retroviral transduced Tconvs expressing one of the indicated recombinant TCRs were stained with MOG:I-A<sup>b</sup>-PE, NF-M:I-A<sup>b</sup>-APC (open histograms) and control CLIP:I-A<sup>b</sup>-PE/APC tetramers (filled histograms). Results from one representative of three independent experiments are shown. **(G)** IL-2 production by recombinant TCR-engTconvs. TCR-engTconvs expressing each of the MOG/NFM cross-reactive or MOG-reactive TCRs, or control engTconvs were stimulated with MOG (35–55) or NF-M(15–35) peptides at the indicated concentrations, and the proportion of IL-2-producing cells were quantified in the Thy1.1<sup>+</sup> population by flow cytometry analysis. Data are from three independent experiments each performed in triplicates (mean  $\pm$  SEM).

### 3.2. Functional characterization of engineered Tregs expressing recombinant TCR

We characterized the expression and function of TCR-engTregs for the four recombinant TCRs. Polyclonal CD4<sup>+</sup>TCR<sup>+</sup>CD25<sup>hi</sup> T cells were isolated by FACS and expanded using anti-CD3 $\epsilon$  and anti-CD28 antibodies before retrovirus transduction. Expression of FoxP3 by sorted Tregs was assessed before and six days after retrovirus transduction. Transduction efficiency on Tregs was determined by Thy1.1 staining (Fig. 2A). For this study, only experiments in which more than 70% of Tregs were efficiently transduced, and in which more than 97% of cells expressed FoxP3 were used for *in vitro* and *in vivo* experiments (Suppl. Fig. 1). We also assessed whether retrovirus transduction altered some key features of Tregs by comparing the expression of cell surface markers and intracellular cytokines in non-transduced vs. transduced engTregs on day six post-transduction. We did not observe notable differences among the five types of Tregs (Suppl. Fig. 2), and detected no changes in surface TCR expression after transduction (Fig. 2A).

We next tested *in vitro* the antigen-specific suppressive function of TCR-engTregs, using as responder cells Tconvs from 2D2 mice, which carry a transgenic TCR specific for both MOG and NF-M [38]. Suppression of 2D2 Tconv proliferation to MOG or NF-M peptides was evaluated at various ratios of TCR-engTregs to 2D2 Tconvs (Fig. 2B). The control-engTregs modestly suppressed 2D2 Tconv proliferation at a ratio of 1:2 (Fig. 2B; top panels), a likely consequence of activation of the control-engTregs for the retroviral transduction. A stronger antigen-specific suppression of 2D2 cell proliferation was observed for each of the four types of TCR-engTregs even at 1:8 Treg:Tconv ratios (Fig. 2B). The magnitude of this antigen-dependent suppression correlated with the avidity of the recombinant TCR for the tested self-antigens. Thus, engTregs expressing the MOG<sup>hi</sup> TCR achieved a 47% antigen-specific suppression of 2D2 Tconvs proliferation at a ratio of 1:2, while this value was 29% for MOG<sup>low</sup> TCR-engTregs. The two bi-specific TCRs also endowed polyclonal Tregs with antigen-specific suppressive capacities. Indeed, engTregs expressing the MOG<sup>low</sup>/NF-M<sup>low</sup> or MOG<sup>low</sup>/NF-M<sup>hi</sup> TCR suppressed 2D2 Tconvs proliferation to 37% and 21%, respectively at the ratio of 1:2 (Fig. 2B; upper & lower panels). Importantly, the MOG/NF-M bi-specific TCR-engTregs additionally suppressed the response of 2D2 Tconvs to NF-M, with a stronger suppressive effect observed with engTregs carrying the MOG<sup>low</sup>/NF-M<sup>hi</sup> TCR (Fig. 2B; upper & lower panels). In contrast, TCR-engTregs bearing a MOG-mono-specific TCR had no effect on NF-M stimulated cultures (Fig. 2B; upper & lower panels). We conclude that TCR-engTregs suppress autoreactive Tconvs in an antigen-dependent manner, so that bi-specific TCR uniquely expand the reactivity profile of TCR-engTregs.

### 3.3. In vivo persistence and stability of transferred engineered Tregs

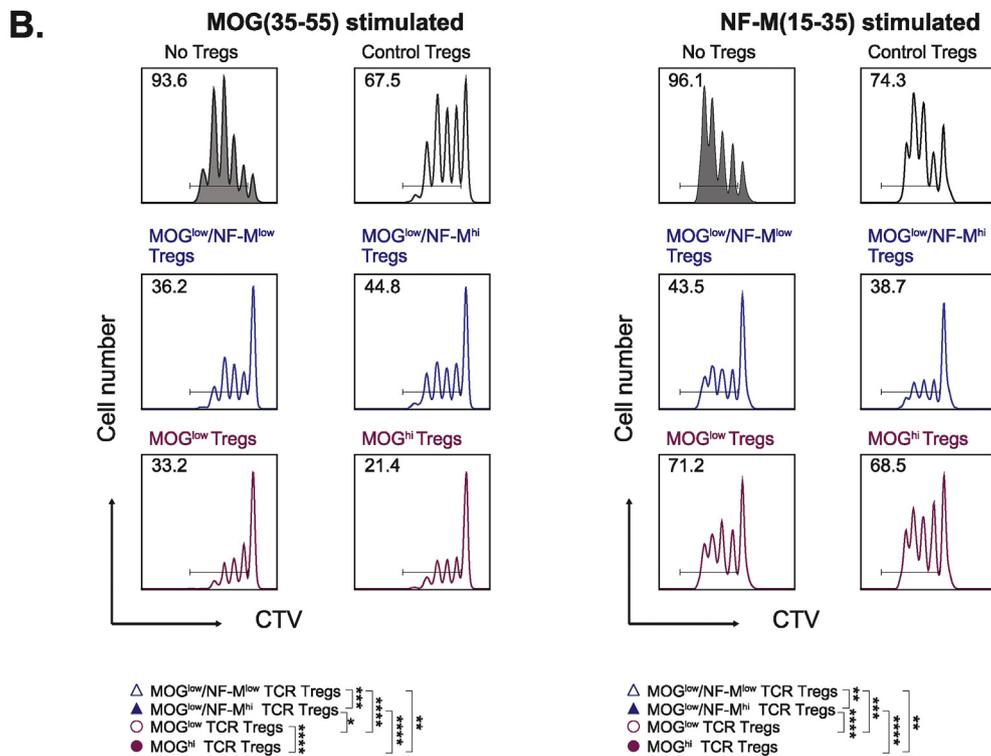
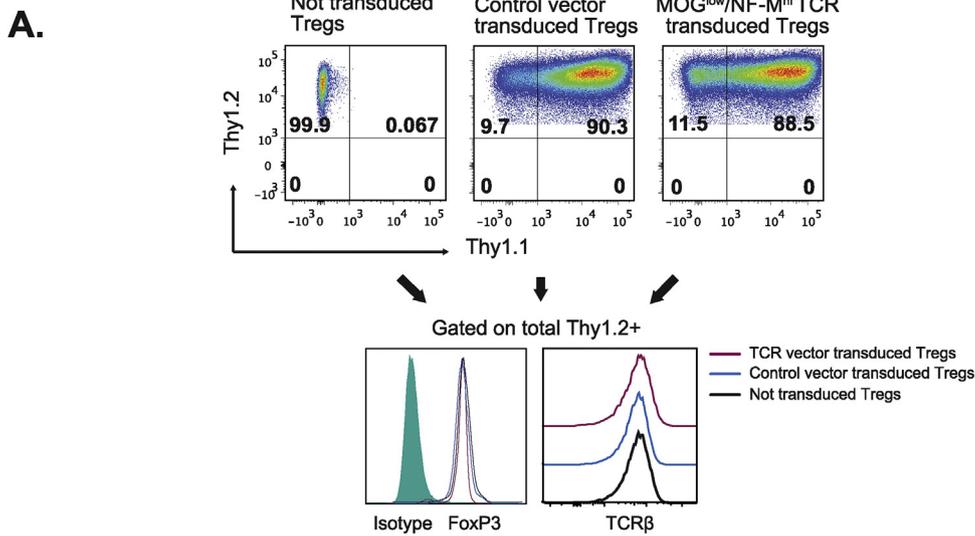
We next evaluated the activation of adoptively transferred engTregs, and their maintenance of FoxP3 expression, in recipient mice

during EAE. TCR-engTregs or control-engTregs, were administered to Thy1.2 C57BL/6 recipient mice one day before EAE induction by immunization with MOG (35–55). Lymph nodes, spleen, blood, spinal cord, and brain samples were then analyzed on days 8, 16, 24 and 32 post-immunization (Fig. 3A). TCR-engTregs were detected in all examined tissues on day 8, and at even higher frequencies on day 16, while their abundance then progressively declined at later time points. This response was antigen-specific because, in contrast, control-engTregs did not display any expansion (Fig. 3B & C). Although there was not a drastic increase in TCR-transduced Treg frequency in the CNS vs. lymphoid tissues, there seems to be a preferential retention of TCR-transduced Tregs in the CNS at day 32 (Suppl. Fig. 3). These findings suggest that TCR-engTregs were stimulated by the MOG (35–55) peptide used for immunization, and subsequently accumulated in the CNS. However, the supra physiological quantity of MOG-specific Tregs used to treat mice may have saturated the local MOG antigen-presentation capacities in the CNS and thereby Treg retention. Importantly, the TCR-engTregs retained high FoxP3 expression in recipient mice for at least 32 days (Fig. 3D).

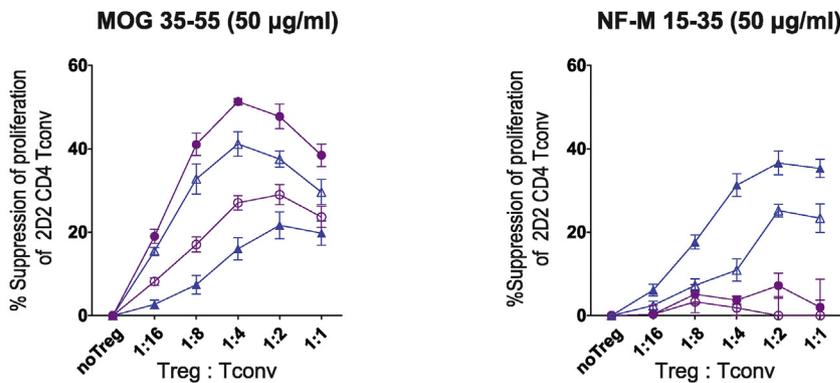
### 3.4. TCR cross-reactivity and functional avidity determine the protective function of engineered Tregs during MOG-induced EAE

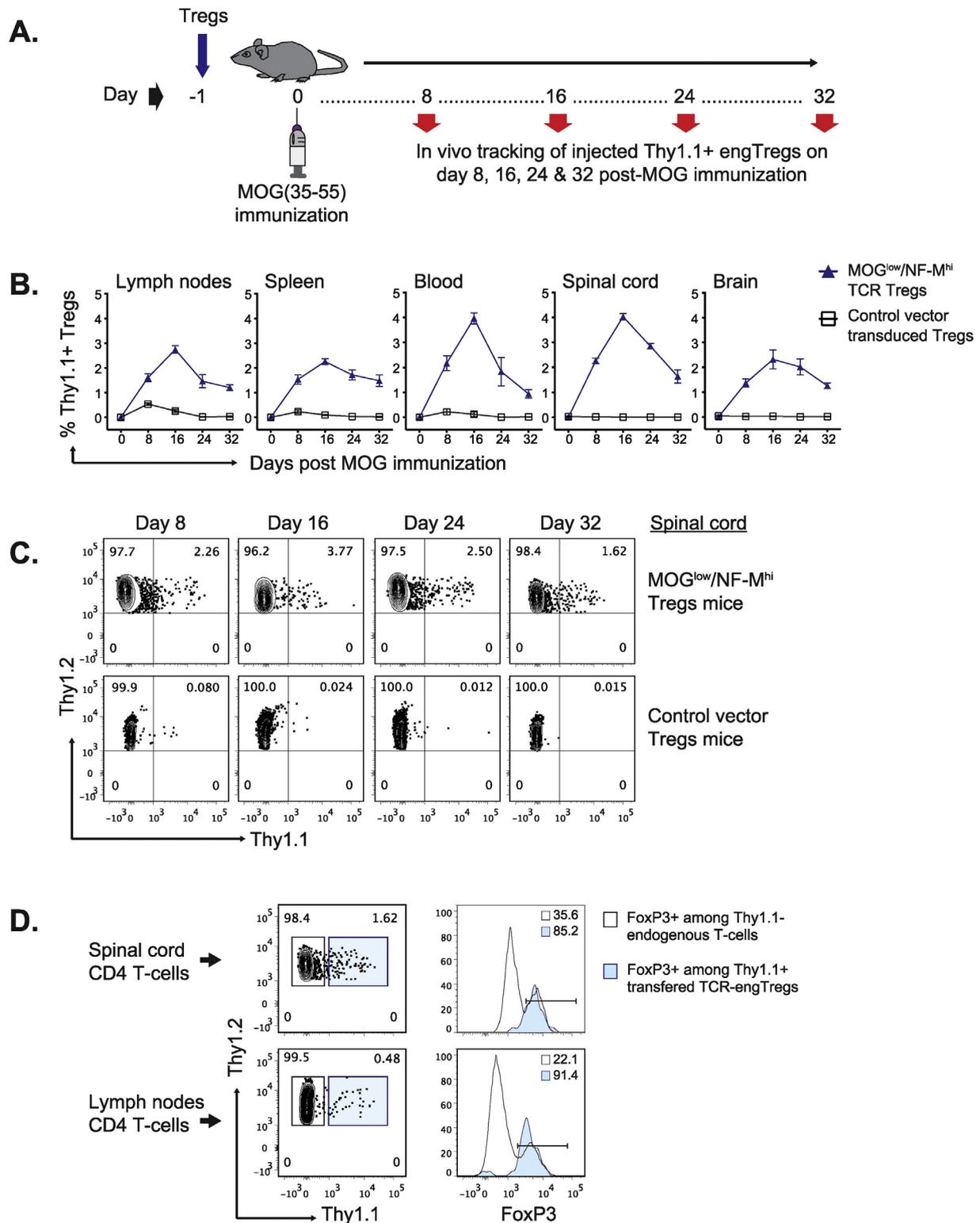
We next tested whether the protective function of TCR-engTregs was influenced by the bi-vs. mono-specificity of their recombinant TCR, or their functional avidity towards their cognate antigen, during EAE. In a preventive disease setting, the adoptive transfer of 10<sup>6</sup> TCR-engTregs one day before EAE induction resulted in a less severe disease and a faster recovery in recipient mice, compared to mice that received control-engTregs or PBS (Fig. 4A; Table 2). The properties of the recombinant TCR determined the protective efficacy of the transferred Tregs. High avidity TCR either for NF-M (MOG<sup>low</sup>/NF-M<sup>hi</sup> Tregs) or for MOG (MOG<sup>hi</sup> Tregs) conferred better protective function to transduced Tregs compared to low avidity TCRs (Fig. 4A; left & middle panels). Notably, among the two-high avidity TCRs, the bi-specific TCR-engTregs were more protective than TCR-engTregs expressing the mono-specific TCR (Fig. 4A; right panel). These data suggest that both the functional avidity and the cross-reactivity of the self-reactive TCRs impacted the protective function of TCR-engTregs in EAE.

We then assessed the *in vivo* protective function of TCR-engTregs in a therapeutic setting by injecting these cells on day 9 post-immunization. The engTregs expressing the TCRs with high avidity to NF-M (bi-specific) or MOG (mono-specific), reduced disease severity when administered in recipient mice after onset of EAE signs (Fig. 4B, Table 3). The bi-specific MOG<sup>low</sup>/NF-M<sup>low</sup> TCR-engTregs also provided benefit, whereas the MOG<sup>low</sup> TCR-engTregs did not. These data suggest that the engTregs with high avidity and/or bi-specific TCRs are more effective for the treatment of autoimmune diseases.

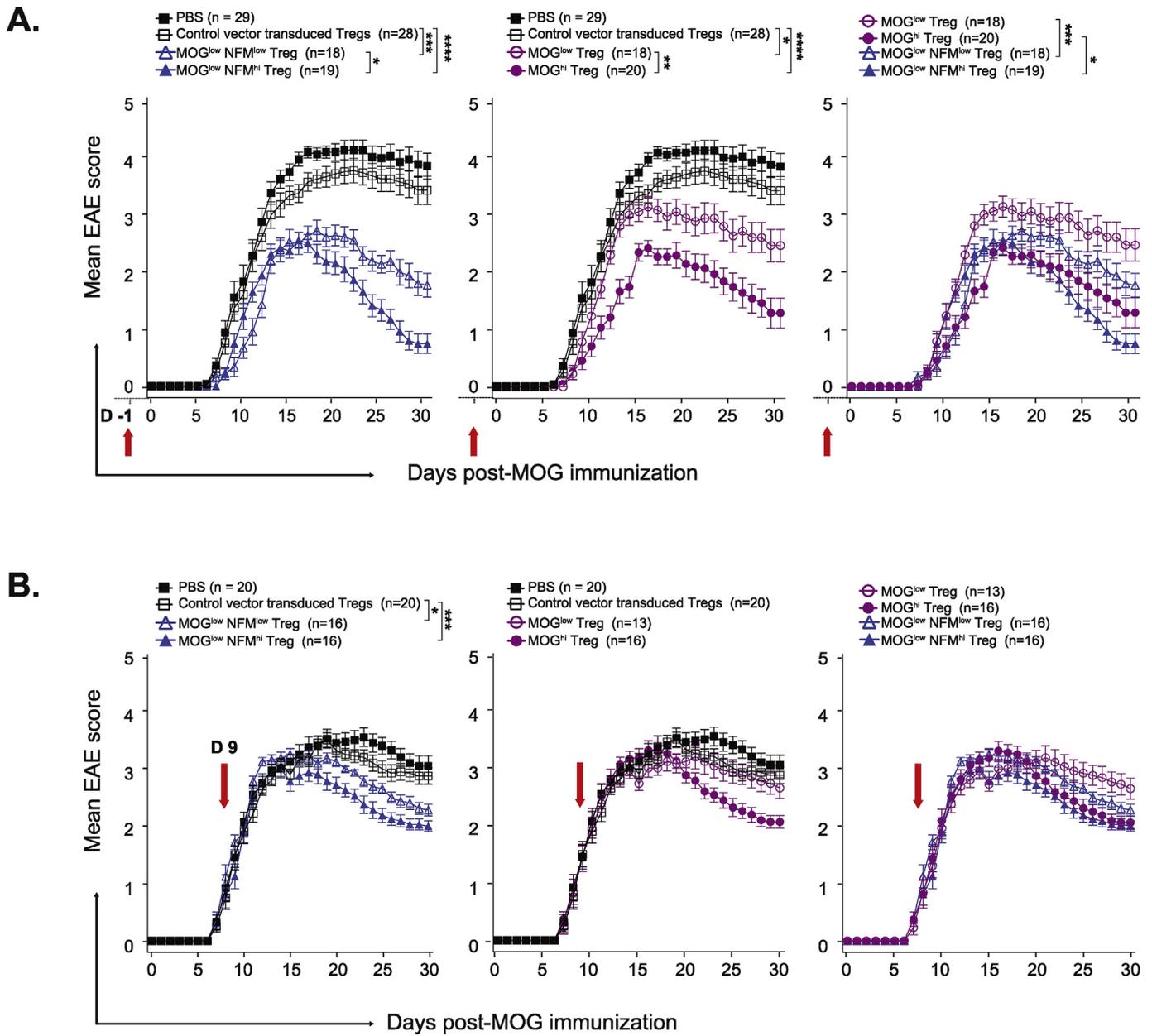


**Fig. 2.** TCR-engineered Tregs mediate antigen-specific immunosuppression in vitro. **(A)** Representative flow cytometry plots assessing transduction efficiency of Tregs based on Thy1.1 staining (upper panels) as well as levels of FoxP3 and TCR expression (lower panels) among not transduced versus control vector or TCR vector (MOG<sup>low</sup>/NF-M<sup>hi</sup> TCR) transduced FACS sorted CD4<sup>+</sup> TCR<sup>+</sup> CD25<sup>hi</sup> cells on day 6 after transduction. **(B)** CD4 Tconvs from 2D2 mice were isolated and labeled with CellTrace Violet (CTV) dye. Labeled 2D2 Tconvs were activated with MOG or NF-M peptide-loaded APC in the absence (no Tregs) or presence of TCR-engTregs or control-engTregs at different ratios. After 72 h, the suppressive property of Tregs was determined by assessing inhibition of CTV dilution by flow cytometry analysis. **Top:** Representative histograms showing Treg-mediated immunosuppression as measured by CTV dilution. Labeled 2D2 CD4 Tconv cells were activated with MOG (left panels) or NF-M (right panels) and cultured alone or with the indicated Tregs at a Treg:Tconv ratio of 1:2. Representative results from one of three independent experiments. Number indicates the percentage of 2D2 Tconvs that underwent at least one cell division. **Bottom:** Graph showing the antigen-specific suppression of proliferation of 2D2 Tconvs mediated by TCR-engTregs, obtained after subtraction of the antigen-independent suppression induced by control-engTregs. Graphs show mean ± SEM of three pooled independent experiments each performed in triplicates.





**Fig. 3.** In vivo persistence of adoptively transferred TCR-engineered Tregs. (A) Experimental scheme: TCR-engTregs expressing Thy1.1 and the  $MOG^{low}/NF-M^{hi}$  cross-reactive TCR, or control-engTregs expressing only Thy1.1, were administered to C57BL/6 mice one day before EAE induction by immunization with MOG (35–55). Lymph nodes, spleen, blood, spinal cord, and brain samples were collected on day 8, 16, 24 and 32 post-immunization. Flow cytometry analyses were performed to quantify the frequency of Thy1.1<sup>+</sup> transferred Tregs in the harvested tissues. (B) Kinetics of the expansion and persistence of  $MOG^{low}/NF-M^{hi}$  cross-reactive TCR-engTregs versus control-engTregs in C57BL/6 mice. Mice were sacrificed at the indicated time points (n = 2/3 per group and per timepoint) and expression of Thy1.1 among total CD4 T cells was analyzed in organs of the recipient animals. Graphs show the percentage of Thy1.1<sup>+</sup> cells among CD11b<sup>-</sup>CD45<sup>hi</sup>CD4<sup>+</sup>Thy1.2<sup>+</sup> viable cells (mean ± SEM). (C) Representative flow cytometry dot plots showing Thy1.1<sup>+</sup> staining among total CD4 T cells from the spinal cord of mice injected with  $MOG^{low}/NF-M^{hi}$  cross-reactive TCR-engTregs or control-engTregs. Values indicate the percentage of cells falling in each quadrant. (D) Representative flow cytometry plots showing expression of FoxP3 among transferred Thy1.1<sup>+</sup>Thy1.2<sup>+</sup>  $MOG^{low}/NF-M^{hi}$  TCR-engTregs (filled histogram), and among host Thy1.1<sup>+</sup>Thy1.2<sup>+</sup> CD4 T cells (open histogram), on day 32 post-immunization in the spinal cord and lymph nodes of a representative recipient mouse. Values indicate the percentage of cells in the respective gates.



**Fig. 4.** Protective function of the TCR-engineered Tregs on MOG-induced EAE. C57BL/6 mice received either  $10^6$  recombinant TCR-engTregs, or  $10^6$  control engTregs or PBS one day before immunization with MOG (35–55) in a prophylactic setting (A) or on day 9 post-immunization with MOG (35–55) in a therapeutic setting (B) as shown by arrows. Clinical signs were scored daily until day 30 post-immunization and shown as mean  $\pm$  SEM. The left graphs show EAE score for PBS and control-engTregs treated groups versus the two MOG/NF-M bi-specific TCR-engTregs groups. The graphs in the middle show EAE score for PBS and control-engTregs treated groups versus the two MOG specific TCR-engTregs groups. The right graphs show EAE scores for the two MOG/NF-M cross-reactive TCR-engTregs groups versus two MOG mono-reactive TCR-engTregs groups. N represents the total number of mice used in three independent experiments. Statistical analyses were performed comparing TCR-engTregs versus control-engTregs, then comparing the different TCR-engTregs; only significant differences are shown.

**3.5. TCR-engineered Tregs regulate autoreactive Tconv *in vivo***

To investigate how TCR bi-specificity affects the immunosuppressive function of engTregs *in vivo*, we compared the autoimmune response in mice treated with engTregs expressing either the bi-specific MOG<sup>low</sup>NF-M<sup>hi</sup> TCR or the mono-specific MOG<sup>low</sup> TCR. The engTregs were administered into C57BL/6 mice one day before EAE induction by immunization with MOG (35–55), and the CD4 T cell response was analyzed in recipient mice at disease onset (day 8), peak of disease (day 16) and during recovery (day 32) in lymph nodes, spinal cord, and brain. Remarkably, the administration of MOG<sup>low</sup>NF-M<sup>hi</sup> TCR-expressing engTregs resulted in a profound reduction of CD4 T cells accumulation in the brain of mice at all time points, while

engTregs carrying the MOG<sup>low</sup> TCR had no impact on this parameter (Fig. 5A). In agreement, mice treated with MOG<sup>low</sup>/NF-M<sup>hi</sup> bi-specific TCR-engTregs displayed a reduced frequency of host CD4 T cells producing IFN $\gamma$  and IL-17 in lymph nodes, spinal cord, and brain at all time points, while MOG<sup>low</sup> TCR engTregs did not significantly reduce this response (Fig. 5B and C). These results correlated with reduced accumulation of host MOG tetramer-positive Thy1.2<sup>+</sup> Thy1.1<sup>+</sup> CD4<sup>+</sup> T cells in the brain on day 32 (Fig. 5D). We conclude from these results that the expression of a bi-specific TCR enhances the beneficial effect of engTregs against autoimmune disease.

**Table 2**  
Prevention of MOG or PLP -induced EAE by transfer with recombinant TCR engineered Tregs one day before immunization.

Groups	Mice (Number)	Day of onset (mean $\pm$ SEM)	Max Score (mean $\pm$ SEM)	Cumulative score (mean $\pm$ SEM)	Disease incidence %
MOG (35–55) induced EAE					
PBS only (No Tregs)	29	9.10 $\pm$ 0.34	4.4 $\pm$ 0.09	80.13 $\pm$ 3.80	96.66 (29/30)
Control Tregs	28	9.03 $\pm$ 0.36	4.0 $\pm$ 0.15	71.75 $\pm$ 3.60	93.33 (28/30)
MOG <sup>low</sup> Tregs	18	9.38 $\pm$ 0.25	3.47 $\pm$ 0.16	56.86 $\pm$ 4.43	90 (18/20)
MOG <sup>hi</sup> Tregs	20	10.6 $\pm$ 0.46	2.7 $\pm$ 0.16	37.45 $\pm$ 4.24	100 (20/20)
MOG <sup>low</sup> NF-M <sup>low</sup> Tregs	18	10.61 $\pm$ 0.52	3.08 $\pm$ 0.14	44.97 $\pm$ 2.19	90 (18/20)
MOG <sup>low</sup> NF-M <sup>hi</sup> Tregs	19	9.52 $\pm$ 0.28	2.84 $\pm$ 0.10	36.89 $\pm$ 2.73	95 (19/20)
PLP (178–191) induced EAE					
PBS only (No Tregs)	20	10.35 $\pm$ 0.37	3.4 $\pm$ 0.09	54.3 $\pm$ 1.47	100 (20/20)
Control Tregs	20	11.35 $\pm$ 0.44	3.3 $\pm$ 0.13	51.02 $\pm$ 1.53	100 (20/20)
MOG <sup>low</sup> Tregs	12	10 $\pm$ 0.53	3.2 $\pm$ 0.11	48.79 $\pm$ 2.46	80 (12/15)
MOG <sup>hi</sup> Tregs	14	9.6 $\pm$ 0.40	2.8 $\pm$ 0.16	42.32 $\pm$ 2.22	93.33 (14/15)
MOG <sup>low</sup> NF-M <sup>low</sup> Tregs	14	9.8 $\pm$ 0.44	3.07 $\pm$ 0.12	46.42 $\pm$ 2.10	93.33 (14/15)
MOG <sup>low</sup> NF-M <sup>hi</sup> Tregs	12	10.83 $\pm$ 0.75	2.79 $\pm$ 0.15	39.45 $\pm$ 3.04	80 (12/15)

### 3.6. TCR-engineered Tregs protect recipient mice from EAE induced by an unrelated CNS autoantigen

The observation that bi-specific TCRs confer improved protective function to engTregs in comparison to mono-specific TCR led us to address whether bi-specific TCR-engTregs could also regulate a pathogenic autoimmune response directed towards a CNS autoantigen they do not recognize. To test this possibility, we assessed the protective effect of engTregs expressing the MOG/NF-M or MOG mono-specific TCR in EAE induced with the PLP (178–191) peptide. The TCR-engTregs or control-engTregs were adoptively transferred in recipient mice either one day before EAE induction or on day 9 post-immunization (Fig. 6; Table 2 and 3). In a prophylactic setting, the administered TCR-engTregs did not affect disease initiation, as predicted by the fact that they were not stimulated by the immunizing antigen, but they significantly improved recovery from paralysis, when a bi-specific TCR (MOG<sup>low</sup>/NF-M<sup>low</sup> and MOG<sup>low</sup>/NF-M<sup>hi</sup>), or the MOG<sup>hi</sup> mono-specific TCR was used (Fig. 6A). In contrast, only the two bi-specific TCR conferred a protective function to engTregs in a therapeutic setting (Fig. 6B). These data further highlight the unique therapeutic value of engTregs expressing bi-specific TCR.

## 4. Discussion

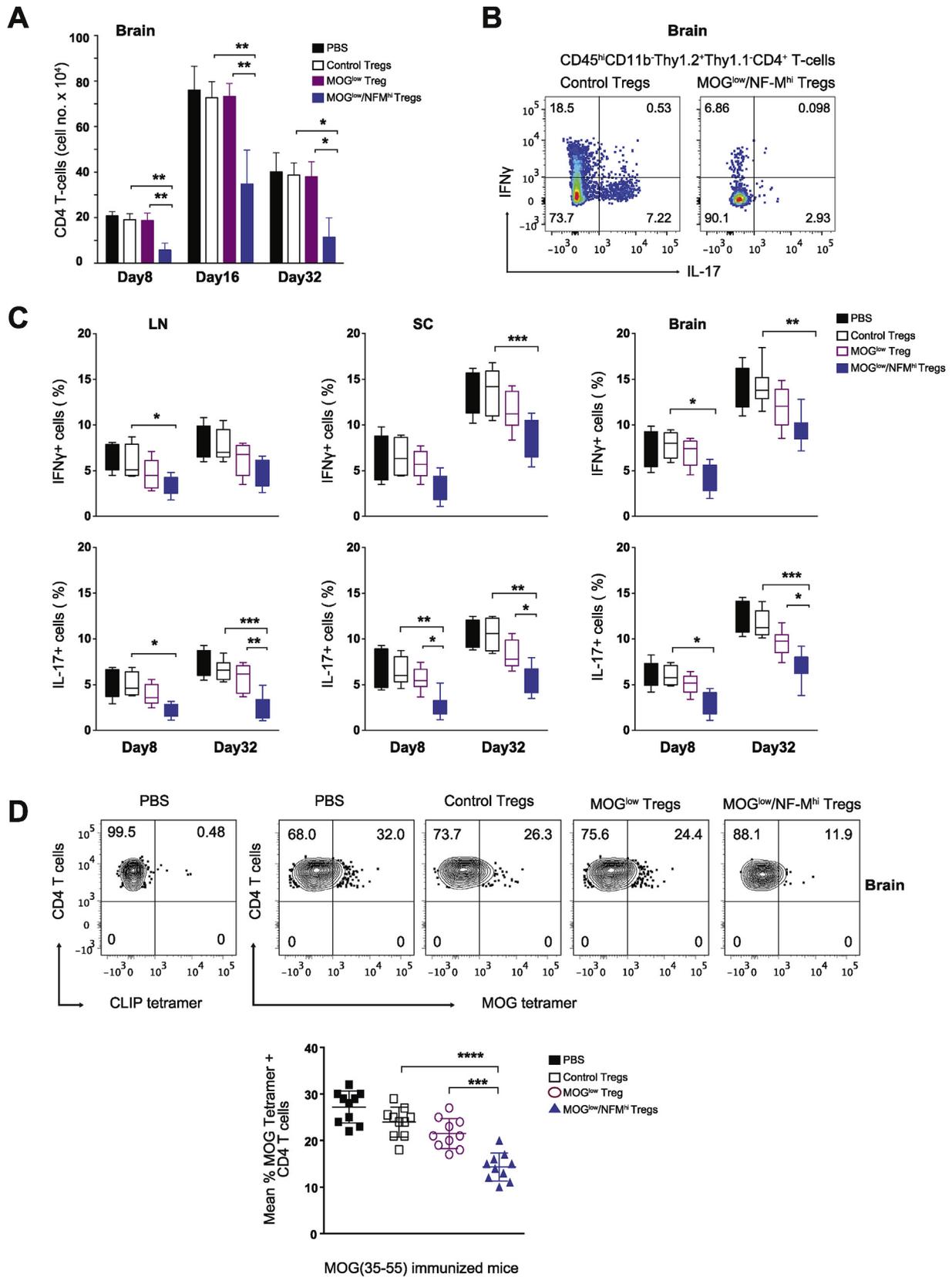
In this study, we engineered, using retroviral transduction, Tregs to express TCR specific for MOG or for both MOG and NF-M. The engTregs exhibited *in vitro* regulatory properties related to the antigenic specificity of the transduced TCR, with potency commensurate with their TCR's avidity. Following their adoptive transfer in an EAE setting, the TCR-engTregs proliferated, migrated to the CNS and retained elevated FoxP3 expression. The TCR-engTregs reduced the severity of MOG-

induced EAE in preventive and therapeutic settings. Importantly, the magnitude of this beneficial effect was dependent on both the avidity of the transduced autoreactive TCR and its bi-reactivity, as assessed by tetramer staining and IL-2 production in response to graded concentrations of self-peptides. A similar trend was also observed when the MOG/NF-M bi-specific and MOG mono-specific TCR-engTregs were used to treat EAE induced by PLP. The therapeutic effect of the transferred TCR-engTregs was associated with reduced T infiltration in the CNS of the recipient mice, and lower production of the pro-inflammatory cytokines IFN- $\gamma$  and IL-17.

Autoreactive Tregs are more efficient at controlling organ-specific autoimmune diseases than polyclonal Tregs and have therefore attracted a lot of attention [20,50]. Strategies have been developed to either induce/amplify *in vivo* these autoreactive Tregs or to isolate and expand them *ex vivo* for subsequent adoptive transfer [51]. However, those approaches are technically challenging and may carry risk of disease exacerbation if pathogenic Tconv are activated. To overcome this problem, we used a strategy to redirect the antigen-specificity of polyclonal Tregs using autoreactive TCR gene transfer [8,28,30–32,52]. The major advantage of this approach is the relative ease to generate large numbers of Tregs carrying the chosen MHC class I or MHC class II:peptide specificity [28,53]. Indeed, in our hands, based on cell surface expression of Thy1.1 protein more than 70% of the engTregs have been efficiently transduced. Tetramer staining and functional *in vitro* and *in vivo* experiments confirm efficient TCR gene transfer. Of note, we detected similar TCR density on the cell surface of transduced and not-transduced T cells. This is probably due to the competition of transferred TCR $\alpha/\beta$  with endogenous TCR $\alpha/\beta$  chains for the limited number of endogenous CD3 chains ( $\gamma, \delta, \epsilon$  and  $\zeta$ ) to form a cell surface TCR/CD3 functional complex [54,55]. Nonetheless, during the expression of the recombinant TCR in engTregs, there is the possibility of mispairing of

**Table 3**  
Prevention of MOG or PLP -induced EAE by transfer with recombinant TCR engineered Tregs on day 9 post-immunization.

Groups	Mice (Number)	Day of onset (mean $\pm$ SEM)	Max Score (mean $\pm$ SEM)	Cumulative score (mean $\pm$ SEM)	Disease incidence %
MOG (35–55) induced EAE					
PBS only (No Tregs)	20	8.30 $\pm$ 0.26	3.9 $\pm$ 0.13	69.27 $\pm$ 2.60	100 (20/20)
Control Tregs	20	8.65 $\pm$ 0.31	3.7 $\pm$ 0.13	65.00 $\pm$ 2.09	100 (20/20)
MOG <sup>low</sup> Tregs	13	8.30 $\pm$ 0.30	3.57 $\pm$ 0.11	62.50 $\pm$ 2.53	86.66 (13/15)
MOG <sup>hi</sup> Tregs	16	8.31 $\pm$ 0.31	3.53 $\pm$ 0.14	58.62 $\pm$ 2.15	100 (16/16)
MOG <sup>low</sup> NF-M <sup>low</sup> Tregs	16	7.87 $\pm$ 0.20	3.53 $\pm$ 0.10	62.46 $\pm$ 0.93	100 (16/16)
MOG <sup>low</sup> NF-M <sup>hi</sup> Tregs	16	8.43 $\pm$ 0.34	3.43 $\pm$ 0.12	54.75 $\pm$ 2.03	100 (16/16)
PLP (178–191) induced EAE					
PBS only (No Tregs)	16	9.75 $\pm$ 0.23	3.71 $\pm$ 0.17	61.90 $\pm$ 3.22	80 (16/20)
Control Tregs	16	10.12 $\pm$ 0.32	3.71 $\pm$ 0.18	59.59 $\pm$ 2.56	80 (16/20)
MOG <sup>low</sup> Tregs	10	10.00 $\pm$ 0.29	3.65 $\pm$ 0.21	57.65 $\pm$ 3.27	83.33 (10/12)
MOG <sup>hi</sup> Tregs	11	10.36 $\pm$ 0.27	3.22 $\pm$ 0.12	49.95 $\pm$ 1.09	91.66 (11/12)
MOG <sup>low</sup> NF-M <sup>low</sup> Tregs	13	10.23 $\pm$ 0.25	3.23 $\pm$ 0.20	49.50 $\pm$ 2.21	86.66 (13/15)
MOG <sup>low</sup> NF-M <sup>hi</sup> Tregs	10	10.50 $\pm$ 0.37	2.85 $\pm$ 0.10	44.15 $\pm$ 2.27	83.33 (10/12)

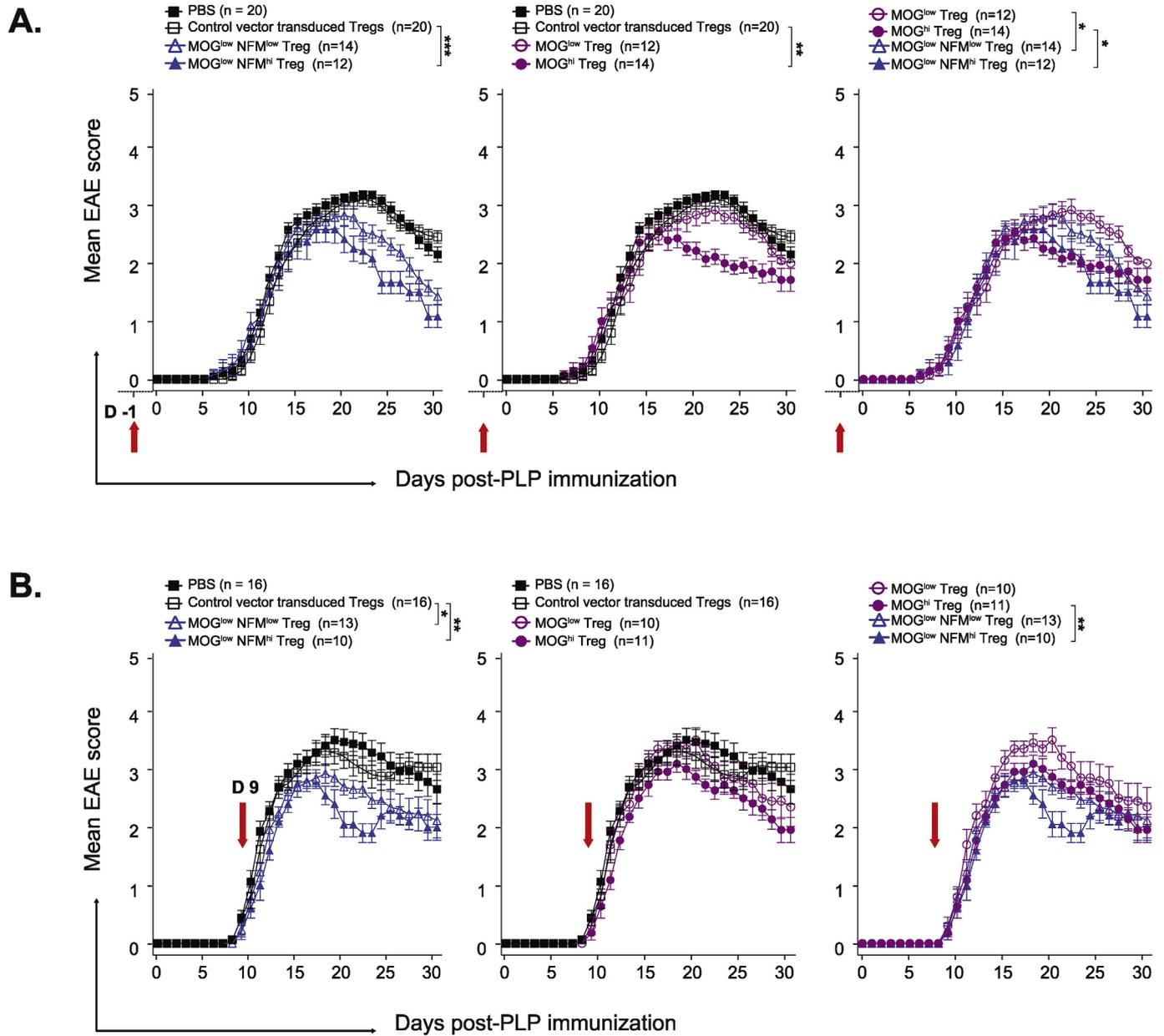


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the exogenous TCR $\alpha$  or  $\beta$  chains with the endogenous TCR chains. However, based on MOG/NF-M:MHC-II tetramer binding, and IL-2 production in response to cognate antigen(s), we assume that most of the recombinant exogenous TCR $\alpha$ / $\beta$  chains were co-expressed, retained

expected functional avidity to the cognate antigen(s), and were able to propagate TCR signaling in the transduced cells. Potential mispairing of exogenous TCR with endogenous TCR chains could be overcome by the addition of a second disulphide bond between the constant regions of

**Fig. 5.** Effect of TCR-engineered Tregs on host MOG-reactive CD4 Tconv cells in EAE. TCR-engTregs expressing a MOG-specific or a MOG/NF-M bi-specific TCR, control-engTregs, or PBS were administered to C57BL/6 mice one day before EAE induction by immunization with MOG (35–55). CD4 T cells were isolated from the lymph nodes (LN), spinal cord (SC) and brain at the indicated time points after immunization. **(A)** The numbers of brain-infiltrating CD4 T cells at first signs of EAE (day 8), at EAE peak (day 16) and during recovery (day 32). Graphs show the number (mean  $\pm$  SEM) of host CD4 T cells (CD45<sup>hi</sup> CD11b<sup>-</sup> Thy1.2<sup>+</sup> Thy1.1<sup>-</sup> CD4<sup>+</sup> cells). **(B)** Brain mononuclear cells were isolated, activated with MOG (35–55) peptide-loaded APCs for 72 h and with phorbol 12-myristate 13-acetate (PMA)/ionomycin for the last 5 h, and stained for intracellular cytokines. A representative dot plot showing IFN $\gamma$  and IL-17 production by brain-infiltrating host CD4 T cells, on day 32 post-immunization, from mice transferred with control-engTreg and MOG<sup>low</sup>/NF-M<sup>hi</sup> TCR-engTregs. Data show cytokine-producing cells gated on CD45<sup>hi</sup> CD11b<sup>-</sup> Thy1.2<sup>+</sup> Thy1.1<sup>-</sup> CD4<sup>+</sup> T cells. **(C)** The frequencies of IFN $\gamma$  and IL-17 producing CD4 T cells isolated and activated from LN, SC and brain as described above. Data represent the percentage of cytokine-producing cells within the host Thy1.2<sup>+</sup> Thy1.1<sup>-</sup> CD4<sup>+</sup> cell population at days 8 and 32 post-immunization. Graphs show mean  $\pm$  SEM values obtained from 2 independent experiments, each involving 2–3 mice. **(D)** Mononuclear cells isolated on day 32 post-immunization were stained with the I-A<sup>b</sup>:MOG (35–55)-PE or control I-A<sup>b</sup>:CLIP-PE tetramers, followed by a cocktail containing  $\alpha$ CD4,  $\alpha$ CD8,  $\alpha$ Thy1.2,  $\alpha$ Thy1.1 mAbs and a viability dye. Flow cytometry analyses were performed to quantify the frequency of viable tetramer + cells among the host Thy1.2<sup>+</sup> Thy1.1<sup>-</sup> CD4<sup>+</sup> T cells. The upper panels show representative flow cytometry dot plots. The lower panels show mean  $\pm$  SEM values obtained from 2 independent experiments, each involving 5 mice.



**Fig. 6.** TCR-engineered Tregs can reduce EAE induced by another myelin antigen, PLP. C57BL/6 mice received 10<sup>6</sup> recombinant TCR-engTregs or control-engTregs, or PBS one day before immunization with PLP (178–191) in a prophylactic setting **(A)** or on day 9 post-immunization with PLP (178–191) in a therapeutic setting **(B)** as shown by arrows. Clinical signs were scored daily until day 30 post-immunization and shown as mean  $\pm$  SEM. The left graphs show EAE score for PBS and control-engTregs treated groups versus the two MOG/NF-M bi-specific TCR-engTregs groups. The graphs in the middle show EAE score for PBS and control-engTregs treated groups versus the two MOG specific TCR-engTregs groups. The right graphs show EAE scores for the two MOG/NF-M cross-reactive TCR-engTregs groups versus two MOG reactive TCR-engTregs groups. N represents the total number of mice used in three independent experiments. Statistical analyses were performed comparing TCR-engTregs versus control-engTregs, then comparing the different TCR-engTregs; only significant differences are shown.

the recombinant TCR $\alpha/\beta$  chains [56–58]. Other limitations relate to the suboptimal regulatory potency of Tregs from MS patients [15], and the reduced migration and/or retention of Tregs in the CNS of MS patients. These may be overcome by adapting the *ex vivo* culture condition and/or by targeting the transferred Tregs *in vivo* [59]. Recently, adding metabolites or Th1-favoring cytokines during the *ex vivo* expansion phase of human Tregs cultures has been shown to modify their expression of homing receptors, thereby promoting migration to inflammatory sites [60]. The use of anti-IL-2 antibody stabilizing IL-2 or of orthogonal IL-2-IL-2 receptor complexes may provide a way to selectively expand the transferred Tregs and to enhance their regulatory functions [61,62].

In agreement with previous work, high avidity MOG-specific TCR-engTregs were more potent at preventing and reversing MOG-induced EAE than TCR-engTregs of lower functional avidity [8]. Similarly, in a mouse model of transplantation, graft survival was increased in recipients of Tregs expressing a high avidity TCR as compared to Tregs expressing the lower avidity TCR [52]. In addition to higher TCR avidity, cross-reactivity to multiple antigens is also likely to be an important contributing factor to the suppressive potency of a transgenic Treg population. Indeed, we demonstrate here that engTregs expressing TCR cross-reactive for two CNS self-antigens have superior protective function over those expressing mono-specific TCR. Our study demonstrates that TCR gene transfer can be used to produce bi-specific Tregs, and underlines that this improves their protective function compared to mono-specific TCR.

Some T cells can express a single  $\beta$  chain and two  $\alpha$  chains and may recognize several antigens. Nonetheless, T cells with a single TCR  $\alpha/\beta$  pair have also been shown to recognize several antigens [37,63]. The cross recognition of MOG and NF-M peptides occurs via a single TCR $\alpha/\beta$  pair and is related to shared amino acid contact residues between the two peptides [40]. Based on theoretical and experimental grounds cross-reacting TCRs have been assumed to be common [37,63,64]. This provides TCR the opportunity to face the enormous number of foreign antigens. We show here that it can be of benefit for immune regulation mediated by autoreactive Tregs.

It has been shown that during infection or repeated exposure to pro-inflammatory cytokines, Tregs can lose FoxP3 expression and in some cases even convert into Tconvs [65,66]. However, in our study, the TCR-engTregs retained FoxP3 expression up to 32 days after transfer. Nonetheless, we found that, when the cycles of *in vitro* expansion were repeated, the TCR-engTregs cultured progressively display a decreasing proportion of FoxP3<sup>+</sup> cells. We currently do not know whether this is due to overgrowth of Tconvs, or the loss of Treg phenotype. It might be possible to limit this problem by introducing exogenous FoxP3 and Id3 genes together with the TCR genes into the retroviral vector to improve the continuous expression of Foxp3 and Treg stability [67]. It should be noted, however, that human Tregs *ex vivo* expanded for 2 weeks retained their Treg phenotype for several weeks - up to one year - post-transfer in an autologous setting, which is reassuring [59,68].

An important issue in the context of autoimmune disease is whether the Tregs used therapeutically need to recognize the autoantigen(s) targeted by the pathogenic T cells or whether Tregs specific for other tissue-specific antigens would be efficacious. Clinical translation would be simplified if the latter were the case, obviating the need of identifying the autoantigens relevant for the disease process. Our data show that the inhibition of EAE was achieved even when the engTregs and the pathogenic T cells recognized different autoantigens within the same tissue, a phenomenon likely related to 'bystander suppression'. These findings are similar to those in which TCR-transgenic or engineered Tregs recognizing one myelin antigen could suppress EAE induced by immunization with a different antigen [22,30,69]. This is particularly relevant because the antigenic specificity of the pathogenic T cells in MS, and the characterization of their TCR, is being progressively understood [1,2]. Bystander suppression likely involves soluble mediators and does not require the Tregs and Tconvs to interact with

the same APC [30]. In some instances [46], but not always [30], Tconvs have been shown to be at least partially resistant to the suppressive effects of Tregs in highly inflammatory settings. Therefore, cellular therapy using eng-Tregs may be more effective if combined with immunomodulatory/immunosuppressive therapies.

As clinical translation would require curing rather than preventing disease, we also tested the impact of transferred engTregs on ongoing EAE. In this therapeutic setting, the significant improvement achieved may be related to the local effect of Tregs within the CNS as demonstrated in other settings [70,71]. Arguing in favor of their effect within the CNS, rather than in secondary lymphoid organs, the high avidity TCR engTregs had no effect on the initial phase of PLP-induced EAE, but significantly improved recovery. Detection of the engTregs at high frequency within the tissue in our study provides credence to this hypothesis. CNS-infiltrating Tregs, most likely upon recognition of auto-antigens released from injured tissue, may both regulate glial activation and neurotoxicity [70] and promote remyelination [71] through the local release of amphiregulin and CCN3, respectively. By contrast, in the prophylactic setting, the beneficial effect of the cellular therapy on EAE severity occurs early during disease development and is marked. It is likely that the effect of TCR-engineered Tregs takes place, at least in part, in secondary lymphoid organs where MOG peptide can reside for prolonged period of time following immunization in complete Freund adjuvant. We, therefore, postulate that the mechanisms and even site of action of the Tregs differ in the therapeutic vs. prophylactic schedule but also when disease is induced by MOG vs. PLP. This is supported by the fact that, in the prophylactic setting, EAE onset is delayed in MOG-induced EAE (with reduced peak EAE score) whereas in PLP-induced EAE disease regulation only occurs after the peak of disease (see Figs. 4A & 6A).

In this context, a salient point of this study is the added value to use engTregs whose TCR recognizes two tissue-relevant autoantigens, which have a unique capacity to promote recovery from disease, even in a therapeutic setting, and for a pathogenic T cell response driven by a distinct autoantigen. This is consistent with the local bystander suppression that can be afforded by Tregs with specificity distinct to that of the pathogenic T cells [30,72]. In conclusion, our study highlights the unique advantages of bi-specific TCR for the development of therapeutic engineered Treg as a platform to treat autoimmune diseases.

#### Author contributions

M.M. conducted all the experiments, prepared the figures and drafted the manuscript; A.S contributed to the development of the project, analyzed the data, and edited the manuscript; S-F provided the retrovirus vector backbone, and protocol to engineer Tregs, contributed in experiment planning, and edited the manuscript; R.L supervised the project, contributed to the development of the project, analyzed the data, interpreted the results and edited the manuscript.

#### Declaration of competing interest

The authors have declared that no conflict of interest exists.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jaut.2020.102401>.

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