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Pathogenicity and Protection Mediated by a Single TCR β in Experimental Autoimmune Encephalomyelitis

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Pathogenicity and Protection Mediated by a Single TCR β in Experimental Autoimmune Encephalomyelitis

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**Pathogenicity and Protection Mediated by a Single TCR β in Experimental
Autoimmune Encephalomyelitis**

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science
From The University of Tennessee

By
Tianhua Wu
August 2018

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ABSTRACT

How the TCR repertoire, together with risk-associated major histocompatibility complex (MHC), imposes susceptibility for autoimmune disease is not fully understood. A small fraction of TCR α or β chains are “public”, and are shared by most individuals. High-throughput sequencing of the mouse TCR β repertoire during myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) identified a public TCR β chain, TCR β 1, which was highly shared among individual mice and preferentially deployed during EAE. Retrogenic expression of TCR β 1 resulted in spontaneous early-onset EAE in mice with high penetrance and severity, despite being paired with a diverse endogenous TCR α repertoire. To further study autoimmunity conferred by this highly reactive beta chain, we generated TCR β 1 transgenic mice. Unexpectedly, TCR β 1 transgenic mice failed to develop spontaneous disease and were also resistant to standard EAE induction by MOG immunization. Despite the absence of disease, ~5% of TCR β 1 transgenic CD4⁺ T cells from unimmunized mice were MOG-specific, and these further expanded in response to MOG immunization. Three independent transfer models, including transfer of *in vitro* activated TCR β 1 splenocytes, transfer of TCR β 1 bone marrow, and a mock retrogenic system, all resulted in EAE in recipient mice, indicating there is no T cell-intrinsic blockade to pathogenesis. MOG-responsive transgenic T cells also expressed higher levels of PD-1 and Lag3 in comparison to those from WT and 2D2 mice, suggesting that they might be more prone to exhaustion. TCR β 1 transgenic T cells secreted higher levels of inflammatory cytokines IFN- γ and IL-17 relative to WT T cells, however these levels were comparable to those in pathogenic 2D2 MOG-responsive T cells. These results suggest that tolerance mechanisms in TCR β 1 transgenic mice prevent T cell pathogenicity and disease. Further studies are needed to fully resolve the mechanisms responsible for protection.

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LIST OF ABBREVIATIONS

CNS	Central Nervous System
EAE	Experimental Autoimmune Encephalomyelitis
IRES	Internal Ribosome Entry Site
MHC	Major Histocompatibility Complex
MOG	Myelin Oligodendrocyte Glycoprotein
MS	Multiple Sclerosis
TCR	T Cell Receptor
Treg	Regulatory T cell

CHAPTER 1. INTRODUCTION

Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis

Multiple sclerosis (MS) is one of the most widespread chronic inflammatory diseases of the central nervous system (CNS). An estimated 2.3 million people worldwide have MS, including 250,000-350,000 patients in the US. MS is characterized by T-cell mediated autoimmune demyelination and axonal damage [1, 2]. MS is a potentially disabling disease, with patients showing variable symptoms such as fatigue, difficulty in walking, and muscle weakness or rigidity [1-3]. In the 150 years since the discovery of MS, the exact cause and nature of the disease remains unclear, and no curative FDA approved medications for MS are currently available [3, 4].

Experimental autoimmune encephalomyelitis (EAE) is an animal model of T-cell mediated autoimmune demyelination characterized by T cell and monocyte infiltration in the CNS. Due to similarities in their key pathological features, EAE is a common animal model for pre-clinical studies of MS [5-8]. There are two common methods for inducing EAE in mice: passive and active EAE induction. Passive EAE induction involves adoptive transfer of encephalitogenic cells into naïve mice [5-8]. Active EAE induction requires immunization with myelin antigens emulsified in complete Freund's adjuvant (CFA) and co-administration of pertussis toxin. Frequently used myelin autoantigens include myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), and proteolipid protein (PLP) [5-9]. MOG is a 28 kDa glycoprotein that accounts for about 0.1% of total CNS myelin [10]. It was first identified as a key autoantigen involved in the autoimmune demyelination response in EAE by immunizing guinea pig with CNS homogenates in complete Freund's adjuvant [11]. Immunization with the MOG₃₅₋₅₅ peptide can induce EAE in C57BL/6J mice [12].

Public T Cell Receptor and TCR β 1

T cell-mediated autoimmunity is dependent on T cell receptor (TCR) recognition of antigenic peptides presented in the context of the major histocompatibility complex (MHC) protein. T cells mature in the thymus, and their survival is dependent on productive recombination of TCR α and β chains that form a heterodimer. Somatic recombination of V (variable), D (diversity) and J (junction) gene segments form the TCR variable domain, and random addition and deletion of nucleotides at the junctions results in a unique sequence. This process of somatic recombination followed by the pairing of individually recombined α and β chains generates highly unique TCR sequences that recognize a diverse array of potential pathogens [13, 14].

Theoretically, the TCR repertoire should be unique among individuals, as there are more potential TCR sequences than there are T cells circulating at any given time. However, due to biases in the recombination process, a small fraction of TCR α and β chains are preferentially formed and are shared by most individuals [13, 14]. These

shared, or “public”, TCRs have been preferentially associated with autoimmune diseases, including MS and EAE [15-17]. Our lab previously performed high-throughput sequencing of the TCR β repertoire of mice with MOG-induced EAE. We identified a public TCR β chain, TCR β 1, which was highly shared among individual mice and preferentially enriched during EAE [18]. Bone marrow cells from TCR $\beta^{-/-}$ mice were transduced with TCR β 1 to generate retrogenic mice. Retrogenic expression of TCR β 1 resulted in spontaneous early-onset EAE in mice, despite being paired with a diverse endogenous TCR α repertoire. This indicated that this single, highly public TCR β chain was sufficient to confer TCR $\alpha\beta$ MOG-reactivity and disease [19].

TCR β 1 Transgenic Mice Are Resistant to EAE

To further study autoimmunity conferred by this highly reactive TCR β chain, we generated TCR β 1 transgenic mice. We hypothesized that TCR β 1 transgenic mice would be a more imitative, spontaneous mouse model for MS, compared to the immunization and cell transfer models of EAE. TCR β 1 retrogenic mice succumbed to spontaneous and severe EAE by approximately 8 weeks after bone marrow transfer [19]. We monitored TCR β 1 transgenic mice for symptoms of EAE or other autoimmune disease for 6 months. No spontaneous neuropathology was observed and further histological analysis did not reveal signs of EAE or other autoimmune disorders such as inflammatory bowel disease, colitis, or immune cell infiltration of other tissues. To determine if immunization could induce disease, similar to wild type B6 mice, we immunized TCR β 1 transgenic and wild type mice with MOG₃₅₋₅₅ to induce EAE. Surprisingly, TCR β 1 mice were completely resistant to standard induction of EAE by immunization (**Figure 1-1**). This difference in phenotype between TCR β 1 retrogenic and transgenic mice presents an opportunity to study the determinants of T cell pathogenicity during EAE, and may provide insights into mechanisms of tolerance and disease induction in EAE.

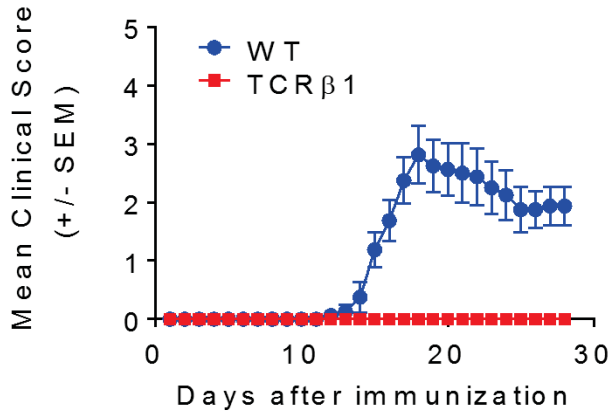


Figure 1-1. TCRβ1 transgenic mice are resistant to standard EAE immunization
 WT (B6) and TCRβ1 transgenic mice were immunized with MOG₃₅₋₅₅ peptide in CFA (s.c.) and pertussis toxin (i.p.) and monitored for signs of disease. Clinical scoring was: 1, limp tail; 2, hind limb paresis or partial paralysis; 3, total hind limb paralysis; 4, hind limb paralysis and body or front limb paresis or paralysis; and 5, moribund.

CHAPTER 2. METHODOLOGY

Mice

B6 [C57BL/6J (B6)], TCR β ^{-/-} [B6.129P2-Tcrbtm1Mom/J (TCR β ^{-/-})], and 2D2 [C57BL/6-Tg (Tera2D2, Tcrb2D2)1Kuch/J] mice [20] were purchased from The Jackson Laboratory (Bar Harbor, ME). TCR β 1 mice were generated in the transgenic core facility at St. Jude Children's Research Hospital. Mice were housed under specific pathogen-free conditions and animal experiments were carried out in compliance with the guidelines of the Institutional Animal Care Committee at St. Jude Children's Research Hospital. Animal care was provided in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited animal barrier facilities at St. Jude Children's Research Hospital.

TCR β 1 Transgenic Mice and Genotyping

TCR β 1 was cloned in pHCD2-VA vector using EcoRI and SnaBI sites. This TCR β is driven under CD2 promoter. The vector was linearized by KpnI and NotI restriction sites to remove vector backbone.

Several μ l of 2 ng/ μ l DNA of the linearized DNA construct was injected into the pronucleus of C57BL/6 zygotes. Approximately 25 injected zygotes were transferred into an infundibulum of a 0.5 dpc (days postcoitum) pseudo pregnant CD-1 foster mother and carried to term. TCR β 1 mice were first generated in the transgenic core facility on a B10.BR background. They were crossed onto a TCR β ^{-/-} background, and finally onto a B6 background, to generate TCR β 1 without endogenous TCR β on a B6 background.

To genotype TCR β 1 transgenic mice, PCR screening of genomic DNA was done with the following primers: (5' – CTCGAGTTGGCTACCCCCTCTCA – 3' and 5' – CGTAGAATTTTTTTTCTTGACCATGG – 3'). In other cases, flow cytometric analysis of V β 8 staining of blood from mice was used to determine genotype.

Flow Cytometry

Red blood cells were lysed with ACK lysis buffer prior to staining. Fc receptors were blocked with FcR blocking reagent (Miltenyi Biotec, San Diego, CA). Cell surface staining was performed for 20 min at 4°C in PBS containing 0.1% sodium azide and 2% (vol/vol) fetal bovine serum (FBS). Monoclonal antibodies (Ab) specific for CD4 (clone RM4-5), CD8 (clone 53-6.7), TCR β (clone H57-597), CD3 (clone 17A2), CD25 (clone PC61), or CD69 (clone H1-2F3) were purchased from BD Biosciences (San Jose, CA). The fluorochrome conjugation for each antibody was variable between experiments, but the clone number was consistent. For intracellular Foxp3 staining, cells were first stained with surface markers, fixed, permeabilized with the Foxp3 Staining Buffer Set

(eBioscience, San Diego, CA), and stained for intracellular Foxp3 (clone FJK-16s). Flow cytometric analysis was performed on an LSRFortessa (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

EAE Immunization

MOG₃₅₋₅₅ peptide (MEVGWYRSPFSRVVHLYRNGK) was synthesized by St. Jude Hartwell Center for Biotechnology and HPLC purified prior to use. Each mouse was s.c. (subcutaneous) immunized with 100 µg MOG₃₅₋₅₅ peptide emulsified in complete Freund's adjuvant (Difco, Detroit, MI) containing 0.4 mg heat-killed *Mycobacterium tuberculosis* H37RA (Difco). Two hundred ng of pertussis toxin (List Biological Laboratories, Campbell, CA) was administered i.p. (intraperitoneal) on day 0 and day 2. Clinical scoring was as follows: 1, limp tail; 2, hind limb paresis or partial paralysis; 3, total hind limb paralysis; 4, hind limb paralysis and body or front limb paresis or paralysis; 5, moribund or deceased.

Adoptive EAE Transfer

Donor mice were immunized s.c. with 100 µg MOG₃₅₋₅₅ peptide emulsified in complete Freund's adjuvant (Difco, Detroit, MI) containing 0.4 mg *Mycobacterium tuberculosis* H37RA (Difco). Spleens from immunized mice were extracted 10 days later and processed through a 40 µm filter (Falcon™) to obtain a single cell suspension. Cells were incubated with ACK lysis buffer (Quality Biological) for 3-4 minutes at room temperature and buffer was neutralized with 10 ml of PBS with 2% FBS. After centrifugation at 1500 RPM for 5 minutes, cells were cultured in presence of MOG₃₅₋₅₅ (100 µg/ml), IL-6 (20 ng/ml), and IL-23 (20 ng/ml) in a concentration of $3.5-4 \times 10^6$ cells/ml. After 5 days, 5×10^6 cultured cells/mouse were injected i.v. (intravenous) into 8-12 week old irradiated (450 rad) TCRβ^{-/-} recipient mice to induce adoptive EAE. All cytokines were obtained from Peprotech.

Generation of Mock Retrogenic and Retrogenic Mice

Murine stem cell virus (MSCV)-GFP and MSCV-TCRβ1-GFP retroviral vectors, in which the GFP is linked through an IRES to generate a polycistronic construct, were used to generate GPE86 viral producer cell lines. (MSCV)-GFP viral supernatants and MSCV-TCRβ1-GFP viral supernatants were used to transduce hematopoietic progenitor cells (HPCs) from TCRβ1 transgenic mice and TCRβ^{-/-} mice respectively as described previously [21, 22]. Bone marrow transduced with MSCV virus were used to generate mock retrogenic mice. Bone marrow transduced with MSCV-TCRβ1 virus were used to generate retrogenic mice. Transduced bone marrow cells ($\sim 2 \times 10^6$ per mouse) were injected via the tail vein into irradiated TCRβ^{-/-} (450 rad) recipient mice. Transduction efficiency (around 40%) was confirmed by flow cytometry for GFP expression.

Cell Proliferation Assays

Spleens were isolated from TCR β 1, WT B6, or 2D2 mice. Red blood cells were lysed with ACK lysis buffer and splenocytes were cultured at 1×10^5 cells per well in 96-well plates in the presence of 2 μ g/ml soluble anti-CD28 (BD Biosciences) and stimulated with 100 μ g/mL MOG₃₅₋₅₅ peptide for 72 hr. For 3 H thymidine incorporation, plates were pulsed with 1 μ Ci/well 3 H thymidine (PerkinElmer, Boston, MA) and incubated for another 8 hr before scintillation counting. The proliferative response was measured as the 3 H thymidine incorporation expressed as mean C.P.M (counts per minute) of triplicate cultures. For quantitative assessment of proliferation, the CellTrace Violet Cell Proliferation kit (Invitrogen) was used according to the manufactory's instruction. Cells were labeled with 5 μ M CellTrace violet dye for 7 min at 37°C in dark and cultured with indicated stimuli for 72 hr. Cells were then stained with surface markers, and proliferation was measured by cell trace violet dye dilution by flow cytometry. For positive and negative controls in these experiments, cells were stimulated with plate bound 3 μ g/ml anti-CD3, or given no stimulation, respectively.

Cytokine Analysis

Cultures were prepared as in cell proliferation assays, and culture supernatants were collected after 72 hr and analyzed for IL-2, IL-4, IL-10, IFN- γ , and IL-17A by using Milliplex MAP mouse cytokine immunoassay kit (Millipore, Billerica, MA) on a Luminex (Bio-Rad) instrument.

Statistics

Means and standard error of the mean (SEM) were calculated in PRISM software (GraphPad, La Jolla, CA). Plots demonstrate mean \pm 1 SEM. Two-tailed Student's t-test was applied to compare between two groups. One-way ANOVA was applied for multiple groups. A value of $p < 0.05$ was considered statistically significant.

CHAPTER 3. RESULTS

TCR β 1 Transgenic Mice Have Abnormal Treg Compartment

Standard induction of EAE with MOG₃₅₋₅₅/CFA emulsion failed to cause EAE in TCR β 1 transgenic mice. Normal T cell compartments are essential to immune response to antigens and abnormal T cell compartments may cause failure of an expected response to MOG₃₅₋₅₅ in TCR β 1 transgenic mice.

Thus we first characterized the major T cell compartments in the spleen and lymph nodes of TCR β 1 transgenic mice to determine if an abnormal composition of T cell subsets may influence susceptibility to disease. TCR β 1 mice have normal percentages of CD4⁺ and CD8⁺ T cells in both organs relative to WT B6 mice (**Figure 3-1**). Moreover, the numbers of CD4⁺ and CD8⁺ T cells in spleens of TCR β 1 mice are comparable to WT B6 mice (**Figure 3-1**). The percent of Foxp3⁺ regulatory T cells (Treg) in TCR β 1 mice was reduced by 50% in both spleen and lymph nodes, relative to WT B6 mice (**Figure 3-1**). The absolute number of Foxp3⁺ regulatory T cells (Treg) was also reduced in spleens of TCR β 1 mice in comparison to WT B6 mice (**Figure 3-1**).

TCR β 1 Transgenic Mice Have MOG-reactive T Cells

TCR β 1 retrogenic mice had high numbers of MOG-reactive T cells and develop spontaneous early-onset EAE [19]. However, TCR β 1 transgenic mice did not develop spontaneous EAE and further failed to develop disease upon active induction with MOG₃₅₋₅₅/CFA emulsion. It is possible that MOG-reactive T cells are deleted during T cell maturation, before they reach the peripheral lymphoid organs, and a lack of MOG-reactive T cells *in vivo* may lead to inability of TCR β 1 transgenic mice to develop EAE. Thus, we next asked if TCR β 1 transgenic mice have endogenous MOG-reactive T cells.

Splenocytes were harvested from unimmunized TCR β 1 transgenic mice and stimulated with MOG peptide *in vitro*, and ³H-thymidine incorporation was used as a readout of proliferation. Upon stimulation with MOG, TCR β 1 transgenic T cells proliferated in a dose dependent manner (**Figure 3-2a**). This result was confirmed using a CellTrace violet proliferation assay, and it was calculated that approximately 5% of the initial CD4⁺ population was proliferating in response to MOG (**Figure 3-2b, c**). We also observed a population of CD8⁺ TCR β 1 T cells that proliferate in response to MOG stimulation (**Figure 3-2d**). Splenocytes from MOG-immunized TCR β 1 transgenic mice also proliferated in response to MOG, with greater numbers (around 12% of input CD4⁺ T cells) proliferating upon MOG stimulation than unimmunized mice. These results indicate that MOG-reactive T cells were present, and activated and expanded upon MOG immunization (**Figure 3-3**). The proliferation assays confirm the existence of antigen-responsive cells in TCR β 1 mice, and resistance to EAE induction is not due to lack of MOG-reactive T cells.

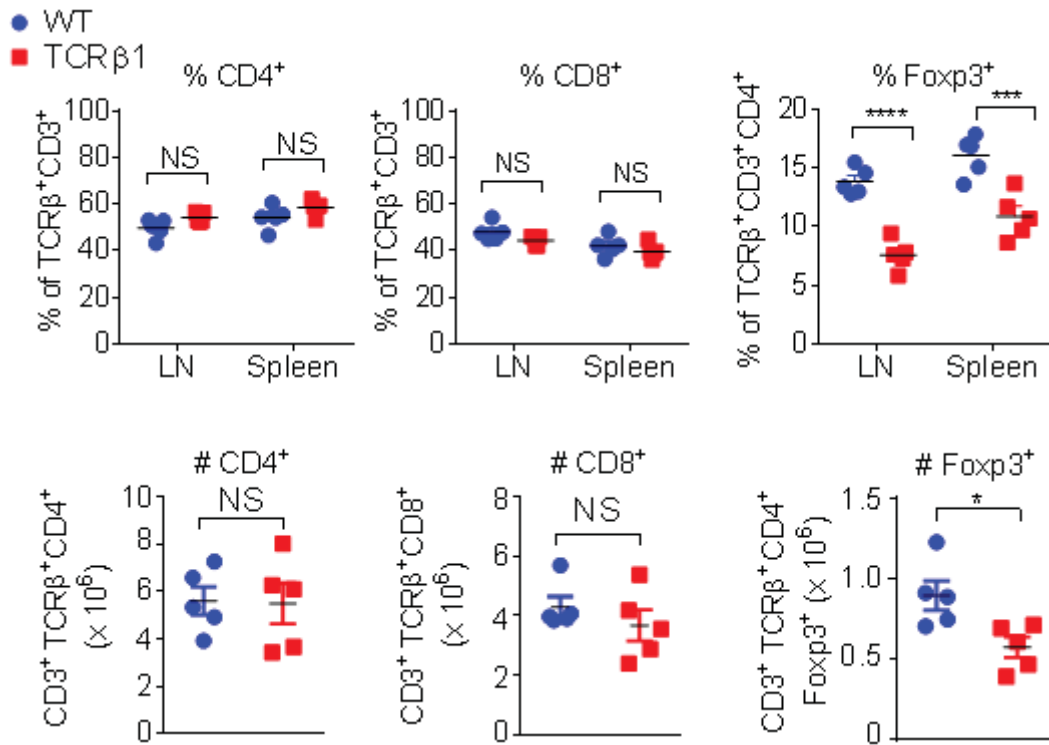


Figure 3-1. Reduced Treg in spleen and lymph nodes of TCRβ1 transgenic mice
 Spleen and lymph nodes from naïve TCRβ1 or WT (B6) mice were harvested, and analyzed with flow cytometry. Upper panels show percentages of CD4⁺ T cells, CD8⁺ T cells, and CD4⁺Foxp3⁺ T cells (Treg) in spleen and lymph nodes. Plots shown results from individual mice (dots) and mean values (bars). Lower panels display absolute numbers of CD4⁺ T cells, CD8⁺ T cells, and CD4⁺Foxp3⁺ T cells (Treg) in spleen. (*p<0.05, ***p<0.001, ****p<0.0001).

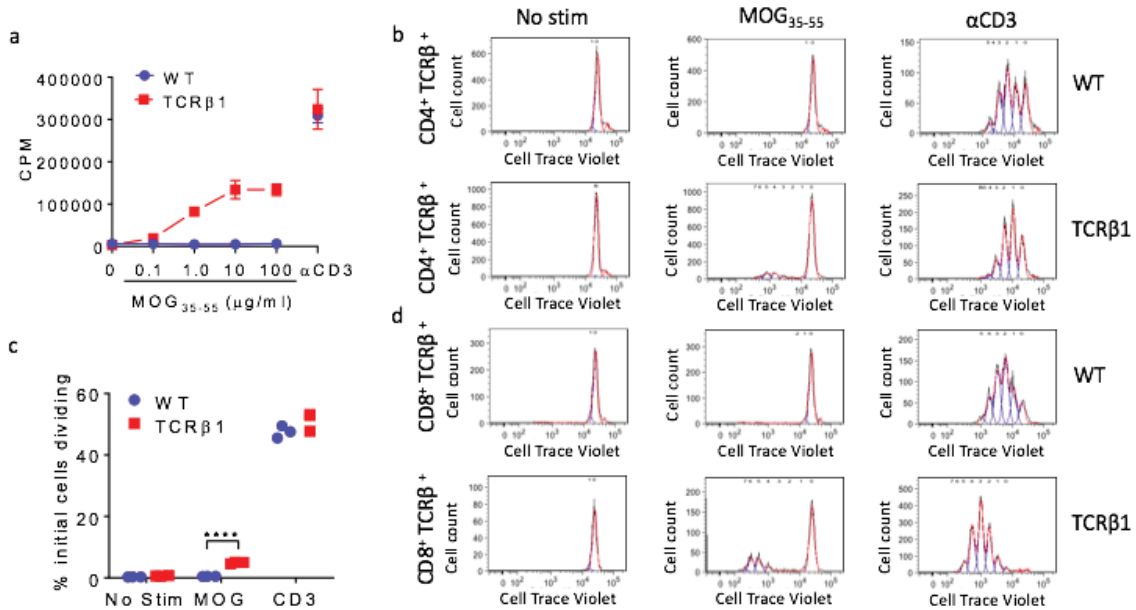


Figure 3-2. TCRβ1 transgenic mice have MOG-specific T cells

(a) Splenocytes from naïve TCRβ1 or WT (B6) mice were stimulated with MOG₃₅₋₅₅ (100 μg/ml) or αCD3 for 72hr. Proliferation was monitored by on day 3 by ³H-thymidine incorporation. (b-d) Splenocytes from TCRβ1 or WT mice were labeled with CellTrace Violet and stimulated with MOG₃₅₋₅₅ (100 μg/ml) or αCD3 for 72 hr. (b) Proliferation of CD4⁺ T cells as monitored by CellTrace Violet dilution. (c) The percent of the initial CD4⁺ T cell population undergoing division was calculated by dividing each division peak by 2ⁿ, where n=division number, to estimate initial cell numbers forming each peak. (d) Proliferation of CD8⁺ T cells as monitored by CellTrace Violet dilution. (****p<0.0001).

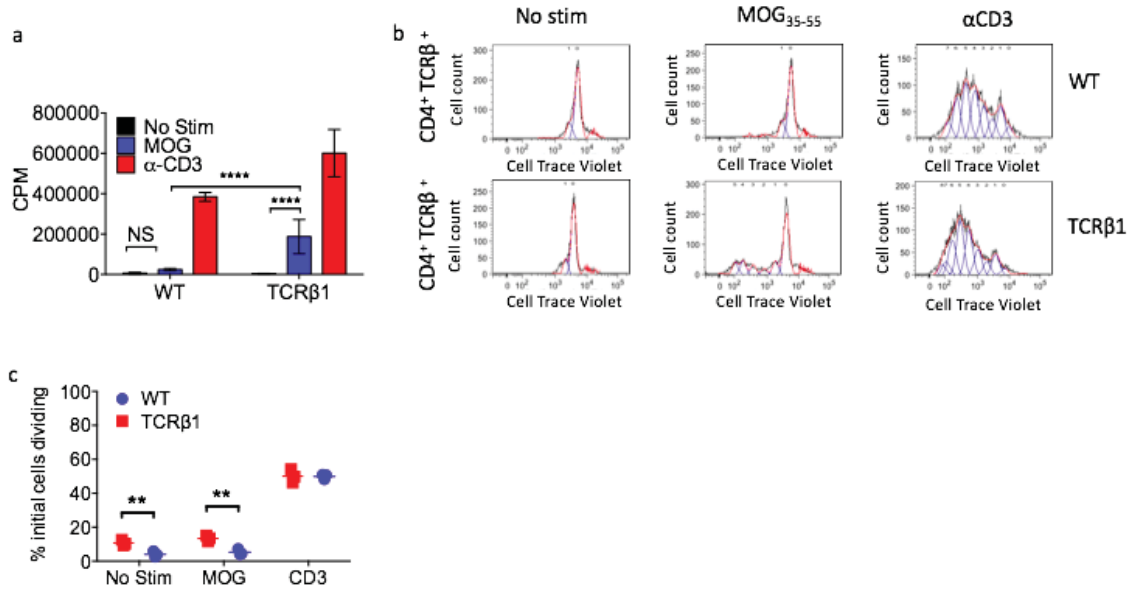


Figure 3-3. TCRβ1 transgenic T cells are MOG-specific and further expand in response to MOG immunization

(a) TCRβ1 and WT mice were immunized with MOG₃₅₋₅₅ peptide. Splenocytes were isolated 10 days later and stimulated with MOG₃₅₋₅₅ (100 μg/ml) or αCD3 for 72 hr. Proliferation was monitored on Day 3 by ³H-thymidine incorporation. (b-c) Splenocytes from immunized TCRβ1 or WT mice were labeled with CellTrace Violet and stimulated with MOG₃₅₋₅₅ (100 μg/ml) or αCD3 for 72 hr. (b) Proliferation of CD4⁺ T cells was monitored by CellTrace Violet dilution and (c) the percent of the initial CD4⁺ T cell population undergoing division was calculated. The percent of initial cells dividing in response to indicated stimulus was calculated by dividing each division peak by 2ⁿ, where n=division number, to estimate initial cell numbers forming each peak. (**p<0.01).

TCR β 1 Cells Are Able to Mediate EAE Disease

T cells originate from hematopoietic stem cells in the bone marrow. The lymphoid progenitors of these cells migrate to the thymus to become mature functional T cells. The matured T cells then exit the thymus and circulate in the periphery. Abnormal T cell development may leave T cells in TCR β 1 transgenic mice intrinsically unable to become pathogenic cells and cause EAE in mice. Thus we next asked if TCR β 1 T cells can be manipulated to make them pathogenic, or if they are intrinsically unable to cause disease.

We utilized three separate methods to investigate this. First, bone marrow was transferred directly from TCR β 1 transgenic mice into sub-lethally irradiated TCR β ^{-/-} mice and disease progression was monitored. Recipient mice exhibited mild EAE symptoms, indicating that TCR β 1 bone marrow can produce pathogenic T cells under these conditions (**Figure 3-4a**). Although a WT bone marrow transfer control was not present in the experiment shown, EAE symptoms do not develop after transfer of WT bone marrow into irradiated mice.

Second, we asked if mature TCR β 1 T cells from transgenic mice can become pathogenic after culture in conditions that have been shown to increase EAE pathogenicity. Stimulation of MOG reactive T cells with MOG peptide, IL-6 and IL-23 has been shown to generate encephalitogenic T cells that cause disease in recipient mice [23, 24]. To determine if TCR β 1 T cells can be activated to generate pathogenic cells, WT or TCR β 1 mice were immunized and spleens were harvested 10 days later and stimulated *in vitro* with MOG, IL-6, and IL-23 for 5 days. These cultured T cells were then transferred into sub-lethally irradiated TCR β ^{-/-} mice (450 rad), and disease progression was monitored (**Table 3-1** and **Figure 3-4b**). Mice that received either WT or TCR β 1 T cells cultured under these conditions developed rapid and severe EAE. Disease incidence and area under the curve were not significantly different between groups.

Third, we directly compared retrogenic TCR β 1 mice [19], which we previously reported to develop severe, spontaneous EAE, and “mock” retrogenic TCR β 1 mice. For retrogenic mice, bone marrow from TCR β ^{-/-} mice were transduced with MSCV-TCR β 1-GFP viral supernatant. For “mock” retrogenic mice, bone marrow from TCR β 1 transgenic mice was transduced with MSCV-GFP viral supernatant. The transduced bone marrow was injected into irradiated TCR β ^{-/-} mice. Mock retrogenic mice had earlier onset of disease, but maximal severity and area under the curve were not significantly different (**Table 3-2** and **Figure 3-4c**). Taken together, these data indicate that there is no intrinsic block to TCR β 1 T cells becoming pathogenic under the right conditions.

TCR β 1 T Cells Differentiate and Produce Effector Cytokines

In early studies, EAE was thought to be a prototypical Th1 autoimmune disease due to its strong association with IFN- γ . However, IFN- γ knockout mice or IFN- γ

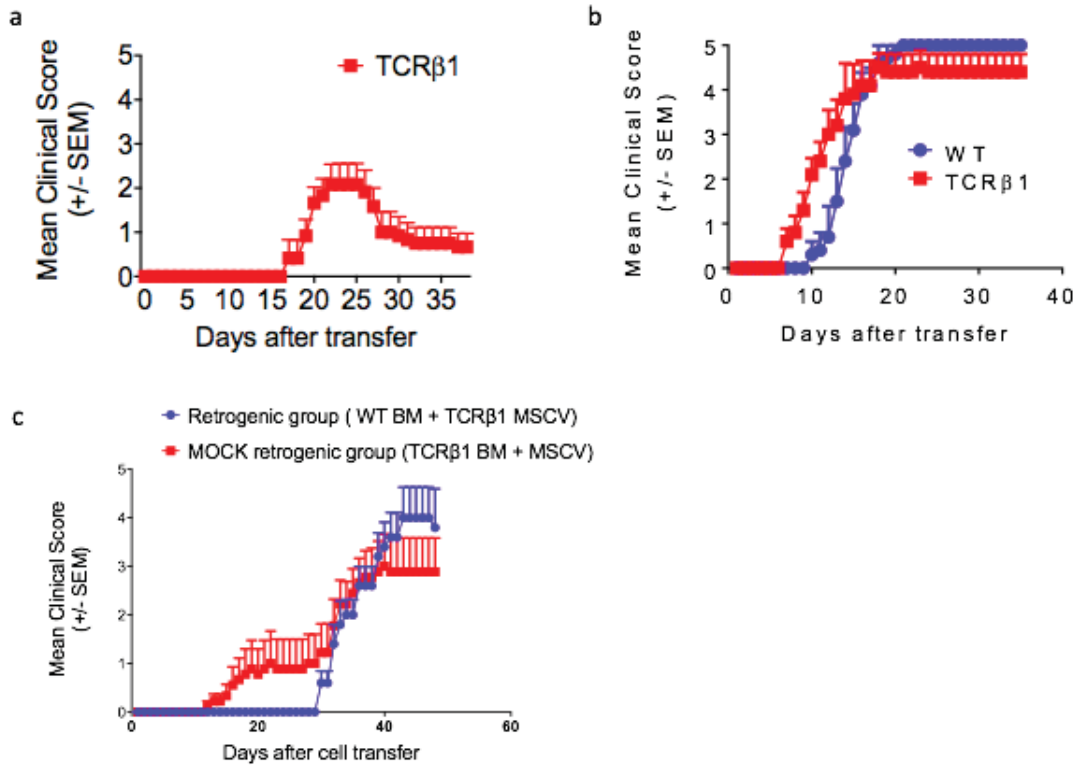


Figure 3-4. TCRβ1 transgenic T cells are not intrinsically non-pathogenic
 (a) Adoptive transfer of bone marrow cells from TCRβ1 transgenic mice or (b) Activated CD4⁺ T cells from TCRβ1 transgenic and WT mice into TCRβ^{-/-} recipients. (c) Clinical scoring of retrogenic and mock retrogenic TCRβ1 mice.

Table 3-1. Summary of adoptive EAE transfer experiment

Measurements	WT group		TCRβ1 group	
	Mean	SEM	Mean	SEM
Day of disease onset	13.0	0.8	7.8	0.5
Area under the curve	103.0	3.3	112.6	5.8
Mortality	5/5	N/A	3/5	N/A
Disease incidence	5/5	N/A	5/5	N/A
Maximum peak disease score	5.0	0.0	4.7	0.2

Day of disease onset, integrated disease score (area under the curve [AUC]), mortality rate, disease incidence, and maximum peak disease score are listed for adoptive EAE transfer experiment.

Table 3-2. Summary of making retrogenic and mock retrogenic mice experiment

Measurements	Retrogenic group		Mock retrogenic group	
	Mean	SEM	Mean	SEM
Day of disease onset	31.2	0.8	26.5	3.1
Area under the curve	51.9	7.0	59.8	15.8
Mortality	3/5	N/A	4/9	N/A
Disease incidence	5/5	N/A	8/9	N/A
Maximum peak disease score	4.0	0.6	3.9	0.5

Day of disease onset, integrated disease score (area under the curve [AUC]), mortality rate, disease incidence, and maximum peak disease score are listed for adoptive EAE transfer experiment.

neutralizing antibody treatment showed that EAE was exacerbated [25-27]. These results suggested that IFN- γ may also play a protective role in EAE. More recently the important role of Th17 cells and IL-17 in EAE has been revealed [28-30]. Cells that co-express IL-17 and IFN- γ have also been reported to be highly pathogenic in EAE [31, 32]. If TCR β 1 T cells do not differentiate into these cytokine-producing encephalitogenic cells (Th1, Th17 or Th1/Th17), TCR β 1 transgenic mice may be resistant to EAE.

We then determined if TCR β 1 transgenic T cells could differentiate into cytokine-producing cells. Because there are very few WT cells that are responsive to MOG, 2D2 transgenic T cells were also analyzed as positive control for MOG-responsive T cells. 2D2 transgenic mice [20] develop EAE disease when immunized with MOG peptide, contrasting with the TCR β 1 transgenic mice. Splenocytes from immunized TCR β 1 transgenic mice were harvested and stimulated with MOG peptide *in vitro*, and cytokine production was characterized by Multiplex ELISA. The secreted cytokine analysis shows that TCR β 1 transgenic T cells produced more IFN- γ and IL-17 than WT T cells, however these levels were not significantly different from those seen with 2D2 transgenic T cells, which are fully pathogenic (**Figure 3-5**). TCR β 1 and 2D2 T cells both expressed higher levels of IL-10 than WT. Interestingly, higher levels of both IL-2 and IL-4 were observed in cultures with 2D2 T cells, relative to TCR β 1 transgenic T cells. Overall, this data indicates that TCR β 1 T cells are capable of producing the two cytokines critical for EAE development, IFN- γ and IL-17, upon MOG stimulation.

TCR β 1 Transgenic T Cells Express High Levels of Exhaustion Markers

Activated T cells are regulated by costimulatory pathways, and negative signals may suppress autoimmune T cell function and pathogenicity [33, 34]. For example, the programmed death-1 (PD-1) molecule is found on activated CD4⁺ and CD8⁺ T cells, and ligation of the PD-1 receptor leads to diminished proliferation and IL-2 production, and limits inflammatory disease in EAE [35]. Besides PD-1, exhausted T cells also express other cell surface inhibitory molecules, such as lymphocyte activation gene 3 protein (LAG3) and CTLA4. Co-expression of these inhibitory surface markers is an indication of exhaustion. The expression level of these inhibitory surface molecules is positively correlated with the severity of exhaustion [33]. It is possible that activated T cells in TCR β 1 transgenic mice exhibit a higher expression level of these related exhaustion markers. Early exhaustion of MOG-reactive T cells in TCR β 1 transgenic mice may leave them unable to promote EAE.

We harvested splenocytes from immunized WT.TCR β 1transgenic mice and 2D2 mice, labeled them with cell trace violet, stimulated them with MOG peptide *in vitro*, and determined the expression of exhaustion markers PD-1, Lag3, CTLA4, and KLRG on CD4⁺ MOG-responsive TCR β 1 T cells. Our results show that CD4⁺ MOG-responsive T cells from TCR β 1 transgenic mice have higher PD1 expression, compared with both WT mice and 2D2 MOG-responsive cells (**Figure 3-6**). CD4⁺ MOG-responsive T cells in TCR β 1 transgenic mice also had significantly higher expression of Lag3 compared with

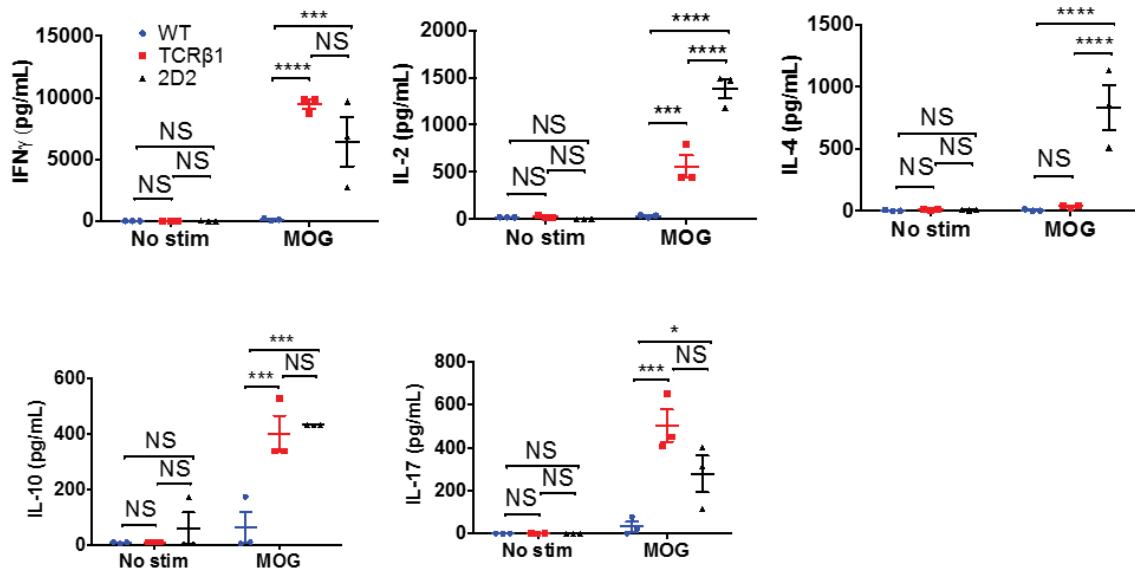


Figure 3-5. Cytokine expression profiles in MOG-stimulated TCRβ1 cultures
 TCRβ1, WT, and 2D2 mice were immunized with MOG₃₅₋₅₅ peptide. Splenocytes from immunized TCRβ1, WT and 2D2 mice were isolated 10 days later and labeled with CellTrace Violet and cultured without or with 100 μg/ml MOG₃₅₋₅₅ and secreted IFN-γ, IL-2, IL-4, IL-10, and IL-17 were measured at 72 hr by Multiplex ELISA assay. Mean value of each individual mouse was plotted. Data are representative of two experiments (*p<0.05, ***p<0.001, ****p<0.0001).

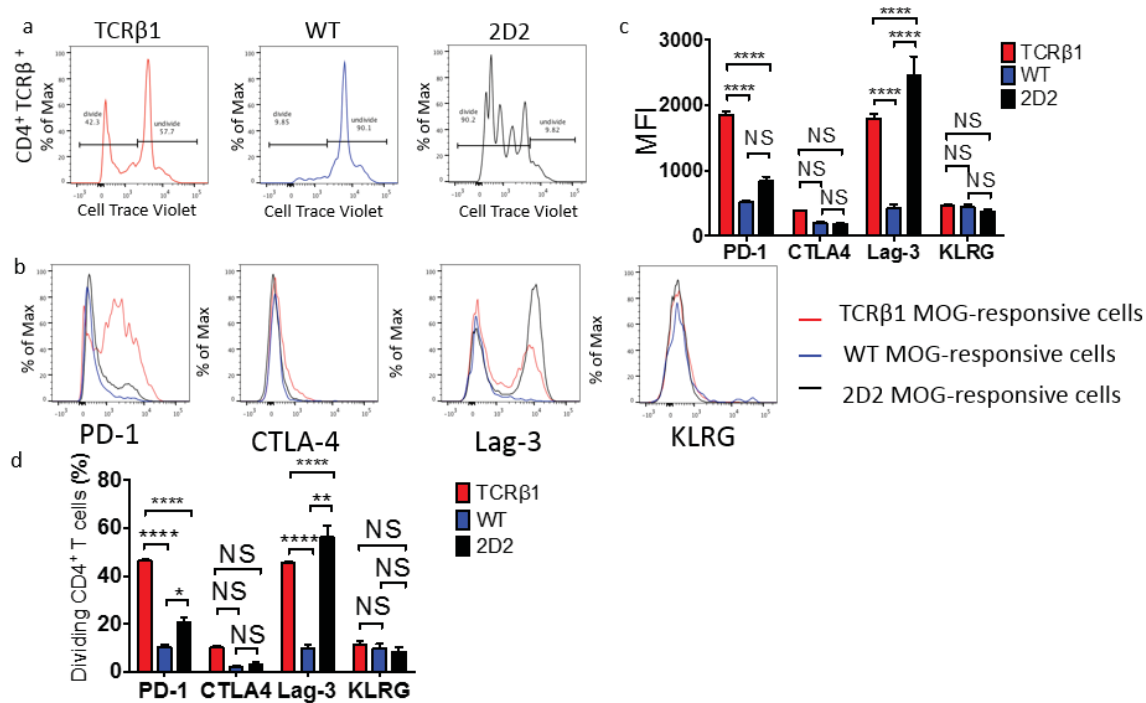


Figure 3-6. TCRβ1 transgenic T cells express higher levels of exhaustion markers TCRβ1, WT mice and 2D2 mice were immunized with MOG₃₅₋₅₅ peptide and splenocytes from immunized TCRβ1 or WT or 2D2 mice were isolated 10 days later. Experiments were done in triplicates for individual mouse, 3 mice per each group. Splenocytes were labeled with CellTrace Violet and stimulated with MOG or αCD3 for 72 hr. Expression level of exhaustion markers (PD-1, CTLA-4, Lag-3, KLRG) on CD4⁺ T cells were determined by flow cytometry. (a) Representative histogram of MOG-responsive CD4⁺ T cells. (b) Representative histograms showing expression of exhaustion markers in MOG-responsive CD4⁺ T cells (left gate in a Red line: TCRβ1 transgenic mice; blue line: WT mice; black line: 2D2 mice). (c) Summary data showing expression of exhaustion markers as MFI. (d) Summary data showing percentages of the MOG-responsive CD4⁺ T cells which is positive for exhaustion marker (PD-1, CTLA-4, Lag-3, KLRG) respectively. (*p<0.05, **p<0.01, ****p<0.0001).

WT mice upon MOG stimulation. However, the expression level of Lag3 on MOG-responsive T cells in TCR β 1 transgenic mice was significantly lower than 2D2 T cells. The flow cytometry data also showed that the expression levels of CTLA4 and KLRG were comparable between TCR β 1 transgenic mice, WT mice and 2D2 mice upon MOG stimulation. But the percentage of CD4⁺ MOG-responsive T cells in TCR β 1 transgenic mice expressing CTLA4 was significantly higher than WT and 2D2 mice. Higher expression level of these exhaustion-associated markers may indicate that TCR β 1 transgenic T cells face early exhaustion.

CHAPTER 4. DISCUSSION

Expression of public TCR β chain, TCR β 1, was sufficient to induce spontaneous and severe EAE in retrogenic mice. However, our results show that TCR β 1 does not lead to the development of spontaneous EAE in a transgenic setting. Furthermore, TCR β 1 mice were not susceptible to induction of active EAE. The comparison of transgenic and retrogenic mice has enabled us to investigate the determinants of this altered pathogenicity of a single TCR β chain, and may provide insights into mechanisms of tolerance and disease induction in EAE.

TCR β 1 transgenic mice had percentages and numbers of CD4⁺ and CD8⁺ T cell comparable to WT C57BL/6 mice in the spleen and lymph node, but Foxp3⁺ regulatory T cells were significantly reduced in both number and percentage in TCR β 1 transgenic mice. Regulatory T cells have a well characterized role in suppression of effector CD4⁺ T cells and controlling cytokine production in MOG-induced EAE models [36, 37]. Our result suggests that resistance to EAE in TCR β 1 transgenic mice is not likely associated with increased suppression by Treg. However, this should be further investigated as these Tregs may have increased suppressive function.

It is also possible that other regulatory or protective cell populations are over-represented or highly active in TCR β 1 transgenic mice. CD8⁺ T cells play a controversial role in EAE, with some studies suggesting they are pathogenic in EAE, and others showing them to play a suppressive role [38, 39]. Given our observation that some CD8⁺ T cells in TCR β 1 mice are MOG-responsive, this should be further investigated. Immunosuppressive Tr1 cells have also been reported to ameliorate EAE in mice and MS in humans [40]. Invariant NKT cells have also been shown to suppress EAE [41]. Regulatory B cells also have been reported to induce regulatory CD4⁺ T cell responses by inducing IL-10 in mice with EAE [42]. Another cell subset which responsible for peripheral tolerance are myeloid derived suppressor cells (MDSCs), which have been shown to be a new negative regulator for EAE [43]. These potential suppressive populations should be further characterized in the future. In addition, to analysis of potentially suppressive populations, there is the fundamental question of whether TCR β 1 cells are simply non-pathogenic, or protective against disease. The active immunization model and adoptive EAE transfer model will be useful in adoptive transfer experiments to address this question.

Thymocytes with high affinity for autoantigen are clonally deleted through negative selection. Potentially autoreactive T cells can escape thymic deletion, and be exported to peripheral lymphoid organs where they may induce autoimmune disease [44-46]. We have shown here that autoreactive TCR β 1 T cells are not deleted during development and are indeed exported to peripheral organs. We confirmed that TCR β 1 conferred responsiveness to self-antigen in TCR β 1 transgenic mice, and that these MOG responsive T cells were further expanded upon immunization. Therefore, this single TCR β chain promotes reactivity to disease-associated autoantigens.

We were able to manipulate TCR β 1 transgenic T cells in three different models to confer pathogenicity and promote EAE in mice. First, transfer of TCR β 1 transgenic bone marrow was able to induce mild EAE in TCR β -deficient recipient mice. Second, splenocytes from TCR β 1 transgenic mice were capable of inducing early and severe EAE in recipient mice, after culture in cytokines known to promote T cell pathogenicity in the MOG EAE model. Third, transduction of TCR β 1 bone marrow with MSCV empty vector induced disease and mimicked the results initially observed in the retrogenic system. These three models all suggest that TCR β 1 transgenic T cells have the potential to become pathogenic, and there is no intrinsic blockade for TCR β 1 to confer pathogenicity in mice. This implies that the inability to induce EAE in these mice is a product of some tolerance generated in transgenic host environment.

We then proposed that TCR β 1 transgenic T cells may not be able to produce cytokines which are essential for pathogenesis. To test this hypothesis, multiplex assay was performed. The multiplex ELISA data showed that TCR β 1 transgenic T cells secreted significantly more IFN- γ compared with WT mice. IFN- γ has been reported to ameliorate EAE by limiting myelin lipid peroxidation [30]. Thus the high concentration of IFN- γ produced by TCR β 1 transgenic T cells may possibly protect the mice from disease development. Future experiments using IFN- γ neutralizing antibodies would be useful to further investigate this.

Another hypothesis is that MOG-reactive T cells in TCR β 1 transgenic mice face early exhaustion. In order to test this, we determined the expression level of exhaustion markers of MOG-reactive T cells. The expression of PD-1 and Lag3 was significantly higher in MOG-reactive T cells in TCR β 1 transgenic mice in comparison with WT mice and 2D2 mice. Though the expression level of CTLA4 was comparable between WT mice and TCR β 1 mice, the percentage of CTLA4⁺ MOG-reactive T cells were significantly higher in TCR β 1 transgenic mice than WT mice. This indicates that MOG-reactive T cells in TCR β 1 transgenic mice may be more prone to early exhaustion upon stimulation with MOG₃₅₋₅₅. It would be useful to treat mice with anti-PD-1 antibodies to resolve the effects of exhaustion on TCR β 1 T cells during EAE.

One other possibility we have not addressed is whether the high affinity of TCR β 1 chain to MOG₃₅₋₅₅ leads to the protection against EAE in mice. We previously isolated CNS-infiltrating TCR β 1⁺ TCRs from WT mice and identified TCR α sequences from them. These were paired with TCR β 1 in retroviral constructs. TCR 342 α showed the highest level of MOG responsiveness *in vitro* [19]. We have generated TCR342 α transgenic mice, and crossed them with TCR β 1 transgenic mice. Similar to TCR β 1 transgenic mice, the TCR β 1/TCR342 α transgenic mice do not develop spontaneous EAE and are resistant to active immunization with MOG₃₅₋₅₅. There are several reasons why the high affinity of TCR β 1 and the TCR β 1/TCR342 α pair may inhibit the development of autoimmunity. T cells with high affinity TCR could lead to early exhaustion or apoptosis. Autoreactive T cells clones bearing high affinity TCRs are held in the periphery by functional inactivation. T cells with high affinity TCRs have also been reported to preferentially upregulate the inhibitory mediators IL-10, CTLA4, TIGIT, and GITR [47-49]. Lower affinity TCR has been reported to be capable of passing negative selection

and may have a greater potential to cause an experimental autoimmune disease [50]. TCR β 1 has high affinity for MOG antigen which could possibly inhibits development of EAE in transgenic mice. Further experiments also need to be done to determine if the high affinity of TCR β 1 for MOG-IA^b leads to co-receptor independent T cell activation, which could explain why we see CD8⁺ transgenic T cells responding to MOG stimulation. Such co-receptor independent cells may be non-pathogenic or have protective functions. Experiments may include defining effector responses of T cells from TCR β 1 to MOG-derived altered peptide ligands (APLs) that have lower TCR affinity *in vitro* and *in vivo*. For instance, immunization of TCR β 1 transgenic mice with lower affinity MOG APLs could be used to determine if priming with lower affinity antigen will induce susceptibility to EAE.

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