Cellular Immunology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Research paper

ESC-derived thymic epithelial cells expressing MOG prevents EAE by central and peripheral tolerance mechanisms

Min Su[a,b], Yujun Lin[a], Cheng Cui[a], Xiaohong Tian[a], Xiuling Lu[c], Zhixu He[b,d,*], Laijun Lai[a,e,*]

[a] Department of Allied Health Sciences, University of Connecticut, Storrs, CT, USA
[b] Tissue Engineering and Stem Cell Research Center, School of Basic Medical Sciences, Guizhou Medical University, Guizhou, China
[c] Department of Pharmaceutical Sciences, University of Connecticut, Storrs, CT, USA
[d] Pediatric Department of the Affiliated Hospital, Guizhou Medical University, Guizhou, China
[e] University of Connecticut Stem Cell Institute, University of Connecticut, Storrs, CT, USA

ARTICLE INFO

Keywords:
Multiple sclerosis
Experimental autoimmune encephalomyelitis
Thymic epithelial cells
Embryonic stem cells
Tolerance induction
T cells

ABSTRACT

Experimental autoimmune encephalomyelitis (EAE) is an animal model for multiple sclerosis (MS), and is induced by immunization with disease-causative self-antigens such as myelin oligodendrocyte glycoprotein (MOG). We have previously reported that transplantation of MOG expressing thymic epithelial progenitors (TEPs) derived from 129S6SvEv Tac mouse embryonic stem cells (mESCs) prevented the development of EAE. In this study, we expand our previous studies to show that transplantation of MOG expressing mESC-TEPs derived from C57BL/6 mice also prevents EAE development. Furthermore, by using a MOG-specific T cell receptor (TCR) transgenic mouse model, we demonstrate that both central and peripheral tolerances are involved in the prevention of EAE induced by MOG expressing mESC-TEPs. Our results suggest that transplantation of human ESC-TEPs expressing MOG may provide an effective approach for the induction of MOG-specific immune tolerance, thereby the prevention and treatment of MS.

1. Introduction

MS is a devastating autoimmune disease of the central nervous system [1–6]. EAE, induced by immunization with disease-causative self-antigens such as MOG, is the most commonly used animal model for human MS [3,7]. MS is currently incurable and remains a major cause of disability in both young and older populations [1,2]. Therefore, development of new strategies to prevent and treat MS is in an urgent need.

We have reported that mESCs (TC-1 line) derived from 129S6SvEv Tac mice can be selectively induced to generate TEPs *in vitro*. When placed *in vivo*, these mESC-derived TEPs differentiate into TECs, reconstitute the normal thymic architecture, and support T cell generation in mice [8,9]. We have also demonstrated that transplantation of the mESC-TEPs expressing MOG prevented the development of EAE in syngeneic 129S6SvEv Tac mice [10]. However, C57BL/6 (B6) mice are the most commonly used animal strain in EAE induction. Recently, we established a new protocol to induce B6 mESCs to differentiate into TEPs *in vitro* that further develop into TECs to support T cell development *in vivo* [11].

In this study, we extend our previous studies to demonstrate that transplantation of B6 MOG/mESC-TEPs into B6 mice also prevents the development of EAE. Furthermore, we also investigate the mechanisms by which transplantation of MOG/mESC-TEPs prevents EAE development by using a MOG-specific TCR transgenic mouse model (2D2 mice). Our data show that transplantation of MOG/mESC-TEPs into the mice results in the deletion of MOG-specific autoreactive T cells and the generation MOG-specific regulatory T cells (Tregs). In addition, we show that deletion of Tregs in the mice partly abrogates the EAE preventive effect induced by MOG/mESC-TEP transplantation. Our results suggest that both central tolerance and peripheral tolerance are involved in the prevention of EAE in MOG/mESC-TEP-transplanted mice.

Abbreviations: EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; MOG, myelin oligodendrocyte glycoprotein; TEPs, thymic epithelial progenitors; TECs, thymic epithelial cells; ESCs, embryonic stem cells; mESCs, mouse embryonic stem cells; TCR, T cell receptor; Tregs, regulatory T cells; B6, C57BL/6J; i.t., intrathymic; AP, alkaline phosphatase; H & E, hematoxylin-eosin; LFB, luxol fast blue; BSI, Bielschowski silver impregnation; DN, double negative; DP, double positive; SP, single positive

* Corresponding authors at: Tissue Engineering and Stem Cell Research Center, Guizhou Medical University, Guiyang 550004, China (Z. He). Department of Allied Health Sciences, University of Connecticut, 1390 Storrs Road, Storrs, CT 06269, USA (L. Lai).
E-mail addresses: hzx@gmc.edu.cn (Z. He), laijun.lai@uconn.edu (L. Lai).

http://dx.doi.org/10.1016/j.cellimm.2017.10.007
Received 11 August 2017; Received in revised form 28 September 2017; Accepted 17 October 2017

2. Materials and methods

2.1. Mice

Four- to 6-week-old female B6 and C57BL/6-Tg (Tcra2D2,Tcrb2D2) 1Kuch/J (2D2 TCR) mice were purchased from Jackson laboratory (Bar Harbor, ME, USA). All animal experiments were performed to minimize animal suffering and discomfort in accordance with NIH guidelines and approved by the University of Connecticut Animal Care and Use Committee.

2.2. Generation of mESCs expressing MOG and induction of the differentiation of the mESCs into TEPs

B6 mESCs were cultured in ESGRO Complete Plus Serum-free Clonal Grade Medium with GSK3β inhibitor supplement (Millipore, Temecula, CA). The mESCs were transfected with a pEF1a-IRES-AcGFP vector with or without MOG gene using Lipofectamine® 2000 Transfection Reagent (Invitrogen, Carlsbad, CA) [10]. It has been reported that the transfection efficiency of this reagent in mESCs was more than 70% [12]. The mESCs were then screened in medium containing 200 μg/ml of G418 (Clontech Laboratories, Inc., Mountain View, CA) to obtain mESC lines that stably expressed the vector with MOG (MOG/mESCs) or without MOG (control mESCs).

The MOG/mESCs and control mESCs were first induced to differentiate into definitive endoderm, and then TEPs in the presence of BMP-4, FGF 7, FGF10, and EGF (BFFE), as well as rFOXN1 and rHOXA3 protein as we previously described [11].

2.3. Immunomagnetic cell separation

Single-cell suspensions from MOG/mESC-derived cells were stained with rat anti-mouse EpCAM1 antibody (eBioscience Inc., San Diego, CA), and followed by anti-rat IgG MicroBeads (Miltenyi Biotec, Auburn, CA). $EpCAM1^+$ cells and $EpCAM1^-$ cells were isolated using a magnetic-activated cell sorter immunomagnetic separation system (Miltenyi Biotec).

2.4. Induction and assessment of EAE

A total of 200 μg of mouse encephalitogenic peptide MOG_{35-55} (GL Biochem, Shanghai, China) in 100 μl of PBS was emulsified in 100 μl of complete Freud's adjuvant (CFA) (Sigma-Aldrich, St Louis, MO, USA) supplemented with 400 μg Mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, MI). Mice were injected subcutaneously with the MOG35-55 in CFA on day 0 and intraperitoneally with 500 ng of purified Bordetella pertussis toxin (Sigma-Aldrich, St. Louis, MO) on days 0 and 2.

The mice were observed for clinical scores based on the following scale: 0, normal; 0.5, partially limp tail; 1, paralyzed tail; 2, loss in coordinated movement, hind limb paresis; 2.5, one hind limb paralyzed; 3, both hind limbs paralyzed; 3.5, hind limbs paralyzed, weakness in forelimbs; 4, forelimbs paralyzed; 5, moribund or dead. As required by animal ethics, mice with a score of 4 and beyond were euthanized.

2.5. Intrathymic (i.t.) injection

Mice were anesthetized and injected with cells in 10–30 μl PBS into the thymus posterior to the upper sternum using a 26–28 gauge needle [13].

2.6. Western blot analysis

Cells or tissues were collected and lysed. Equal amounts of denatured proteins were loaded onto a 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA), electrophoresed and transferred onto a PVDF membrane (Invitrogen, Carlsbad, CA). The membranes were blocked with 5% nonfat milk in TBST (mixture of Tris-Buffered Saline and Tween 20), and then incubated with primary antibody against MOG (Abcam, Cambridge, MA) or actin (Santa Cruz Biotechnology, Inc., Dallas, TX) overnight at 4 °C. After washing with TBST, the membranes were incubated with HRP-conjugated secondary antibody and developed with a SuperSignal West Pico chemiluminescence substrate (Thermo Scientific, Rockford, IL).

2.7. Flow cytometric analysis

The single-cell suspension was stained with fluorochrome-conjugated antibodies directly or indirectly as described [14]. For intracellular staining, the cells were first permeabilized with a BD Cytofix/Cytoperm solution for 20 min at 4 °C. The following antibodies were used: CD4, CD8, CD25, FOXP3, EpCAM1, Vα3.2 and Vβ11 (BioLegend, or BD Biosciences, San Diego, CA), Keratin (K) 5 (Covance, Dallas, TX), and K8 (US Biological, Salem, MA). The samples were analyzed on a FACSCalibur or LSRFortessa X-20 Cell Analyzer (BD Biosciences).

2.8. Histopathology

Spinal cords were removed from mice and fixed with 10% formaldehyde for 24 h. Segments of the tissues were embedded in paraffin, and sections (4–7 μm) were prepared. The sections were stained with hematoxylin-eosin (H & E), Luxol fast blue (LFB) and Bielschowski silver impregnation (BSI) to assess inflammation, demyelination, and axonal damage, respectively. All histological stained sections were semiquantitatively scored blind as described [6,15].

2.9. Immunofluorescence staining

Cultured cells were fixed with 4% paraformaldehyde, permeated with 0.25% Triton X-100, and blocked with 5% BSA. The cultured cells were incubated with primary antibodies. The following primary antibodies were used: anti-SSEA1, Sox2 (Cell Signaling Technology, Inc., Danvers, MA), and MOG (Abcam, Cambridge, MA). After washing, the sections were incubated with fluorochrome-conjugated secondary antibody, counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) and observed under a Nikon A1R Spectral Confocal microscope (Nikon, Kanagawa, Japan).

2.10. Cytokine production detection

Splenocytes were stimulated with MOG *in vitro* for 3 days. Samples of supernatant were collected and measured for cytokine content using ELISA kits for IFNγ, TNFα, and IL-17A (Biolegend) according to the manufacturer's instructions.

2.11. Statistical analysis

P-values were based on the two-sided Student's T test. A confidence level > 95% ($p < .05$) was determined to be statistically significant.

3. Results

3.1. Characterization of B6 mESCs expressing MOG and induction of the mESCs to differentiate into TEPs in vitro

We have demonstrated that transplantation of MOG expressing TEPs from TC-1 mESCs that were derived from 129S6SvEv Tac mouse prevented the development of EAE in syngeneic mice [10]. However, because B6 mice are the most commonly used animal strain in EAE induction, and because 2D2 TCR mice that will be used in the studies of MOG tolerance mechanisms are in the B6 background, we established