Clinical Immunology 173 (2016) 109–116

Contents lists available at ScienceDirect

Clinical Immunology

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

MicroRNA 182 inhibits CD4$^+$CD25$^+$Foxp3$^+$ Treg differentiation in experimental autoimmune encephalomyelitis

Cong Wan [a,b,1], Chang-Yun Ping [a,1], Xiao-Yu Shang [a,f,1], Jiang-Tian Tian [b,e], Si-Han Zhao [a], Lei Li [c], Shao-Hong Fang [b,e], Wei Sun [d], Yan-Feng Zhao [c], Zhao-Ying Li [a], Yan-Wen Xu [a], Li-Li Mu [a], Jing-Hua Wang [a], Qing-Fei Kong [a], Guang-You Wang [a], Hu-Lun Li [a,b], Bo Sun [a,*]

[a] Department of Neurobiology, Harbin Medical University, No.194 XueFu Road, Harbin, Heilongjiang 150081, China
[b] The Key Laboratory of Myocardial Ischemia, Harbin Medical University, Ministry of Education, Heilongjiang Provence 150086, China
[c] Department of Neurology, The Second Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang 150086, China
[d] Department of Neurology, The First Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang 150086, China
[e] Department of Cardiology, The Second Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang 150086, China
[f] Administration Center of Parks and Gardens, Xiamen City, Huli District, China

ARTICLE INFO

Article history:
Received 15 October 2015
Received in revised form 19 September 2016
accepted with revision 20 September 2016
Available online 21 September 2016

Keywords:
MicroRNA 182
Treg cells
Experimental autoimmune encephalomyelitis
Foxo1

ABSTRACT

MicroRNA 182 has been found to have a distinct contribution in the clonal expansion of activated- and functioning of specialized-helper T cells. In this study we knocked down microRNA 182 in vivo and induced experimental autoimmune encephalomyelitis (EAE) to determine the influences of microRNA 182 in the Treg cells functional specialization through Foxo1 dependent pathway in the peripheral lymphoid organs. Down-regulation of microRNA 182 significantly increased the proportions of Foxp3$^+$ T cells in the peripheral lymph nodes and spleen. In vivo study verified a positive correlation between microRNA 182 levels and symptom severity of EAE, and a negative correlation between microRNA 182 and the transcriptional factor Foxp3. In vitro polarization study also confirmed the contribution of Foxo1 in microRNA 182 mediated down-regulation of Foxp3$^+$ T cells. Together, our results provide evidence that during the development of EAE, microRNA 182 repressed Treg cells differentiation through the Foxo1 dependent pathway.

1. Introduction

MicroRNAs have been recognized to play critical roles in post-transcriptional regulation of gene expression [1–3]. In the immune system, microRNAs were highly expressed [4] and participated in autoimmunity as the regulators [3,5]. Notably, the miR-155 was known highly expressed in Treg cells and miR-125a-deficient Treg cells demonstrated reduced immune-regulatory function [6]. MiR-146a was also found expressed in Treg cells and plays an important role in Treg cell-mediated immunological tolerance [7]. microRNA 182 of the microRNA-182-96-183 cluster was found to be essential in regulating CD4$^+$T cells, and promotes activation of Th lymphocytes by IL-2 [1,8]. A recent report further verified that microRNA 182 regulates clonal expression by post-transcriptional regulating clonal expansion [8,9]. In breast cancer cell, microRNA 182 down-regulated the expression of Foxo1 mRNA [8]. MicroRNA 182 targets the 3′ untranslated region (UTR) of Foxo1 transcripts and their expressions were negatively correlated in Th cells [10]. However, participation of microRNA 182 directed regulation of Foxo1 in autoimmune disorders such as MS/EAE, still remains to be established.

In 2009 [11,12], studies provided new sight into the role of the forkhead box (Fox) transcription factor in various cellular processes. In mammals, members of the Foxo subfamily include Foxo1, Foxo3, Foxo4, and Foxo6 [13], which participate in regulating processes such as cell cycle progression, proliferation, energy metabolism, differentiation, apoptosis, and stress resistance [13–15]. In the immune system, Foxo1 regulates T cell and B cell mediated immune responses [16,17]. Recently, researchers have demonstrated that Foxo1 is essential for the induction of Foxp3 expression in Treg cells [18,19]. In addition, Foxo1 can inhibit T-bet expression and differentiation of effector CD4$^+$T cells – Th1 and Th17 indirectly [20].

In this study, we observed an elevated level of microRNA 182 and decreased Foxo1 at acute phase of Experimental Autoimmune Encephalomyelitis (EAE). Then we demonstrated that overall knockdown of microRNA 182 in C57BL/6 mice was accompanied by increased regulatory T cell-polarizations in peripheral lymphoid organs after myelin

* Corresponding author at: Department of Neurobiology, Harbin Medical University, Harbin, Heilongjiang 150086, China.
E-mail addresses: lihulun@aliyun.com (H.-L. Li), sunbo@hrbmu.edu.cn (B. Sun).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.clim.2016.09.008
1521-6616/

oligodendrocytic glycoprotein35–55 (MOG_{35-55}) immunization. In addition, our in vitro experiments based on the $CD4^+$T cells, revealed the opposing effects of microRNA 182 and its putative target Foxo1, abetting that microRNA 182 and Foxo1 act antagonistically in the specialization of regulatory T cells. Finally, the proliferation experiment of $CD4^+$T cells from C57BL/6 mice confirmed that the microRNA 182 inhibited Treg cell specialization through Foxo1 dependent pathway.

2. Material and methods

2.1. Animals

Female C57BL/6 mice, 6–8 weeks old, were purchased from Peking Vital River Laboratory Animal Ltd. (Beijing, China). MicroRNA 182 transgenic mice constructions were cooperated with The Key Laboratory of Myocardial Ischemia (Chinese Ministry of Education, Harbin, Heilongjiang, China). All mice were kept in specific pathogen-free environments. Genetic engineering of microRNA 182 was achieved by infection of zygotes by lentiviral vectors.

The specific process is as follows:

Female C57BL/6 mice, 4–6 weeks old, were purchased from Peking Vital River Laboratory Animal Ltd.; each mice was injected pregnant mare's serum gonadotropin (PMSG) 5 IU; 48 h later, each mice was injected human chorionic gonadotropin (hCG) 5 IU, then put together with mature male mice (>2 months old) into the same cage immediately; female mice with visible vaginal plug were selected for breeding, and zygotes were collected surgically. (SF. 4A-a). Harvested zygotes were maintained in M2 culture medium; after cleaning these zygotes with the hyaluronic acid enzyme solution, cleaned zygotes were then maintained in M16 culture medium. Indicated lentiviral vectors were injected into the zygotes (SF. 4A-b&c), and transplanted into the fallopian tube of surrogate mother mice (Female C57BL/6 mice, 6–8 weeks old, post-mating with male mice undergone vasectomy). Surrogate mother mice gave birth to the F-0 generation “transgenic mice” (SF. 4A-d&e), after detecting and screening by fluorescent lamp (SF. 4A-f&g) and qPCR analysis (SF. 4B), we could acquire the transgenic mice. Animals were inbred for >3 generations. Successful construction was identified by significantly lower expression of microRNA 182 compared with the WT mice in qPCR genotyping (SF. 4B).

The following plasmids were engineered to lentiviruses for experiments in this study: mmu-miR-182-inhibitor (microRNA 182-inhibitor) (CGGUGUGAGUUCUACCAUUGCCAAA), and the negative control (UUCUCCGAACGUGUCACGUTT; ACGUGACACGUUCGGAGAATT) (synthesized by Shanghai GenePharma Co., Ltd.).

2.2. Induction and clinical evaluation of EAE

Mice were immunized subcutaneously with 200 μg MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK) emulsified in complete Freund's adjuvant (Sigma) supplemented with heat-inactivated Mycobacterium tuberculosis H37 RA (Difco Laboratories, Detroit, MI). 50 μl of the emulsion were injected in each axillary fossa. 200 ng of pertussis toxin (LIST BIOLOGICAL LABORATORIES, INC.) were administered intravenously at the time of immunization and 48 h later.

Clinical score was assessed daily according to the following scoring criteria: 0, no detectable signs of EAE; 1, limp tail; 2, hind limb weakness or impaired gait; 3, complete hind limb paralysis; 4, paralysis of fore and hind limbs; and 5, moribund or death. 0.5 was added to the lower score when clinical signs were intermediate between two grades of disease.

2.3. Preparation of mononuclear cells from lymph nodes and spleen

Mononuclear cells (MNC) were obtained from the axillary lymph nodes, spleen and thymus isolated from both EAE and CFA groups on 7, 14 and 21 days post immunization. Cells from lymph nodes and thymus were washed in PBS, then filtered through a 40 μm cell strainer (BD Biosciences), and centrifuged at 700*g* for 10 min at 4 °C, then cultured in DMEM medium (Hyclone, Logan, UT) containing 10% fetal bovine serum (Gibco, Paisley, UK), and 1% penicillin–streptomycin (Gibco). Red blood cells from spleen were lysed in ACK lysis buffer for 5 min at room temperature, centrifuged at 200*g* for 10 min and then followed the same steps as lymph nodes.

2.4. $CD4^+$ T cell purification and sorting

$CD4^+$ T cell from lymph nodes, spleen and thymus of mice were isolated using the MagCellect™ Mouse $CD4^+$T Cell Isolation Kit (R&D systems) according to manufacturer's instruction.

2.5. $CD4^+CD25^+$ Regulatory T cell purification and sorting

$CD4^+CD25^+$ Regulatory T Cell from lymph nodes, spleen and thymus of mice were isolated using the MagCellect™ Mouse $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (R&D systems) according to manufacturer's instruction.

2.6. Flow cytometry

EAE and CFA mice were sacrificed on 7, 14 and 21 days post immunization. Lymph node, spleen and thymus MNC were harvested and prepared as described above. FACSCalibur™ System (BD Biosciences) was used for profiling of $CD4^+$T-cell subsets. The following antibodies were used for cell surface staining: PERCP-anti-CD4 (BD), FITC-anti-CD4 (BD), PE-anti-CD25 (eBioscience).

For intracellular cytokine staining, mononuclear cells were stimulated with PMA and ionomycin (Sigma) in the presence of Brefeldin A (GolgiStop; BD) for 4 h before staining. After incubated with surface marker, samples were treated with fixation/permeabilization buffer (eBioscience) and then stained with PE-anti–IFN-γ (eBioscience), PE-anti–IL-17 (eBioscience) and PE-anti-IL-4 (eBioscience).

Intranuclear staining for APC-anti-Foxp3 was performed with the mouse regulatory T cell staining kit (eBioscience) according to the manufacturer's instructions. Flow cytometry was performed with FACSCalibur™ (BD Biosciences) and data were analyzed using FlowJo software (Tree Star).

2.7. Quantitative PCR

Total RNA from sorted $CD4^+$T cells and $CD4^+CD25^+$ Regulatory T Cell were extracted with TRIzol reagent RnaEx (GENEray) following the manufacturer's protocol. After microRNA was reverse transcribed with the All-in-One™ miRNA First-Strand cDNA Synthesis Kit (GeneCopoeia, Inc.) and mRNA with the M-MLV Reverse Transcriptase (Invitrogen), TransStart® Top Green qPCR SuperMix and CFX96TM Real-Time System (C1000 Touch Thermal Cycler; BIORAD) were used to assay the microRNA 182, Foxo1and Foxp3 expression using sequence specific primers. Primers of microRNA 182 and Foxo1 were purchased from GENECOPIEA, and Foxp3 were synthesized by Invitrogen (sequences available upon request).

MiRNA was normalized to U6 (mouse), and other mRNAs were normalized to β-actin for qPCR analysis.

Fold difference was determined by ddCq method.

2.8. $CD4^+$T cells transduction in vitro

$CD4^+$T cells were isolated from lymph node of EAE mice on 7 days post immunization, then transfected with LV-mmu-miR-182-5p-inhibition (anti-LV-microRNA 182) (Viral Products were purchased from SHANGHAI GENECHEM Co., Ltd.) separately according to the manufacturer's protocol. Under the same condition, anti-LV-microRNA 182-NC was used as the negative control. All cells were treated with MOG_{35-}