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Interleukin 2 Ameliorates Autoimmune Neuroinflammation by Modulating the Balance of T Helper 17 Cells and Regulatory T Cells in Mouse

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Abstract. ***Objective***. Multiple sclerosis (MS) is a progressive autoimmune-mediated inflammation of the central nervous system (CNS), and experimental autoimmune encephalomyelitis (EAE) is a suitable model to study the pathogenesis of MS. IL-2 has been considered as both a T cell growth factor and an anti-inflammatory cytokine. In the present study, we investigated the effects of a low dose IL-2 treatment on mouse EAE therapy. ***Method.*** The expression of IL-2 and IL-2 receptor were predicted using public microarray data and verified by real-time PCR and ELISA in mouse EAE model. Mice were injected with Myelin Oligodendrocyte Glycoprotein (35-55)(MOG_{35-55}) subcutaneously to induce EAE model. IL-2 treatment was initiated during 5 consecutive days from day 15 post MOG_{35-55} immunization. Flow cytometry was applied to investigate the proportions of Th17 and Treg cells. ELISA was used to detect the concentrations of IL-17a, IFNr, IL-10 and TGFb. ***Results***. In this study, we showed that the IL-2 treatment ameliorates the clinical severity of EAE. Flow cytometry results indicated that the therapeutic effect was related to a reduction of Th17 cells and an expansion of Treg cells in the EAE spinal cord. *In vitro* experiments also confirmed the anti-inflammatory effect of IL-2 in EAE-reactivated T cells. ***Conclusion***. Low-dose IL-2 is a potential therapeutic strategy for EAE and MS.

Key words: interleukin 2, experimental autoimmune encephalomyelitis, T helper cell 17, regulatory T cell.

Introduction

Multiple sclerosis (MS) is a progressive autoimmune-mediated inflammation of the central nervous system (CNS) of young adults, affecting approximately 2.3 million people worldwide [1]. Its pathological feature is demyelination, which ultimately leads to neurological impairment. The underlying cause of MS is still unknown, but is believed to be related to virus infection, low vitamin D, smoking and genetic susceptibility [2]. Discovering the detailed mechanism and developing new therapies based on new discoveries could be a major approach to help individuals afflicted with MS. Myelin Oligodendrocyte Glycoprotein (35-55)(MOG_{35-55}) induced experimental autoimmune encephalomyelitis (EAE) is a suitable model to study the pathogenesis of MS, which reflects important aspects of MS and is wildly used throughout the world [3]. In mouse EAE models, CD4+ T cells infiltrate the CNS and start an inflammatory cascade by secreting cytokines and chemokines.

Th17 cells are a subset of CD4 T cells secreting interleukin-17 (IL-17) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which are involved in the pathogenesis of many autoimmune diseases [4-6]. It is now apparent that Th17 cells mediate host defense and play a crucial role in the pathogenesis of EAE[7]. In the pathogenesis of CNS autoimmune inflammation, immunization with myelin antigen MOG_{35-55} induces proliferation and transfer of Th17 cells [8]. However, Treg cells produce anti-inflammatory cytokines, IL-10 and TGF-β, to maintain immune homeostasis and ameliorate autoimmune diseases [9]. The balance between Treg cells and Th17 cells is impaired in MS [9]. Thus, several new studies focused on the rebalance of Th17/Treg ratio in EAE mice [10,11].

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Interleukin-2 (IL-2) is a typical four-α helix type I cytokine belonging to γc family cytokines [12]. Early studies shown that IL-2 is a T cell growth factor secreted by antigen-activated T cells which is crucial to T cell proliferation and the generation of memory T cells [13]. Thus, the administration of high doses of IL-2 is applied to stimulate antitumor T cell responses in patients with cancer [14]. High-dose IL-2 can induce deleterious side effects. It is now well accepted that low-dose IL-2 treatment preferentially activates Treg cells in SLE, graft-versus-host disease and type I diabetes [15-17]. IL-2 is now known to own a wide range of influence on immune balance. However, the effect of IL-2 on MS treatment remains elusive.

In this study, we evaluated the low expression of IL-2 and high expression of IL-2 receptor in the mouse EAE model. Then, we showed that the IL-2 treatment ameliorates the clinical severity of EAE. Flow cytometry results indicated that the therapeutic effect was related to a reduction of Th17 cells and an expansion of Treg cells in the EAE spinal cord. *In vitro* experiments also confirmed the anti-inflammatory effect of IL-2 in EAE-reactivated T cells.

Materials and Methods

Microarray Data. Microarray data are attained in GEO DataSets under accession no. GSE38010. RNA expression was analyzed using Human Genome U133 Plus 2.0 arrays (Affymetrix) with R software (version 3.5.1).

EAE induction and IL-2 treatment. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Jilin University. 7-8-week-old C57/B66 mice were housed under pathogen-free conditions and maintained in compliance with the guidelines of the Department of Laboratory Animals. EAE was induced in C57/BL6 mice using $MOG_{35–55}$ (R&D Systems) emulsified in Complete Freund's Adjuvant (InvivoGen). Mice were injected subcutaneously at 2 side using MOG_{35-55}. 50,000 IU/injection IL-2 treatment was initiated during 5 consecutive days from day 15 post immunization. Mice were monitored every 2 days as follow: 0, no clinical signs; 1, limp tail; 2, limp tail and weakness of hind legs; 3, limp tail and complete paralysis of hind legs; 4, limp tail, complete hind leg and partial front leg paralysis; 5, moribund and dead.

RNA extraction and Real-time PCR. Spinal cords were isolated from EAE mice on day 28 after immunization. Total RNA was isolated from spinal cord using Trizol (Invitrogen). RNA was re-transcribed to cDNA with PrimeScript RT reagent Kit Perfect Real Time kit (TAKARA) according to manufacturer's protocol. For quantitative real-time PCR, cDNA was subjected to real-time PCR using SYBR Green Mixture purchased from Thermo Fisher Scientific on an ABI prism 7500 instrument (Applied Biosystems). The sequences of real-time PCR primers are as follows: IL-2, forward primer: 5'-CAGCTCGTGTATCTCTGACGC-3'; reverse primer, 5'- CCCAGCAGTCAACTTTATCTTCC-3'; IL-2rg, forward primer: 5'-TACCAGACATTTGTTGTCCAGC-3'; reverse primer, 5'- GCCCGTGGGATCACAAGATT-3'; IL-2rb, forward primer: 5'- CCAATGTCTCTTGCATGTGGA -3'; reverse primer, 5'- CCCGAGGATCAGGTTGCAG -3'; The data were analyzed using the comparative cycle threshold method to obtain relative quantitation values.

Isolation of spinal cord lymphocytes and splenocytes. Spinal cords and spleens were isolated from EAE mice on day 28 after immunization. A single-cell suspension was achieved by pressing the spleen or spinal cord with 1 mL syringe piston and wash the tissue through a 70 μm nylon cell strainer (BD Biosciences). The single cell suspension resuspended in red blood cell (RBC) lysis buffer to acquire splenocytes. Splenocytes were cultured in RPMI 1640 medium (Corning) supplemented with 10% FBS, penicillin and streptomycin (Corning).

Enzyme-linked immunosorbent assay (ELISA). To identify the IL-2 induced cytokines secretion in splenocytes, we incubate isolated splenocytes of EAE mice, 10 ug/ml MOG_{35-55} and 0-50 ng/ml murine IL-2 for 7 days. The supernatant was collected for ELISA assay. The concentration of IL-2, IL17, IFNr, IL-10, TGF-β were detected by Ready-Set-Go ELISA kit (Thermo) according to the manufacturer's protocol.

Flow cytometry. Cells were stimulated with PMA and ionomycin for 4 hours before flow cytometry detection. For surface protein detection, 1×10^6 suspended cells were incubated with anti-mouse CD 3 (17A2) and CD4(GK1.5) antibody for 15 min. For intracellular IL17a and Foxp3 detection, cells were permeabilized with Fixation Buffer and intracellular staining Perm Wash Buffer and stained with anti-mouse IL17a (TC11-18H10.1) and Foxp3 (MF-14) antibody according to manufacturer's protocol. All flow cytometry reagents were purchased from BioLegend company.

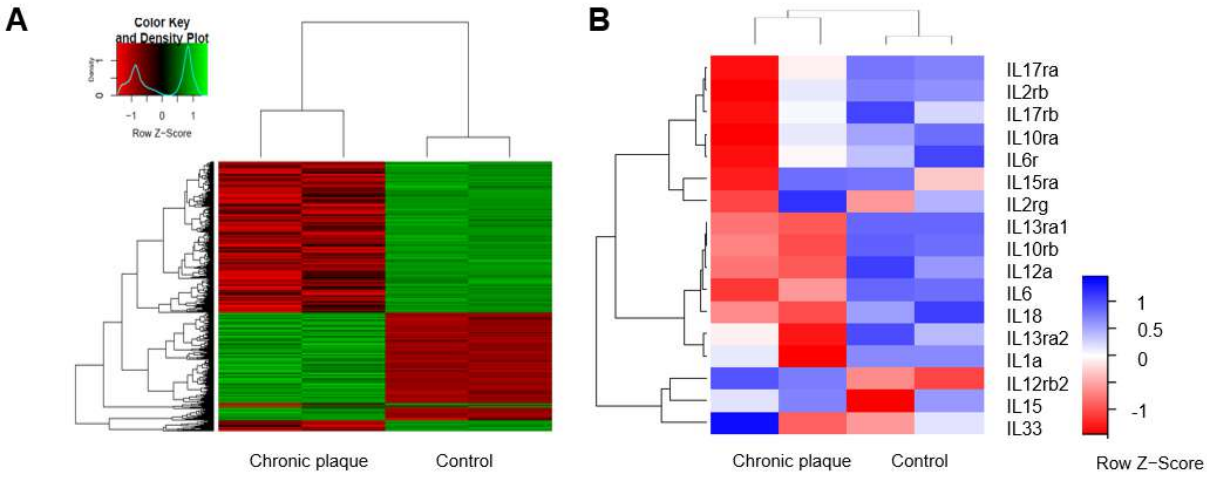

Figure 1. *Identification and comparison of MS patient transcriptomic landscapes.* ***(A)*** *Global transcriptomic expression levels of microarray analysis of MS chronic plaque and normal tissue.* ***(B)*** *Comparative differential RNA expression of interleukin related gene in MS chronic plaque and normal tissue.*

Statistical analysis. Levels of significance for comparison between two groups were determined by Student t test. A p value <0.05 was considered statistically significant. All error bars represent SEM.

Results

mRNA expression profiles of human MS lesions. We analyzed the mRNA expression dataset of MS brain tissue from May H. Han and colleagues' previously published work [18]. Tissues containing chronic plaque and control were analyzed. Microarray analysis identified 6601 RNA targets. The data showed significant differences in mRNA expression between chronic plaque and control tissue **(Figure 1A)**. Furthermore, we analyzed the interleukin related differential gene expression in two kinds of tissues. The data showed more interleukin related differential genes including pro-inflammatory and anti-inflammatory cytokines expressed in chronic plaques **(Figure 1B)**.

IL-2 and IL-2 receptor expression in EAE mice. To identify the mRNA expression shown in microarray data of MS, we detected the mRNA expression of IL2, IL2rb and IL2rg in spinal tissue of EAE mice using real-time PCR. As shown in **Figure 2A**, the IL-2 expression has been significantly downregulated in EAE mouse tissues. While, the IL-2 receptor expressions vary between IL2rb and IL2rg. RNA expression of IL2rb is similar between normal and EAE tissues. IL2rg expression in EAE tissue is significantly higher than normal tissue **(Figure 2B)**. Moreover, the ELISA data also indicated the lower concentrations of IL-2 in serum of EAE mice **(Figure 2C)**. These data identified the differential expression of IL2 and IL2 receptor in the pathology of EAE.

IL-2 treatment attenuates the clinical symptoms in EAE mice. To study the function of IL-2, we injected 50,000 IU/injection IL-2 or PBS into mice from day 15 after MOG_{35-55} immunization. Over the 50 days after immunization, clinical assessment of EAE demonstrated that IL-2 treatment had decreased mean disease severity scores compared with PBS control **(Figure 3A)**. This result suggests that IL-2 treatment reduced disease progression in EAE mice.

IL-2 treatment suppresses Th17 cell differentiation and promotes the generation of Foxp3+ Treg cells during EAE. To further clarify the impact of IL-2 treatment on the pathogenesis of EAE, we performed flow cytometry of spinal cord cells to identify the proportions of CD4 T cell subsets. The results indicated that the proportion of IL17+ Th17 cells was significantly decreased in IL-2 treated mice compared to PBS control **(Figure 3B)**.

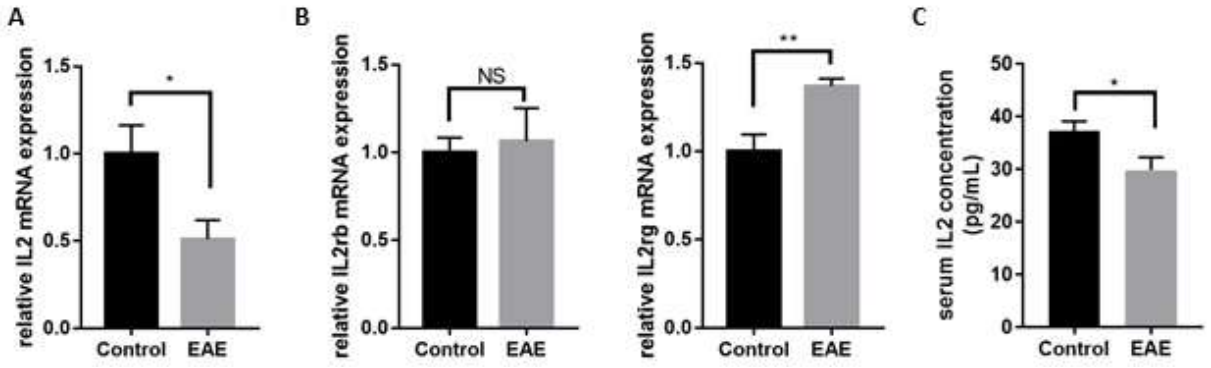

Figure 2. *IL-2 and IL-2 receptor expression in mouse EAE model.* ***(A)*** *IL2 expression of spinal cord of EAE mouse was detected by real-time PCR.* ***(B)*** *IL2rb and IL2rg expression of spinal cord of EAE mouse was detected by real-time PCR.* ***(C)*** *IL-2 concentration in serum of EAE mice was detected by ELISA. *: P<0.05, **: P< .01.*

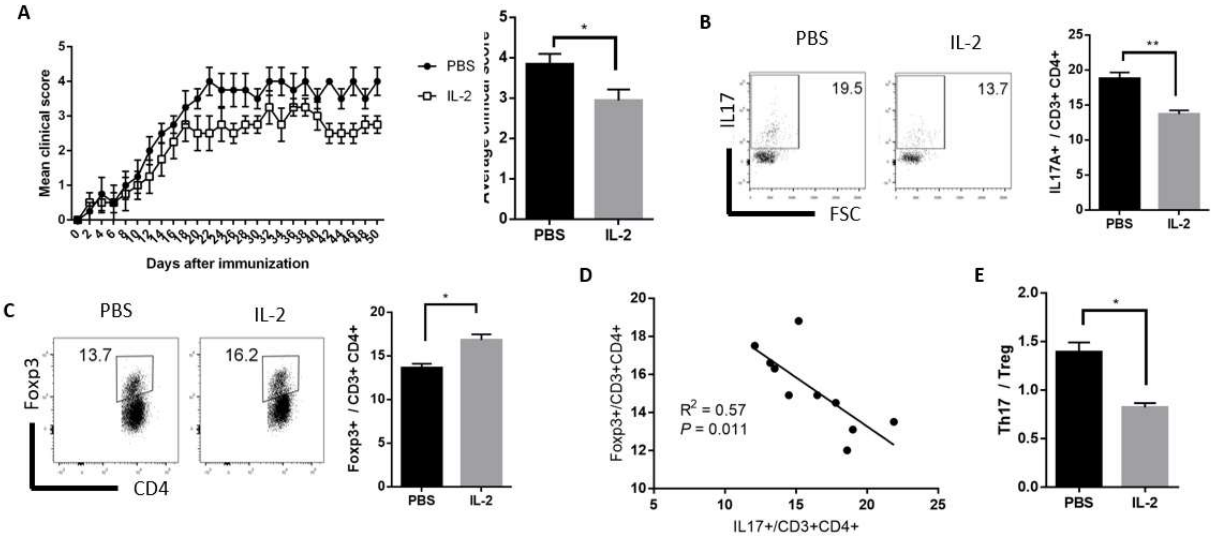

Figure 3. *IL-2 treatment results in ameliorated clinical score, impaired Th17 cell and enhanced Treg differentiation during EAE.* ***(A)*** *Mean clinical scores ± SEM of PBS or IL-2 treated mice immunized with myelin peptide MOG_{35-55} and CFA. At day 22, mononuclear cells were enriched from the spinal cords and counted, and the proportions of* ***(B)*** *Th17 and* ***(C)*** *Treg cells of EAE mouse spinal cord were detected with flow cytometry.* ***(D)*** *Line correlation between Th17 and Treg cells was analyzed with Pearson correlation coefficient.* ***(E)*** *The ratios of Th17/Treg cells were analyzed with the flow cytometry data. *: P<0.05, **: P<0.01.*

Furthermore, the proportion of Foxp3+ Treg cell was significantly increased in IL-2 treated mice **(Figure 3C)**. To confirm the relationship between Th17 and Treg cells, we analyzed the line correlation using GraphPad with Pearson correlation coefficients. As shown in **Figure 3D**, the result indicated a significantly negative correlation between Th17 and Treg cells. Similarly, the ratio of Th17/Treg was significantly decreased after IL-2 treatment **(Figure 3E)**. Taken together, these findings indicated that IL-2 treatment rebalanced the Th17/Treg ratio in EAE mice.

IL-2 selectively suppresses Th17 cell differentiation and promotes the generation of Foxp3+ Tregs in vitro. To investigate the direct impact of IL-2 on CD4 T cells, we isolated the CD4 T cells from spleens of EAE mice. Splenocytes were stimulated with 10 ug/ml MOG_{35-55} and 0-50 ng/ml murine IL-2 for 7 days. The supernatants of splenocytes were isolated for ELISA detection. Our data showed that IL-2 significantly decreased the production of IL-17a and IFNr at the concentrations of 20 and 50 ng/ml **(Figure 4A&B)**. Meanwhile, IL-2 induced significantly higher level of IL10 even in the

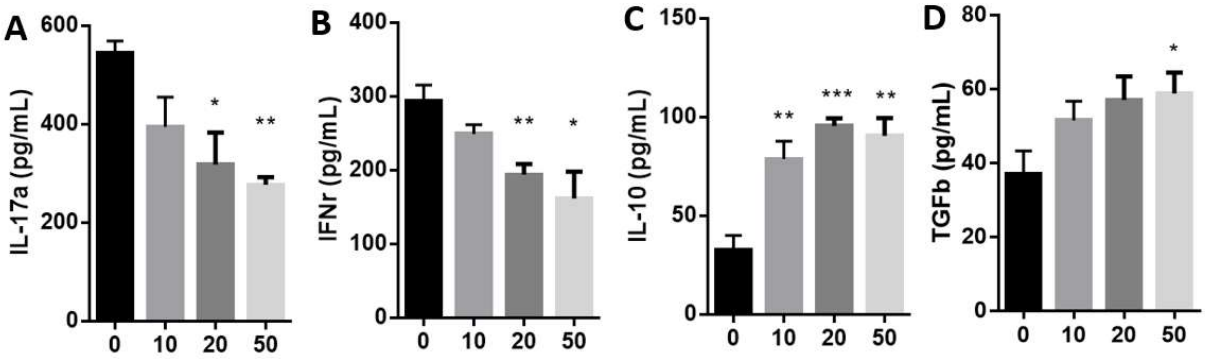

Figure 4. *IL-2 selectively suppresses pro-inflammatory cytokine secretion and promotes the generation of anti-inflammatory cytokine in vitro. EAE mice were sacrificed on day 28 after immunization. Splenocytes were isolated and stimulated with MOG_{35-55} and increasing concentrations of IL-2 (0-50 ng/ml) for 72 h. Pro-inflammatory cytokines IL-17* ***(A)*** *and IFNr* ***(B)*** *were measured by ELISA. Anti-inflammatory cytokine IL-10* ***(C)*** *and TGF-β* ***(D)*** *were measured by ELISA. *: P<0.05, **: P<0.01, ***: P<0.001.*

low concentration (10 ng/ml) **(Figure 4C)**. Furthermore, IL-2 also induced another anti-inflammatory cytokine, TGFb, in MOG_{35-55} induced splenocytes **(Figure 4D)**. Taken together, these data indicated that IL-2 inhibited the production of pro-inflammatory cytokines, IL-17a and INFr, and promoted the secretion of anti-inflammatory cytokines from Treg cells, IL-10 and TGFb.

Discussion

Recent MS treatment has been shown to reduce disease activity and progression in patients. While these disease-modifying agents were considered as the symptomatic treatments [19]. It was reported that immediate side effects of disease-modifying treatment included peripheral neuropathy, sinusitis and dysgeusia [20]. Further studies investigating and attenuating the side effects of disease-modifying treatment are needed.

The Th1 and Th2 cytokines are a key determinant of susceptibility or resistance in MS [21]. Pro-inflammatory cytokines, INF-r, IL-17 and TNF-a, are essential in the pathogenesis of EAE. In recent years, more and more studies have shown that IL17 secreted by Th17 plays a central role in the progression of EAE [22,23]. IL-17 expression is also increased in MS patients [24]. IL-2 treatment decreased the expression of IL-17 and IFN-r from MOG-activated splenocytes in EAE. Treg cells play a crucial role in the prevention of autoimmunity. Treg cells were recruited into the brain and maintained their suppressive function in the inflamed brain of MS patients [25]. Our study showed that IL-2 treatment increased the frequency of Treg cells and the expression of anti-inflammatory cytokines, IL-10 and TGFb. Thus, these results suggest that the therapeutic effect of IL-2 may alleviate the EAE by modulating the balance of Th17 and Treg cells.

MS therapy is initiated after the onset of the MS symptoms. Therefore, we started IL-2 treatment at day 15 post-immunization when the symptoms appeared. Our investigation demonstrated that the IL-2 treatment reduced the clinical score and the frequency of Th17 cells in EAE. This result suggests IL-2 as a potent therapy for further MS therapy. In some studies, the different doses of IL-2 induce divergent effects in clinical therapy [26-28]. Our results did not show the opposite effect of the relatively high dose IL-2 (50 ng/mL) *in vitro*. Further studies of the high dose IL-2 treatment in EAE therapy *in vivo* are needed.

In summary, this study confirmed the low expression of IL-2 and high expression of IL-2 receptors in mouse EAE model. We then showed that the IL-2 treatment ameliorates the clinical severity of EAE. Flow cytometry results indicated that the therapeutic effect was related to a reduction of Th17 cells and an expansion of Treg cells in the EAE spinal cord. *In vitro* experiments also confirmed the anti-inflammatory effect of IL-2 in EAE-reactivated T cells. Additional studies are needed to disclose the detailed mechanism of the EAE pathology and the therapeutic effect of IL-2 in EAE and other autoimmune diseases.

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