

Mature dendritic cells cause Th17/Treg imbalance by secreting TGF- β 1 and IL-6 in the pathogenesis of experimental autoimmune encephalomyelitis

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Abstract

Multiple sclerosis (MS) is generally acknowledged to be an autoimmune disease, but its etiology remains unknown. The most intensively studied animal model of MS is experimental autoimmune encephalomyelitis (EAE). Dendritic cells (DCs), the professional antigen presenting cells (APCs), have gained increasing attention because they connect innate and adaptive immunity. The aim of this study was to determine the role of mature DCs in the pathogenesis of EAE. It was found that the number of mature DCs in the EAE spleen increased compared to the control group ($p < 0.05$). And there was an imbalance between Th17 (effector) and Treg (regulatory) in EAE. The data showed that mature DCs can regulate the differentiation of Th17 and Treg in EAE. In addition, there was a significant difference in secretion of TGF- β 1 and IL-6 between mature DCs from mice with EAE and controls. The present data suggest that mature DCs cause an imbalance between Th17 and Treg by secreting TGF- β 1 and IL-6 in the pathogenesis of EAE disease. Thus, targeting DC may be an effective strategy for treating MS.

Key words: EAE, Treg, Th17, DCs.

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Introduction

Multiple sclerosis (MS) is a chronic neurodegenerative and autoimmune disease of the central nervous system (CNS) that affects about 2.5 million people worldwide [1]. Multiple sclerosis disabilities are reflected in physical and cognitive impairments, including pain, depression, and bladder dysfunction. Untreated, the disease may progress, significantly interfering with lifestyle and career plans, and ultimately shortening lifespan [2]. Because the etiology of MS is unknown, current therapies are generally immunosuppressive and lack antigen (Ag) specificity; thus, they are only partially effective and their long-term use is associated with considerable side effects [3].

Experimental autoimmune encephalomyelitis (EAE) is an artificially induced demyelination disease of the CNS that resembles MS in its clinical, histopathological, and immunological features [4]. It is induced in susceptible laboratory animals by immunization with proteins from

the CNS, such as myelin proteolipid protein, myelin oligodendrocyte glycoprotein (MOG), or myelin basic protein associated with complete Freund's adjuvant (CFA) [5, 6]. MOG-induced EAE in C57BL/6 mice is amongst the most frequently used mouse models for MS studies [4, 7]. Much of our actual knowledge came from investigations done with EAE in mice.

Previous studies suggest that reactive CD4⁺ T cells play a crucial role in the pathogenesis of MS [8, 9]. Naive CD4⁺ T cells require two signals to successfully differentiate into effector T cells. The first signal determines the specificity of the response through binding of peptide: MHC (major histocompatibility complex) class II (MH-CII) complexes to the T cell receptor (TCR) expressed on CD4⁺ T cells. The second signal is delivered in the form of costimulatory signals and serves to lower the signaling threshold of the TCR required to successfully activate the CD4⁺ T cell [3]. The two signals are supplied by antigen-presenting cells (APC). Dendritic cells (DCs),

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considered the most potent APCs of the immune system, play a crucial role in regulating both innate and adaptive immunity [10, 11]. They are also key players in maintaining immune tolerance: once ablated, autoimmunity ensues, demonstrating the involvement of DCs in maintaining immune tolerance under steady-state conditions [12]. DCs are increasingly being recognized for their potent regulatory ability to limit inflammation, with possible therapeutic applications in the treatment of autoimmune and inflammatory diseases [13-16].

In the present study, we sought to determine the role of mature DCs in the pathogenesis of MS. Using EAE, a widely used animal model of MS, we examined the number of mature DCs, as well as the relative numbers of Th17 (effector) and Treg (regulatory) T-cell subsets, the expression of Foxp3 and Ror- γ t mRNA, in the spleen and brain, IL-17A mRNA in the brain, and test the cytokine profiles at different stages of EAE.

Material and methods

Mice

Eighty female, 10-12-week-old C57BL/6 mice were purchased from the Shanghai SLAC Laboratory Animal Co. Ltd and maintained under pathogen-free conditions. All experiments were performed in accordance with guidelines and with the approval of the Animal Care and Use Committee of Fu Jian Medical University School.

Reagents

MOG₃₅₋₅₅ peptide (MEVGWYRSPFSRVVHLYRNGK) with a purity > 99%, determined by high-performance liquid chromatography (HPLC), was synthesized by Shanghai Sangon. Complete Freund's adjuvant (CFA), 4% paraformaldehyde solution and pertussis toxin were purchased from Sigma Company (USA). Fetal bovine serum (FBS) was obtained from HyClone (USA). High-glucose Dulbecco's modified Eagle medium (DMEM) was purchased from Gibco (USA). Mouse CD4⁺CD25⁺FoxP3⁺Treg, CD4⁺IL-17⁺ Th17, CD80-PE, CD86-PE and CD11c⁺CD83⁺ DC detection kits were obtained from BD Biosciences (USA). Red blood cell lysate was purchased from Tiangen Corporation (China). Anti-IL-6-antibody and anti-TGF- β 1 monoclonal antibody was purchased from R&D System (USA). An enzyme-linked immunosorbent assay (ELISA) detection kit was purchased from Wuhan Boster Company (China). TRIzol reagent was purchased from Invitrogen (USA). SYBRGreen RT-PCR kit was purchased from Takara (Japan).

Induction and clinical evaluation of EAE

EAE was induced by injecting mice ($n = 40$) subcutaneously (into the flanks) with 100 μ l of an emulsion containing 300 μ g of MOG₃₅₋₅₅ peptide supplemented with 100 μ l

of complete CFA. Pertussis toxin (400 ng per mouse) was injected intraperitoneally on days 0 and 2. Mice in the control group ($n = 24$) were injected with an emulsion containing 100 μ l of phosphate-buffered saline (PBS) and 100 μ l of CFA on day 0. Mice in the normal group ($n = 16$) received nothing. Clinical signs of EAE were recorded on a daily basis and graded on a previously described standard scale [17], as follows: 1, limp tail; 2, loss in coordinated movement and hindlimb weakness; 2.5, partial hindlimb paralysis; 3, complete hindlimb paralysis; 3.5, both hindlimb paralysis and forelimb weakness; 4, forelimb paralysis; 5, moribund state. EAE mice were divided into early onset (day +16), peak (day +23) and chronic stage (day +30) based on their clinical symptoms.

Histopathology

At the peak stage (day 27) of EAE, mice were perfused with PBS followed by a 4% paraformaldehyde solution in PBS. The brain and spinal cord were harvested and fixed with 4% paraformaldehyde. The spinal cord and brain were divided into 10 to 12 transverse segments and five coronal slabs, and embedded in paraffin. Eight-micron-thick sections of CNS tissues were prepared for staining with Luxol-fast blue, and 4- μ m-thick sections were prepared for hematoxylin and eosin (H&E) staining.

Flow cytometry analysis

Single-cell suspensions of lymphocytes and DCs were prepared from EAE and control mice spleens by first grinding spleens, then separating cells based on their different suspension and adherent properties. Thereafter, cells were resuspended in PBS and analyzed by flow cytometry using a FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions, and analyzed using Cellquest software (BD Biosciences).

Cell culture

DCs isolated from peak-stage EAE mice spleens (day 27, $n = 8$) and CFA group mice (day 27, $n = 8$) were incubated for 2 days in complete medium (high-glucose DMEM, 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 292 mg/ml glutamine) at 37°C; then, the supernatants were collected and used for ELISA.

Co-culture and neutralization

Lymphocytes from normal mice ($n = 8$) and DCs from peak-stage EAE mice spleens (day 27, $n = 8$) were co-cultured in complete medium for 2 days; then, Th17 and Treg in harvested lymphocytes were quantified using a FACSCalibur flow cytometer on day 2, according to the manufacturer's instructions. Anti-mouse IL-6 (5 μ g/ml)/anti-mouse IL-6 (5 μ g/ml) and anti-TGF- β 1 (10 μ g/ml) monoclonal antibody was added to DCs from peak-stage

EAE mice spleens (day 27, $n = 4$) for two hours and then lymphocytes from normal mice ($n = 4$) were co-cultured in complete medium for 2 days; then, Th17 and Treg in harvested lymphocytes were quantified using a FACSCalibur flow cytometer on day 2, according to the manufacturer's instructions. The population of Th17 and Treg in lymphocytes from normal mice spleens was also analyzed as normal.

ELISA

The concentrations of transforming growth factor β 1 (TGF- β 1) and IL-6 in cell culture supernatants and sera of mice were determined by a sandwich ELISA, according to the manufacturer's instructions.

RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA from splenocyte lymphocyte and brain of two group mice was extracted using TRIzol according to the manufacturer's instructions. cDNA (500 ng) was reverse transcribed using AMV Reverse Transcriptase (TaKaRa, Japan). Quantitative PCR was performed using the SYBR Green I Master kit (TaKaRa, Japan) and a Light-Cycler 480 Detection System, following the manufacturer's recommendations (Roche Applied Science, Meylan, France). Specific primers for Foxp3, Ror- γ t, IL-17A and β -actin (Table 1) were designed using the Primer 3 software. Briefly, 500 ng cDNA were amplified and the analysis of mRNA expression level was performed using the Roche LightCycler480 software 1.5. Expression levels of transcripts were normalized to the housekeeping gene β -actin. For quantification, values were expressed as the relative mRNA level of specific gene expression as obtained using the $2^{-\Delta C_t}$ method.

Statistical analysis

Differences among groups were determined by analysis of variance (ANOVA), and differences between two groups were analyzed by *t*-test using GraphPad Prism 4.0

(GraphPad Software, Inc., San Diego, CA, USA). A *p*-value < 0.05 was considered statistically significant.

Results

Successful establishment of the EAE model

Despite the use of genetically identical mice, the success rate of EAE establishment in MOG-treated mice is not always 100% and can vary from experiment to experiment. The reasons for this variability are unknown [17]. In our study, the incidence rate was about 80%. As shown in Fig. 1 and Table 2, the clinical signs of the EAE group are consistent with those previously reported [17].

A histological analysis of CNS tissue sections from mice treated with MOG and CFA showed typical signs of inflammation (Fig. 2B, C) and demyelination (Fig. 2D), whereas tissue sections from mice treated with CFA only were normal (Fig. 2A).

Profile of mature DCs in EAE

CD11c, which is strongly expressed by DCs, is used as an identifying marker of mouse DCs [18]. Because it is strongly induced during DC maturation, CD83 is a commonly used marker for mature DCs (19). Thus, the CD11c⁺ CD83⁺ cells in the single cell suspension represented mature DCs. The number of mature DCs in the EAE spleen increased compared to the control group ($p < 0.05$), showing the greatest increase at the peak stage and declining in the chronic stage (Fig. 3A). As we know, mature DCs also express high levels of MHC-II molecules, CD80, CD86. We detected cell-surface expression of positive co-stimulatory molecules: CD80 and CD86 in the single cell suspension at the peak stage from the two groups (Fig. 4). The expression of CD80 in the EAE group increased compared to the control group ($p < 0.05$). The expression of CD86 in the EAE group also increased compared to the control group ($p < 0.05$).

Table 1. Primers used for RT-PCR detection

Target gene	Sequence of primers (5'-3')	Tm (°C)	Amplicon size (bp)
Foxp3	F: CATTGGTTTACTCGCATGTTTCG R: TTCATCTACGGTCCACACTGC	62	123
Ror- γ t	F: CTGCAAGACTCATCGACAAGG R: CACATGTTGGCTGCACAGG	62	125
β -actin	F: CATCCGTAAAGACCTCTATGCCAAC R: ATGGAGCCACCGATCCACA	64	125
IL-17A	F: CATCCGTAAAGACCTCTATGCCAAC R: ATGGAGCCACCGATCCACA	64	121

F – forward primer, R – reverse primer

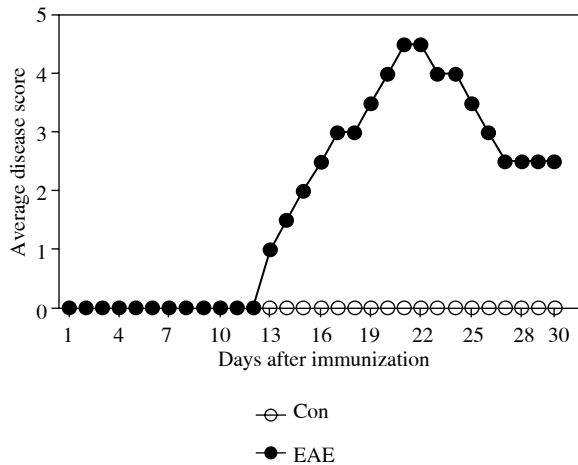


Fig. 1. Mean clinical score was monitored in both EAE and control group mice from day 1 post immunization. EAE, mice were immunized with MOG35–55 peptide; Con, mice treated with CFA only

Imbalance between Th17 and Treg T cells in EAE

T cells were isolated from mice splenocytes and assessed by flow cytometry. The number of Th17 cells increased in the EAE group compared to the control group ($p < 0.05$), showing the greatest increase at the peak stage and decreasing at the chronic stage (Fig. 3B). Treg numbers dramatically decreased in the EAE group compared to the control group at both onset and peak stage ($p < 0.05$), but remained considerably elevated at the chronic stage (Fig. 3C).

Previous studies have shown that the interaction between Treg and Th17 cells are regulated by specific transcription factors Foxp3 and Ror- γ t. This study shows that Foxp3 mRNA expression in spleen of EAE mice dramatically decreased in the early and peak stage of EAE ($p < 0.05$), but increased in the chronic stage of EAE ($p < 0.05$); the Ror- γ t mRNA expression from mice spleen increased in the EAE group compared to the control group ($p < 0.05$), showing the greatest increase at the peak stage and decreasing at the chronic stage (Fig. 5).

Multiple sclerosis as an inflammatory demyelination disease in the CNS is pathologically characterized by multifocal inflammation, demyelination, and neuronal damage [1]. Interleukin 17A, the predominant functional mem-

ber of the IL-17 family, is a pro-inflammatory cytokine produced by Th17 cells. So we detected the expression of Foxp3, IL-17A and Ror- γ t mRNA in the brain. This study shows that Foxp3 mRNA expression in brain of EAE mice dramatically decreased in the early and peak stage of EAE ($p < 0.05$), but increased in the chronic stage of EAE ($p < 0.05$); the IL-17A and Ror- γ t mRNA expression from mice brain increased in the EAE group compared to the control group ($p < 0.05$), showing the greatest increase at the peak stage and decreasing at the chronic stage (Fig. 6).

Cytokines play important roles in immune cell differentiation and polarization into functional subtypes and in directing their biological functions in autoimmunity [20]. So we next assessed secretion of TGF- β 1 and IL-6 – two key cytokines that drive differentiation of CD4⁺ T cells into Treg or Th17 [21] – by ELISA at different stages of EAE (Fig. 7). Interleukin 6 and TGF- β 1 levels in the peripheral blood of the EAE group mice dramatically increased compared to those in the control group ($p < 0.05$). As the disease progressed, Interleukin-6 levels gradually decreased, reaching a concentration at the chronic stage that was not significantly different from that in controls ($p > 0.05$). In contrast, the concentration of TGF- β 1 remained high at the chronic stage in EAE mice compared to the control group ($p < 0.05$).

Mature DCs may cause an imbalance in Th17 and Treg T-cell subsets during the development of EAE

To further determine whether mature DCs directly influence Th17 and Treg differentiation by secreting IL-6 and TGF- β 1 *in vivo*, we co-cultured splenic lymphocytes from healthy mice (Normal group, Nor) and peak-stage splenic DCs from EAE mice (day 27) for 2 days in the presence or absence of anti-IL-6 and anti-TGF- β 1 antibody, and then determined the population of Th17 and Treg in the harvested lymphocytes by flow cytometry (Fig. 8). The number of Th17 in lymphocytes increased substantially after co-culture without any neutralization compared to the normal group ($p < 0.05$), whereas the number of Treg in lymphocytes decreased considerably after co-culture ($p < 0.05$). These results show that mature DCs can induce the differentiation of Th17 and suppress Treg T cells. As shown in Fig. 8, neutralization of IL-6 induced a significant increase in Treg numbers ($p < 0.05$), whereas the number of Th17 showed no change compared

Table 2. Clinical evaluation of EAE (mean \pm SD)

Group	Incidence (%)	Average onset (d)	Mean clinical score	Maximum clinical score	Weight loss (g)
Control	0	0	0	0	-2.10 \pm 0.96
EAE	80	16 \pm 1.20	4.0 \pm 0.6	5.0	3.00 \pm 0.86

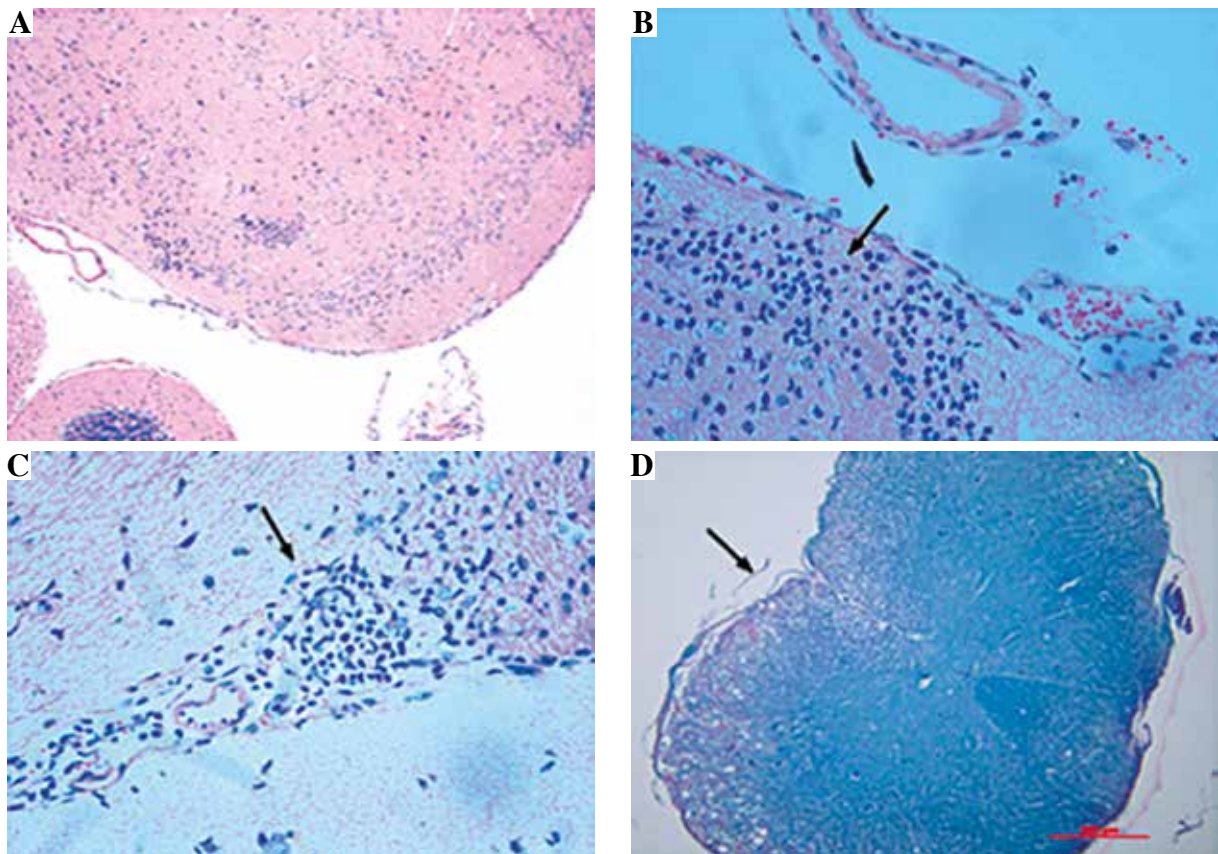


Fig. 2. Spinal cord and brain sections from EAE mice and control (Con) mice. Inflammation and demyelination were assessed by staining sections with H&E and Luxol fast blue. A) Normal tissue sections of brain stained with HE. B, C) H&E staining showing inflammatory cell infiltrates in the white matter of the spinal cord (B) and brain (C). D) Luxol fast blue staining showing demyelination in the spinal cord

to the normal group ($p < 0.05$). Moreover, neutralization of IL-6 and TGF- β 1 did not induce differentiation of Th17 and Treg compared to the normal group ($p < 0.05$). The concentrations of IL-6 and TGF- β 1 in culture supernatants from EAE-derived DCs also increased compared to those from control mice (Fig. 9) ($p < 0.05$).

Discussion

Because DCs are a heterogeneous population of professional APCs they potentially initiate primary immune responses and possess the ability to regulate both innate and adaptive immunity [22, 23]. DCs, which can activate the naive T cells, are the most powerful professional antigen-presenting cells. The differentiation and development of DCs experiences immature and mature stages. Immature DCs (imDCs) are in the peripheral tissues, which are poor in stimulating mixed lymphocyte reaction (MLR) as they express low levels of MHC-II molecules and costimulatory molecules [23]. Due to the dangerous/invasive antigen or inflammatory factors, imDCs switch to the mature

DCs (mDCs). mDCs, expressing high levels of MHC-II molecules, CD80, CD86, and CD83, are ideally situated to meet and initiate effector T cell activation, govern the type of T-cell response, and alter the immune response profile *in vivo* [24, 25]. Recently, large numbers of studies have identified that DCs are involved in the pathogenesis of autoimmune disease [26, 27], we asked whether DCs are involved in the pathogenesis of EAE. In this study, firstly EAE was induced by sensitizing animals with the CNS antigen, MOG, according to Ingunn's method [17]. This animal model is commonly used for MS studies because of histopathological and immunological similarities [28]. Next, we investigated the profile of DCs from EAE, and the results showed that the number of mature DCs increased in the EAE group compared to the control group, showing the greatest increase at the peak stage and declining in the chronic stage. At the peak stage, DCs showed a high expression of co-stimulatory molecules: CD80 and CD86 in the EAE group compared to the control group. These results suggest DCs may be involved in the pathogenesis of EAE.

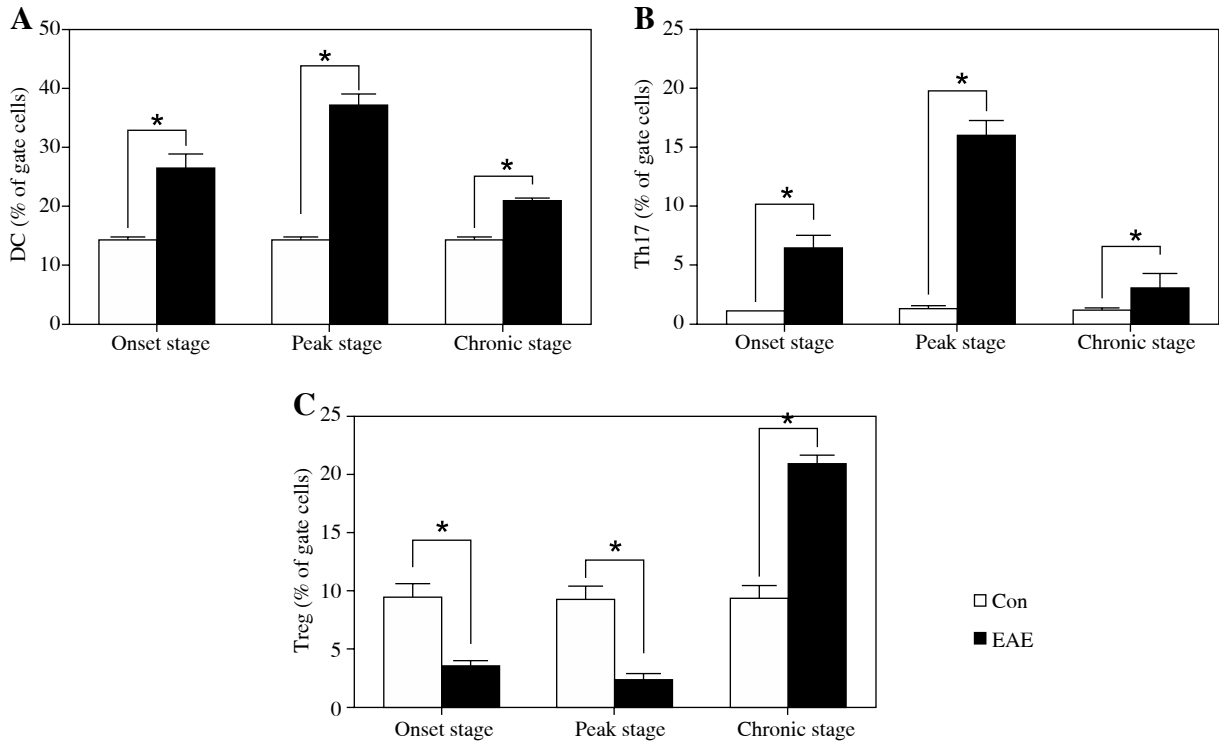


Fig. 3. The percentage of mature DCs, Th17 and Treg T cells in splenocyte, at the onset of EAE symptoms (day +16, onset stage), at the peak of the disease (day +23, peak stage), at the time of EAE stabilization (day +30, chronic stage) in the two groups. Data are expressed as means \pm SEM (* $p < 0.05$ compared with the control group), $n = 8$

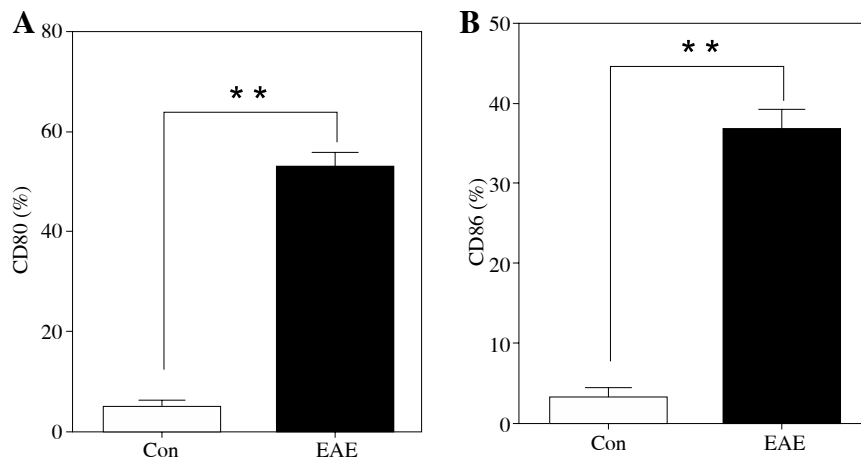


Fig. 4. The percentage of cell-surface expression of positive co-stimulatory molecules: CD80 and CD86 in splenocyte at peak stage in the two groups. Data are expressed as means \pm SEM (** $p < 0.01$ compared with the control group), $n = 8$

A previous study demonstrated that Th17 and Treg play important roles in the development of EAE [29]. Th17, specific expression of Ror- γ t, mediates inflammatory response. Treg, specific expression of Foxp3, maintains cell immune tolerance. In this study, an imbalance between Th17 and Treg was found in EAE. At the onset and peak

stage of EAE, the balance Th17/Treg tilt to Th17, the percentage of Th17 increased, while that of Treg decreased. As the development of the disease, for the weakening of antigen stimulus the percentage of Th17 decreased, while that of Treg, which can reduce inflammatory response, increased. A previous study suggested that DCs can sig-

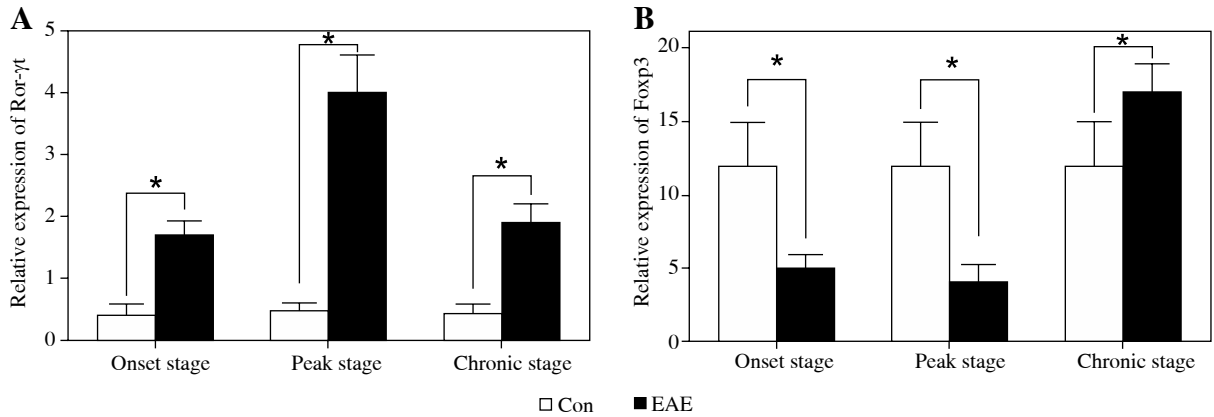


Fig. 5. The expression of Foxp3 and Ror- γ t mRNA in splenocyte at the onset of EAE symptoms (day +16, onset stage), at the peak of the disease (day +23, peak stage), at the time of EAE stabilization (day +30, chronic stage) in the two groups. Data are expressed as means \pm SEM (* p < 0.05 compared with the control group), n = 8

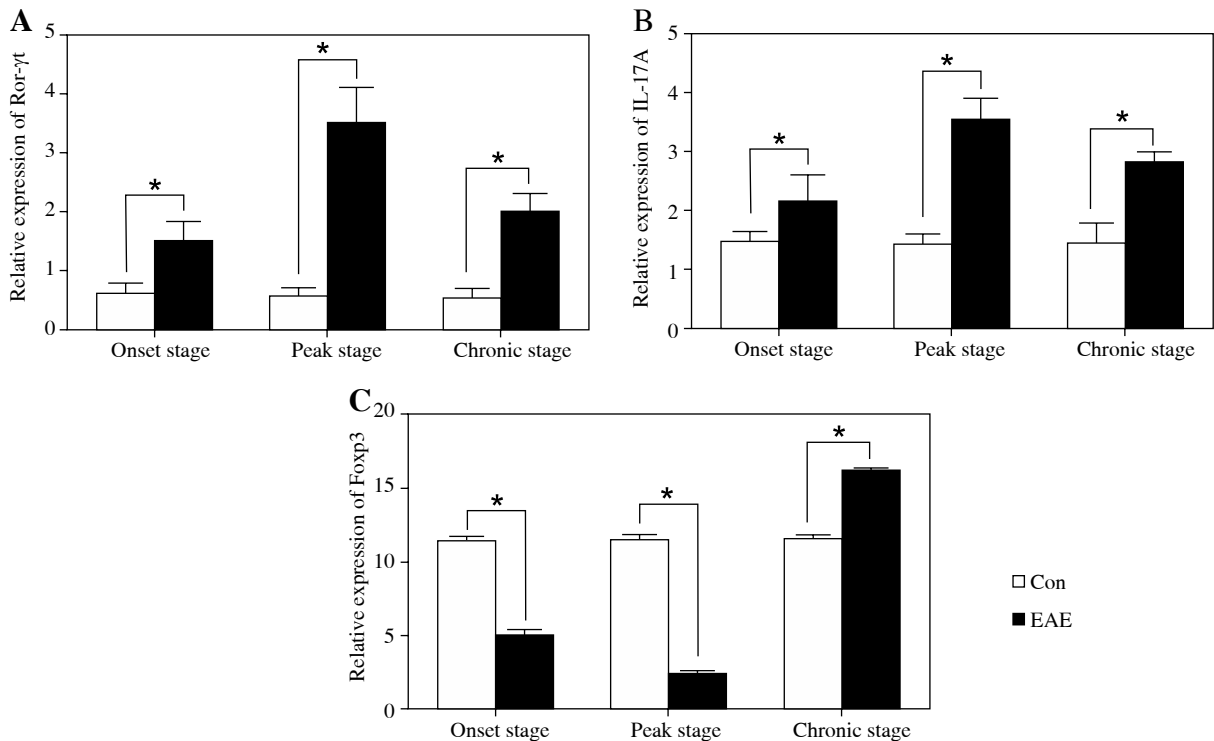


Fig. 6. The expression of Ror- γ t, IL-17A and Foxp3 mRNA in the brain, at the onset of EAE symptoms (day +16, onset stage), at the peak of the disease (day +23, peak stage), at the time of EAE stabilization (day +30, chronic stage) in the two groups. Data are expressed as means \pm SEM (* p < 0.05 compared with the control group), n = 8

nificantly stimulate the naive T cells proliferation and activation, regulating the differentiation of naive T cells to Th17 and Treg [30], so we asked whether DCs can regulate the differentiation of Th17 and Treg in the EAE. Our data from co-culture showed that mature DCs can induce the differentiation of Th17 cells and suppress Treg T cells in mice, which supports the hypothesis that mature DCs maintain the balance between the generation of Th17 and

Treg cells. These results demonstrate that DCs can regulate the differentiation of Th17 and Treg in the EAE.

Next, we will discuss the mechanism involved in the interaction of DCs and T cells in the peripheral organ. Treg T cells and Th17 effectors arise in a mutually exclusive fashion depending on whether they are activated in the presence of TGF- β 1 or TGF- β 1 plus IL-6 [31, 32]. TGF- β 1 has been reported to stimulate naive CD4⁺ T cells

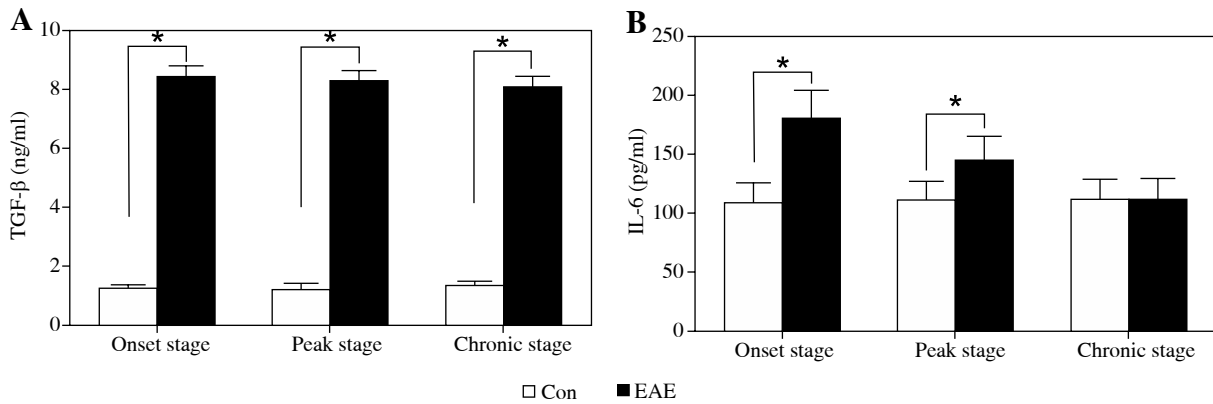


Fig. 7. The concentrations of IL-6 and TGF-β1 in mouse sera at the different stages in the two groups. Data are expressed as means ± SEM (**p* < 0.05 compared with the control group), *n* = 8

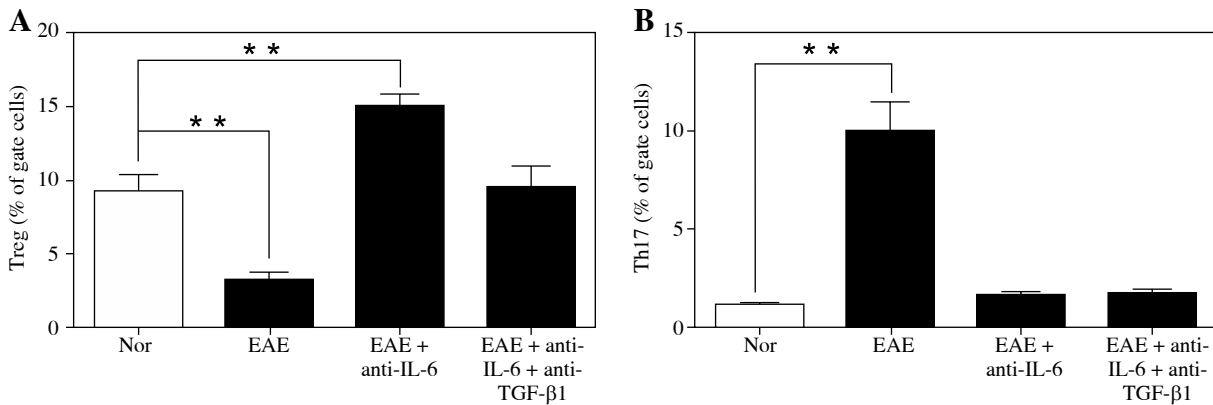


Fig. 8. Co-culture lymphocytes from the spleens of healthy mice and DCs from the spleens of peak-stage EAE mice (day 27) in the presence or absence of anti-IL-6 and anti-TGF-β1 antibody in complete medium for two days. The percentage of Th17 and Treg cells in harvested lymphocytes were analyzed by flow cytometry on day 2. Panel A showed the percentage of Th17 cells in lymphocytes before and after co-culture. Panel B showed the percentage of Treg in lymphocytes before and after co-culture. Nor = lymphocytes from normal mice spleens, EAE = lymphocytes from normal mice spleens co-culture with DCs from the spleens of peak-stage EAE mice, EAE + anti-IL-6 = anti-mouse IL-6 monoclonal antibody was added to DCs from peak-stage EAE mouse spleens for two hours before co-culture. EAE + anti-IL-6 + anti-TGF-β1 = anti-mouse IL-6 monoclonal and anti-TGF-β1 antibody was added to DCs from peak-stage EAE mouse spleens for two hours before co-culture. Data are expressed as means ± SEM (***p* < 0.01 compared with the normal group), *n* = 4

to differentiate into Treg which also produce TGF-β1 [33]. Although TGF-β1 exerts an anti-inflammatory activity via the induction of FoxP3 expression, in combination with IL-6, TGF-β1 stimulates the production of Th17 cells in EAE [30, 34]. DCs have a functional diversity of cytokine production that regulates the polarization of naive T-cells to Th1 or Th2 [35]. So we asked whether mature DCs directly influence Th17 and Treg differentiation by secreting IL-6 and TGF-β1 *in vivo*. Our studies showed that EAE-derived DCs have a high expression of IL-6 and TGF-β1. We also found that exogenously added anti-IL-6

antibody in co-culture, then only induced the differentiation of Treg. When anti-IL-6 and anti-TGF-β1 antibody were added in co-culture, there was no polarization of Th17 and Treg. The results from neutralization of IL-6 and TGF-β1 further confirmed that DCs can regulate the differentiation of Th17 and Treg in the EAE by secreting IL-6 and TGF-β1. In the present study, the high TGF-β1 level in the mature DCs culture supernatant and sera can be speculated to the induction of Th17 differentiation by the combination of IL-6, resulting in the exacerbation of EAE in the early and peak stage [36]. As the disease pro-

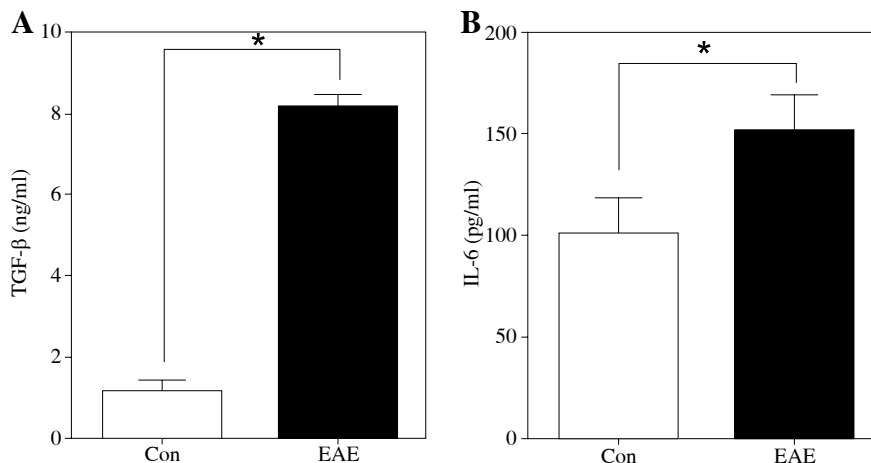


Fig. 9. Culture supernatants were collected, and the levels of the indicated cytokines were measured by ELISA. Data were expressed as means \pm SEM (* p < 0.05 compared with the control group), n = 8

gresses, IL-6 levels gradually decreased, the concentration of TGF- β 1, which can induce Treg, remained high. Taken together, our results strongly suggest that in the early stage of EAE, mature DCs promote the activation of Th17 and inhibit the function of Treg, causing chronic tissue injury by secreting IL-6 and TGF- β 1. These DCs can regulate the differentiation of Th17 and Treg in the EAE.

In summary, mature DCs play a critical role in the pathogenesis of EAE, at their peak incidence, mature DCs are the predominant players, inducing primarily Th17 and resulting in the highest EAE clinical score. As the disease progresses, the number of mature DCs decreases, reducing Th17 generation and allowing Treg function to recover, resulting in partial alleviation of clinical symptoms, suggesting that targeting DC may be an effective strategy for treating of MS.

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The authors declare no conflict of interest.

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