Neurosci. Bull. February, 2016, 32(1):70–82
DOI 10.1007/s12264-015-0010-9

www.neurosci.cn
www.springer.com/12264

ORIGINAL ARTICLE

Programmed Death Ligand-1 on Microglia Regulates Th1 Differentiation *via* Nitric Oxide in Experimental Autoimmune Encephalomyelitis

Jingxia Hu[1,2] · Hao He[2] · Zhengang Yang[3] · Guangming Zhu[2] · Li Kang[2] · Xiuli Jing[2] · Hai Lu[4] · Wengang Song[2] · Bo Bai[4] · Hua Tang[2]

Received: 7 June 2015 / Accepted: 6 October 2015 / Published online: 14 January 2016

Abstract Microglia are considered to be potential antigen-presenting cells and have the ability to present antigen under pathological conditions. Nevertheless, whether and how microglia are involved in immune regulation are largely unknown. Here, we investigated the suppressive activity of microglia during experimental autoimmune encephalomyelitis (EAE) induced by myelin oligodendrocyte glycoprotein, with the goal of understanding their role in regulating the T cell reaction. Using flow cytometric analysis, we found that microglia were characterized by increased cell number and up-regulated programmed death ligand-1 (PD-L1) at the peak phase of EAE. Meanwhile, both the $CD4^+$ T cells and microglia that infiltrated the central nervous system expressed higher levels of PD1, the receptor for PD-L1, accompanied by a decline of Th1 cells. In an *ex vivo* co-culture system, microglia from EAE mice inhibited the proliferation of antigen-specific $CD4^+$ T cells and the differentiation of Th1 cells, and this was significantly inhibited by PD-L1 blockade. Further, microglia suppressed Th1 cells *via* nitric oxide (NO), the production of which was dependent on PD-L1. Thus, these data suggest a scenario in which microglia are involved in the regulation of EAE by suppressing Th1-cell differentiation *via* the PD-L1-NO pathway.

Keywords Microglia · Negative immune regulation · PD-L1 · Nitric oxide · EAE

Introduction

Experimental autoimmune encephalomyelitis (EAE), characterized by primary demyelination of axonal tracts in the central nervous system (CNS), is a well-known experimental model for the human inflammatory demyelinating disease, multiple sclerosis (MS). Mice with EAE usually experience an acute paralytic episode followed by partial recovery [1]. While both Th1 and Th17 cells are involved in the pathogenesis of EAE [2], the contributions of these two T cell subsets differ. Th1 cells induce "classic" paralytic EAE characterized by monocyte-rich CNS infiltrates and strong pro-inflammatory cytokine responses in the CNS, whereas Th17 cells mediate "atypical" ataxic EAE with a relatively mild course in $\sim$ 50% of mice *via* an IL-12-independent pathway [3]. Th1 cells, rather than Th17 cells, are highly pathogenic, can lyse autoantigen-presenting astrocytes and fibroblasts, and are required to facilitate the entry of Th17 cells into CNS lesions during EAE [4]. Th1 cells may play a more important role in the pathogenesis of EAE than Th17 cells. Importantly, previous reports have shown that spontaneous remission is, in part, attributable to the clearance of inflammatory Th1 cells from the CNS [5, 6], indicating the

Jingxia Hu and Hao He have contributed equally to this work.

✉ Bo Bai
bbai@mail.jnmc.edu.cn

✉ Hua Tang
tanghuazhang218@163.com

[1] College of Life Science, Shandong Agricultural University, Taian 271018, China

[2] Institute of Immunology, Taishan Medical University, Taian 271000, China

[3] Department of ENT, Center Hospital of Taian City, Taian 271000, China

[4] Department of Neurobiology, Jining Medical University, Jining 272067, China

existence of negative regulation of Th1 cells in EAE. However, the mechanisms underlying the suppression of Th1 cells during EAE remain poorly understood.

Microglia, the resident macrophages of the CNS, are exquisitely sensitive to brain injury and disease, and play an important role in the maintenance of CNS homeostasis [7]. Increasing evidence has shown that microglia are involved in the regulation of EAE, as modification of microglial activation affects the progression of EAE [8–11]. Microglia not only affect neurons by secreting neurotoxic [12, 13] or neuroprotective molecules [14, 15], but they are also thought to be the principal antigen-presenting cells (APCs) in the CNS and the central player in the pathophysiology of MS [16], considering its high expression of MHC-II and co-stimulatory molecules. This suggests that microglia regulate the T cell-mediated response that occurs in the CNS. This hypothesis is supported by the finding that IFN-γ-stimulated $CD11b^+$ microglia inhibit the differentiation of Th1 cells *in vitro*, suggesting potential suppressive activity [17]. However, the microglia used in this study were sorted from IFN-γ-stimulated cultures of cerebral cortical glia from neonatal mice, and differed from those purified from the CNS of EAE mice. So, whether and how microglia modulate the progression of EAE *via* suppressing Th1 cells need further investigation.

Given the ability of microglia to present antigen to $CD4^+$ T cells [18], co-stimulatory molecules expressed on microglia may affect T-cell differentiation and function. Previous studies have shown that IFN-γ-stimulated microglia express programmed death ligand-1 (PD-L1), an inhibitory co-stimulatory molecule [19]. The receptor of PD-L1, PD1, is known to be expressed by T cells. Therefore, how PD-L1-expressing APCs regulate $PD1^+$ T cells *via* cell-cell contact has attracted much attention. However, since inflammation and infection would upregulate PD1 in microglia, which express PD-L1 as well [20–22], the effect of PD-L1-PD1 interaction on microglia is also a matter of interest.

Moreover, previous reports have indicated that macrophages with higher PD-L1 expression produced more NO [23]. As the resident macrophages of the brain, microglia also upregulate PD-L1 and NO upon LPS/IFN-γ stimulation [24]. This sparked our interest in the relationship between PD-L1 and NO in microglia during EAE. That NO strongly affects Th1 cell differentiation has been demonstrated in NOS2/iNOS KO mice [25–27], so there may be an alternative pathway in microglia that depends on PD-L1-PD1 interaction, and is responsible for modulation of the $CD4^+$ T cell response *via* NO production. We therefore set out to investigate these possibilities.

Materials and Methods

Animals

C57BL/6 (H-2 K^b) mice were purchased from Vital River (Beijing, China). $OVA_{323–339}$ peptide-specific TCR transgenic mice (OT-II mice) were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were housed and cared for according to protocols approved by the Animal Care and Use Committee of Taishan Medical University.

Reagents and Antibodies

Fluorescein-conjugated antibodies specific for the mouse antigens CD4 (GK1.5), CD11b (M1/70), CD11c (N418), CD45 (30-F11), IA/IE (MKS4), CD86 (GL1), PD-L1 (M1H5), PD-L2 (TY25), B7-H2 (MIH12), PD1 (J43), IFN-γ (554412), and iNOS (CXNFT) were from eBioscience (San Diego, CA) or BD Pharmingen (San Diego, CA). Magnetic bead-conjugated anti-mouse antibodies (mAbs) to B220 (RA3-6B2) and CD4 (L3T4) were from Miltenyi Biotec (Bergisch Gladbach, Germany). Purified anti-mouse CD16-CD32 (2.4G2) was from BD Pharmingen and purified anti-CD3 (145-2C11), anti-CD28 (37.51), and anti-PD-L1 (M1H5) were from eBioscience.

RPMI-1640 medium (PAA Laboratories, Linz, Austria) was supplemented with 10% (*v*/*v*) FBS (PAA Laboratories), 2 mmol/L L-glutamine (Gibco Life Technologies, Grand Island, NY), 0.01 mol/L HEPES pH 7.2 (Gibco), 1 mmol/L sodium pyruvate (Gibco), 200 U/mL penicillin-streptomycin (Gibco) and 0.055 mmol/L β-mercaptoethanol (Gibco). Complete Freund's adjuvant (CFA), pertussis toxin (PTx), L-N^{ω}-nitroarginine methyl ester (L-NAME), 4′,6-diamidino-2-phenylindole (DAPI), paraformaldehyde (PFA), collagenase II, Dnase I, polyvinylpyrrolidone, Triton X-100, bovine serum albumin, and normal goat serum were from Sigma-Aldrich (St Louis, MO). Percoll (GE Healthcare, Uppsala, Sweden), *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI), Cytofix/Cytoperm kit (BD Pharmingen, San Diego, CA), Tissue-Tek OCT (optimal cutting temperature) compound (Sakura Finetek, Torrance, CA), and collagenase IV (Gibco Life Technologies) were also used.

Induction and Assessment of EAE

Using the method described by Stromnes [1], mice were injected subcutaneously with 300 μg $MOG_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK) in CFA containing 4 mg/mL *M. tuberculosis* H37Ra at four sites on the back. PTx (400 ng) was given intraperitoneally on days 0 and 2 post-immunization. Clinical EAE status was scored as

follows [1]: 0, no clinical symptoms; 0.5, partially limp tail; 1, completely limp tail; 1.5, limp tail and hindlimb weakness; 2, partial hindlimb paralysis; 2.5, complete unilateral hindlimb paralysis; 3, complete bilateral hindlimb paralysis; 3.5, complete bilateral hindlimb paralysis and partial forelimb paralysis; 4, paralysis of forelimbs and hindlimbs; and 5, death.

Isolation of CNS Mononuclear Cells

Mononuclear cells were isolated from the CNS as previously described [28] with modifications. Briefly, mice were perfused through the left ventricle with cold 0.01 mol/L PBS. The brain and spinal cord were cut into pieces and digested with collagenase II (2 μg/mL) and Dnase I (1 mg/mL) at 37 °C for 1 h. Cells were isolated by passing the tissue through a cell strainer (70 μm), followed by Percoll gradient (70%/40%) centrifugation. Mononuclear cells were removed from the interface, washed, and re-suspended in culture medium for further analysis.

Isolation of Splenocytes

The spleen was digested for 30 min at 37 °C in collagenase IV (1 mg/mL) and the cell suspension was filtered through a cell strainer (70 μm). Red blood cells were lysed with ACK lysing buffer (Gibco Life Technologies).

Flow Cytometry

For cell surface staining, after blocking with 2.4G2, cells were stained with anti-CD45-eFlur650, anti-CD11b-FITC, anti-IA/IE-PE-cy7, anti-CD86-PE, anti-PD-L1-PE, anti-PD-L2-PE, or anti-B7-H2-PE mAbs to analyze microglia or with mouse anti-CD4-FITC or anti-PD1-PE-cy7 antibody to analyze PD1 expression on $CD4^+$ T cells. For iNOS staining, after blocking with anti-FcR mAb (2.4G2), cells were stained with anti-CD45-eFlur650, anti-CD11b-PE, or CD4-percp-cy5.5, fixed and permeabilized with the Cytofix/Cytoperm kit, and then stained with anti-iNOS-FITC. For intracellular cytokine staining, after blocking with anti-FcR mAb (2.4G2) and staining with anti-CD4-FITC, cells were fixed and permeabilized with the Cytofix/Cytoperm kit and then stained with anti-IFN-γ-PE. To assess T-cell proliferation, cells were blocked with anti-FcR mAb (2.4G2) and stained with anti-CD4-FITC mAb. Then PBS was added to each well to a final volume of 300 μL. Finally, cells were collected at high speed for 50 s, and the $CD4^+$ T cells were counted on a BD FACS Aria II Flow Cytometer as described elsewhere [29].

For cell sorting, CNS mononuclear cells were isolated from mice at the peak of EAE. The Fc receptor was blocked with anti-mouse FcR (2.4G2), followed by staining with anti-CD45-eFlur650 and CD11b-PE, and $CD45^{low}$-$CD11b^+$ cells were recognized as microglia [30, 31]. $CD4^+$ T cells were purified from the spleens of 8-week-old OT-II mice *via* positive selection using CD4-coated microbeads. The positive cells were labeled with anti-CD4-FITC and anti-CD62L-PE mAb. The $CD4^+CD62L^{high}$ cells were sorted as naive T cells.

For T cell-proliferation assays, naive T cells were labeled with Cell Proliferation Dye eFluor® 450 (eBioscience). To isolate splenic dendritic cells (spDCs), $B220^-$ splenocytes were collected by negative selection with B220-coated magnetic microbeads. The negative cells were further labeled with anti-CD11c-APC and anti-IA/IE-PE-cy7 mAb, and $CD11c^{hi}Ia^{hi}$ cells were recognized as DCs. Finally, all cells were sorted on the BD FACS Aria II Flow Cytometer.

Proliferation and Inhibition Assays

In a total volume of 200 μL in 96-well round-bottom plates, 1×10^5 OT-II naive $CD4^+$ T cells labeled with eFluor® 450 were co-incubated with microglia (4×10^4 cells/well), splenic DCs (2×10^4/well), or microglia/splenic DCs in the presence of $OVA_{323–339}$ (ISQAVHAAHAEINEAGR, 10 μg/mL) at 37 °C under 5% CO_2 for 72 h. Then viable cell numbers and $CD4^+$ T cell differentiation were counted on the FACS Aria II Flow Cytometer. To analyze the action of microglia on $CD4^+$ T cell differentiation, the co-cultured cells were further re-stimulated with anti-CD3 (10 μg/mL) and anti-CD28 (2 μg/mL) mAbs for 5 h in the presence of GolgiStop (BD Pharmingen). The percentage of $IFN\gamma^+CD4^+$ T cells was analyzed on the FACSAria II. In some experiments, anti-PD-L1 mAb (10 μg/mL) or an iNOS inhibitor (L-NAME, 0.5 mmol/L) was added at the beginning of culture.

Immunohistofluorescent Staining

Mice were perfused through the left ventricle with 0.01 mol/L PBS followed by 0.1 mol/L PBS containing 4% PFA. Spinal cord tissue was collected, post-fixed in 0.1 mol/L PBS containing 4% PFA overnight at room temperature, and embedded in OCT. Sections (20 μm) were processed and stored at −20 °C in anti-freeze solution containing 0.05 mol/L PBS, 30% sucrose, 1% polyvinylpyrrolidone, and 30% ethylene glycol until use.

Double labeling for CD4 and PD1 was carried out in a one-step procedure. Selected sections from anti-freeze solution were blocked with blocking buffer (0.4% Triton X-100, 1% FBS, and anti-mouse FcR (2.4G2) (1:100) in 0.01 mol/L PBS) for 60 min. After incubation with anti-

CD4-FITC and anti-PD1-PE-Cy7 mAbs at room temperature for 2 h, the sections were incubated with DAPI for 5 min, then they were mounted and coverslipped. Images were captured using spinning-disk confocal microscopy (Leica SD AF, Wetzla, Germany).

Systemic Administration of iNOS Inhibitor During EAE

L-NAME dissolved in 0.2 mL sterile saline (125 mg/kg) or saline was injected intraperitoneally into mice with EAE (twice per day) from day 14 until day 22 post-immunization. Animals were sacrificed the day after the last injection and mononuclear cells from the CNS were prepared to analyze the percentage of IFN-γ^{+}CD4^{+} T cells.

Quantitative PCR Analysis of iNOS Expression

RNA was extracted from microglia and CD4^{+} T cells sorted from the CNS at the peak phase of EAE using an RNeasy Mini Kit (Qiagen) according to the manufacturers' recommendations. Aliquots of each RNA sample (1 μg) were reverse-transcribed to produce cDNA using a QuantiTect Reverse Transcription Kit (Qiagen). Quantitative real-time PCR (qPCR) was performed with a Rotor-Gene SYBR Green PCR Kit (Qiagen) on a Rotor-Gene Q cycler (Qiagen). Samples were analyzed in triplicate and experiments were performed at least three times. The absolute number of gene copies was normalized to GAPDH and standardized using a sample standard curve. iNOS primer sequences were as follows: sense strand: 5′ GTT CTC AGC CCA ACA ATA CAA GA 3′, anti-sense strand: 5′ GTG GAC GGG TCG ATG TCA C 3′.

Measurement of NO Production

The nitrite concentration in culture supernatants was measured colorimetrically using the Griess assay. Cell-free supernatant (100 μL) was added to a microtiter plate containing 100 μL 1% sulfanilamide and 0.1% naphthylethylenediamine in 2.5% H_3PO_4. The absorbance value was measured at 550 nm after 10 min.

Statistical Analysis

All experiments were designed and performed in accordance with the ARRIVE guidelines. Data are expressed as mean ± SD and were analyzed with two-tailed Student's *t* test using the GraphPad Prism 5 software package. $P < 0.05$ was considered statistically significant.

Results

Microglial Numbers Increase and PD-L1 Expression is Upregulated During EAE

EAE was induced in C57BL/6 mice by subcutaneous injection of $MOG_{35–55}$; it was characterized by monophasic paralysis followed by spontaneous partial remission [1]. Based on the clinical score and the number of days post-immunization [32, 33], EAE was classified into five progressive stages: the pre-onset phase (score 0, 4–6 days post-injection, dpi); the onset phase (score 0.5–1, 9–11 dpi); the increasing phase (score 1–2.5, 12–16 dpi); the peak phase (score 2.5–3.5, 17–23 dpi); and the remission phase (score 1–0.5, 30–35 dpi). Naive mice were set as controls (Fig. 1A).

To investigate whether microglia are involved in MOG-induced neuroinflammation, we first evaluated the dynamic changes in the numbers of microglia during EAE. The percentage of microglia (CD45^{low}CD11b^{+})[30, 31] among leukocytes gradually decreased until the peak phase, followed by a rebound at remission (Fig. 1B). This change was contrary to that of the cell count, which could be explained by the remarkable infiltration of leukocytes from the beginning and their subsequent retraction at the remission of EAE.

The changes in the numbers of microglia indicated their involvement in the pathogenesis of EAE, and were consistent with previous reports that microglia become activated and participate in EAE [9]. To gain further insights into the function of microglia *in vivo*, flow cytometry was used to assess the expression of antigen presentation-related molecules on microglia following MOG-immunization. Compared with naïve controls, microglia from EAE mice at different stages displayed significant dynamic changes in the expression of MHC class II (Fig. 1C), CD86 (Fig. 1D), and PD-L1 (Fig. 1E). However, PD-L2 and B7-H2 expression was relatively stable (data not shown).

In detail, the expression of MHC II and CD86 was upregulated from the pre-onset phase and reached a maximum at the increasing and onset phases, suggesting that microglia became activated and participated in antigen-presentation. We also found increased expression of PD-L1 on microglia from EAE mice, beginning from the pre-onset phase until the peak phase followed by a decrease thereafter. The start and the peak of PD-L1 upregulation were delayed compared with MHC II and CD86. This discrepancy suggested a functional change in microglia with the development of EAE, considering the PD-L1-mediated suppression of EAE severity and progression demonstrated in PD-L1$^{-/-}$ mice or mice with PD-L1 blockade [34–36].

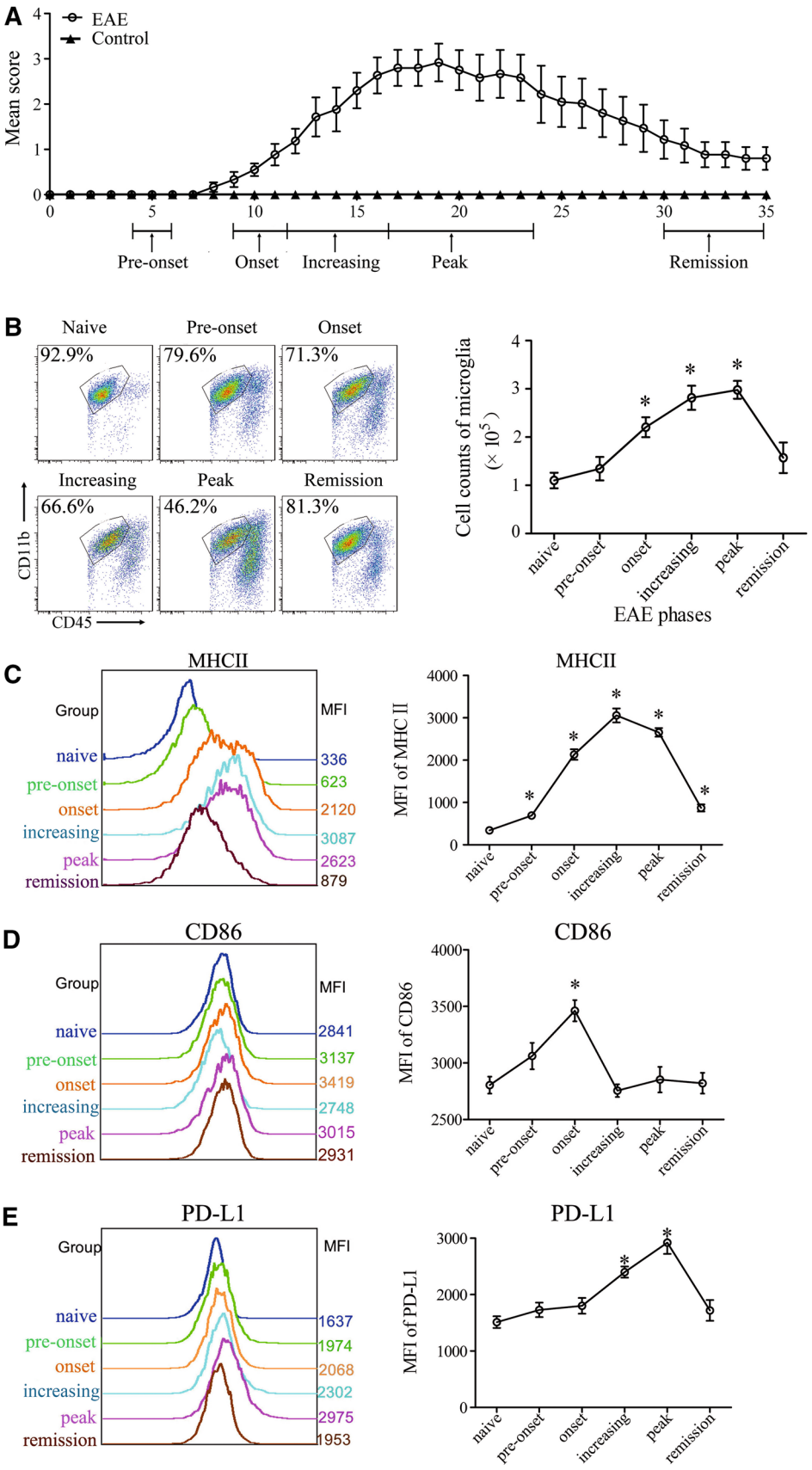


Fig. 1 Changes of quantity and phenotype of microglia during EAE. **A** EAE was established in C57BL/6 mice by immunization with $MOG_{35–55}$/CFA co-injected with PTx. Five progressive stages during EAE were defined according to the clinical score ($n = 12$ mice). **B** CNS mononuclear cells were prepared from the different phases of EAE and stained with anti-CD45 and anti-CD11b mAb for flow cytometric analysis ($n = 5$). The percentage of microglia ($CD45^{low}CD11b^{+}$) and their cell number were calculated. **C–E** $CD45^{low}CD11b^{+}$ microglia from EAE mice at different stages were gated, and the expression of MHC II (**C**), CD86 (**D**), and PD-L1 (**E**) was analyzed by flow cytometry. $^{*}P < 0.05$ compared with control. Data represent the mean ± SD of at least three separate experiments.

Therefore, we further explored the regulatory effect of PD-L1 on microglia.

Infiltrating $CD4^+$ T Cells in the CNS Upregulate PD1 Expression During EAE

Flow cytometry showed that the $CD4^+$ T cells infiltrating the CNS displayed gradual upregulation of PD1, reaching the highest level at the peak phase of EAE followed by a decrease at remission (Fig. 2A). To further confirm the PD1 expression on $CD4^+$ T cells, immunohistofluorescence showed that, at the peak phase of EAE, $CD4^+PD1^+$ T cells occurred mainly in the anterior median fissure, the posterior median sulcus, and the lateral funiculus of the white matter in the spinal cord, while neither $CD4^+$ T cells nor PD1 expression was found in the CNS of naive mice (Fig. 2B).

To explore the relationship between PD-L1 expression and the $CD4^+$ T cell response, dynamic changes in the Th1 cells infiltrating the brain and spinal cord during EAE were assessed. Mononuclear cells prepared from the CNS were stimulated with anti-CD3 and anti-CD28 mAbs for 5 h. Intracellular cytokine staining was performed, and the cells were analyzed by flow cytometry. The percentage of IFN-γ^+CD4^+ T cells (referred to as Th1 cells) in the CNS gradually increased and reached a maximum after the increasing phase, followed by a decrease thereafter (Fig. 2C). Of interest, the decline of Th1 cells was accompanied by a remarkable increase in PD-L1 expression on microglia (Fig. 1E), suggesting that Th1 cell reduction might be associated with microglial PD-L1.

Microglia Sorted from EAE Mice Suppress $CD4^+$ T Cell Proliferation and Th1 Differentiation in a PD-L1-Dependent Manner

To investigate whether microglia negatively regulate $CD4^+$ T cells, proliferation assays were performed by 72-h co-culture of OT-II $CD4^+$ T cells and $OVA_{323–339}$-loaded splenic DCs (spDCs) in the presence or absence of microglia sorted from mice at the peak phase of EAE. The $OVA_{323–339}$-pulsed spDCs but not microglia stimulated the proliferation of $CD4^+$ T cells (Fig. 3A). Interestingly, the spDC-initiated $CD4^+$ T cell proliferation was significantly inhibited when microglia were added. By assessing the intracellular IFN-γ of $CD4^+$ T cells in the co-culture, we further found a significant inhibition of IFN-γ^+CD4^+ Th1 cell differentiation by microglia sorted from EAE mice (Fig. 3B). Therefore, we demonstrated *ex vivo* that microglia from mice at the peak phase of EAE suppress $CD4^+$ T cell proliferation and Th1 cell differentiation.

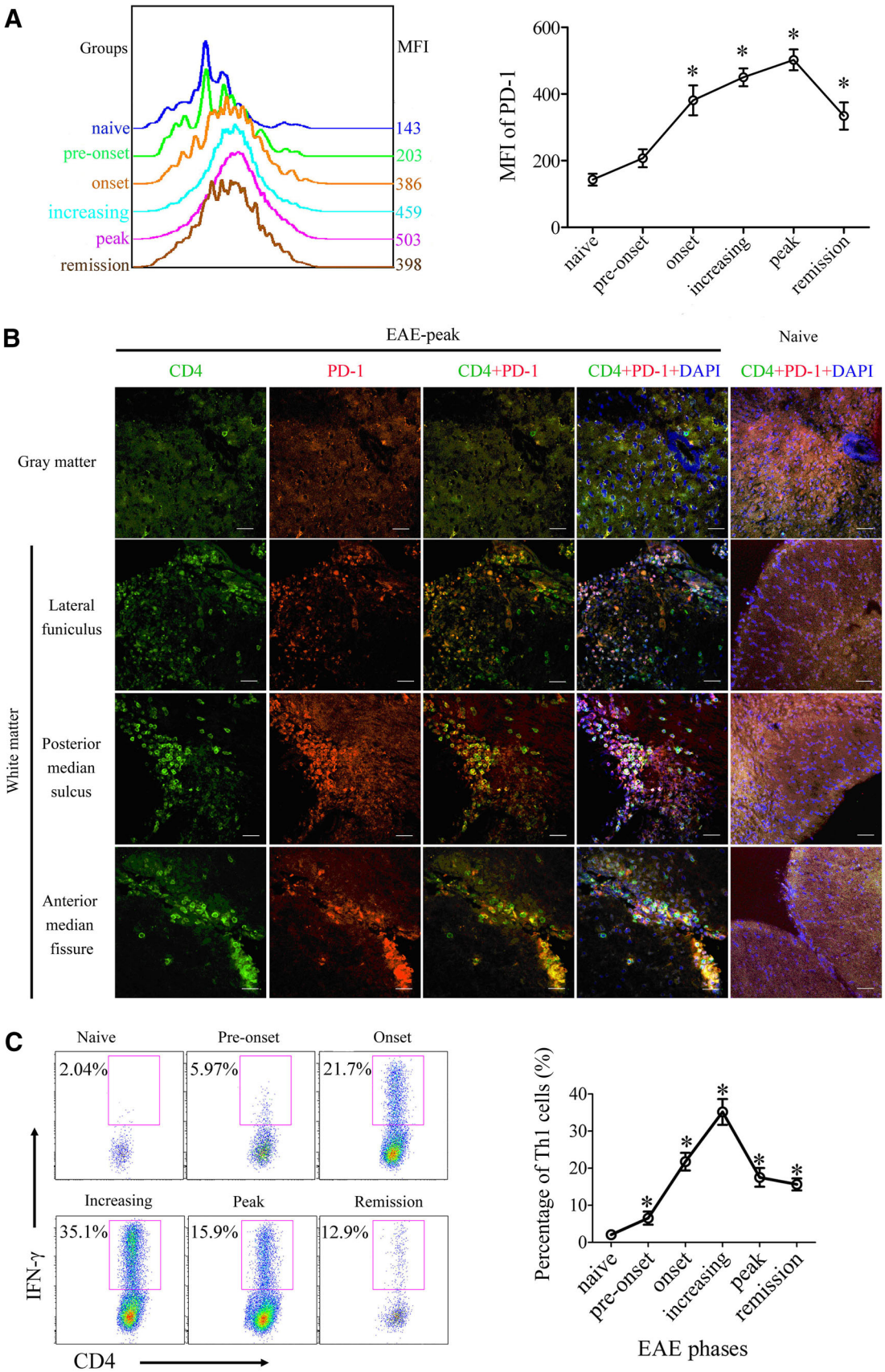
Considering the simultaneous upregulation of PD-L1 on microglia (Fig. 1E) and PD1 on $CD4^+$ T cells (Fig. 2A) in the course of EAE, we speculated that microglia might exhibit inhibitory effects through the PD-L1/PD1 pathway. Thus, we assessed the proliferation and differentiation of $CD4^+$ T cells using co-culture systems in the presence of anti-PD-L1 mAb or isotype control. After administration of PD-L1-blocking mAb, we found that both the $CD4^+$ T cell count and the percentage of Th1 cells were significantly higher in the microglia/$CD4^+$ T group and the microglia/spDC/$CD4^+$ T group than that without PD-L1 blockade, suggesting that microglia inhibit $CD4^+$ T cells in a PD-L1-dependent manner (Fig. 3C, D).

Fig. 2 Expression of PD1 on $CD4^+$ T cells and dynamic changes of Th1 cells in the CNS of EAE mice. **A** Mononuclear cells of the CNS from mice at different phases of EAE were prepared and stained with anti-CD4 and anti-PD1 mAbs. $CD4^+$ T cells were gated to analyze PD1 expression by flow cytometry ($n = 5$). **B** At the peak phase of EAE, immunofluorescence assays were used to analyze $PD1^+CD4^+$ T cells in the *gray* and *white matter* (*scale bars* 200 μm). **C** Mononuclear cells of the CNS from mice at different phases of EAE were stimulated by anti-CD3 (10 μg/mL) and anti-CD28 mAbs (2 μg/mL). Five hours later, cells stained with anti-CD4 and anti-IFN-γ mAbs were analyzed by flow cytometry. The percentage of IFN-γ^+CD4^+ T cells (Th1) was assessed from $CD4^+$ T cells ($n = 5$). $^*P < 0.05$ compared with control. Data represent the mean ± SD of three separate experiments.

Microglia-Mediated Suppression of Th1 Cells Depends on NO

From the data above, we concluded that PD-L1-expressing microglia regulate PD1-expressing Th1 cells *via* cell-cell contact, which has been demonstrated in other disease models [37–39]. However, it remained unclear whether other alternative pathways exist in microglia that depend on PD-L1-PD1 interaction and are responsible for modulation of the $CD4^+$ T cell response. We supposed that microglia secrete certain soluble factors to regulate the $CD4^+$ T cell response. Previous reports have indicated that LPS/IFN-γ stimulation promotes microglia to produce NO [24], an important molecule that strongly affects Th1 cell differentiation [25, 26, 40] and EAE development [41, 42]. This sparked our interest in whether microglia activated during EAE exhibit suppressive activity *via* the production of NO.

qPCR and flow cytometry revealed much higher expression of iNOS on microglia from mice at the peak phase of EAE than that from naive mice (Fig. 4A, B). Further, the concentration of NO in the culture supernatant from the microglia/$CD4^+$ T group or the microglia/spDC/$CD4^+$ T group was significantly higher than that from the spDC/T group (Fig. 4C), suggesting the involvement of NO in microglia-mediated immunoregulation. Confirming the effect of NO on the suppression of $CD4^+$ T cells, addition of the iNOS inhibitor L-NAME into the co-culture


A
Groups
MFI
naive
pre-onset
onset
increasing
peak
remission
143
203
386
459
503
398
MFI of PD-1
600
400
200
0
naive
pre-onset
onset
increasing
peak
remission
B
EAE-peak
Naive
CD4
PD-1
CD4+PD-1
CD4+PD-1+DAPI
CD4+PD-1+DAPI
Gray matter
White matter
Lateral funiculus
Posterior median sulcus
Anterior median fissure
C
Naive
Pre-onset
Onset
2.04%
5.97%
21.7%
Increasing
Peak
Remission
35.1%
15.9%
12.9%
IFN-γ
CD4
Percentage of Th1 cells (%)
40
30
20
10
0
naive
pre-onset
onset
increasing
peak
remission
EAE phases

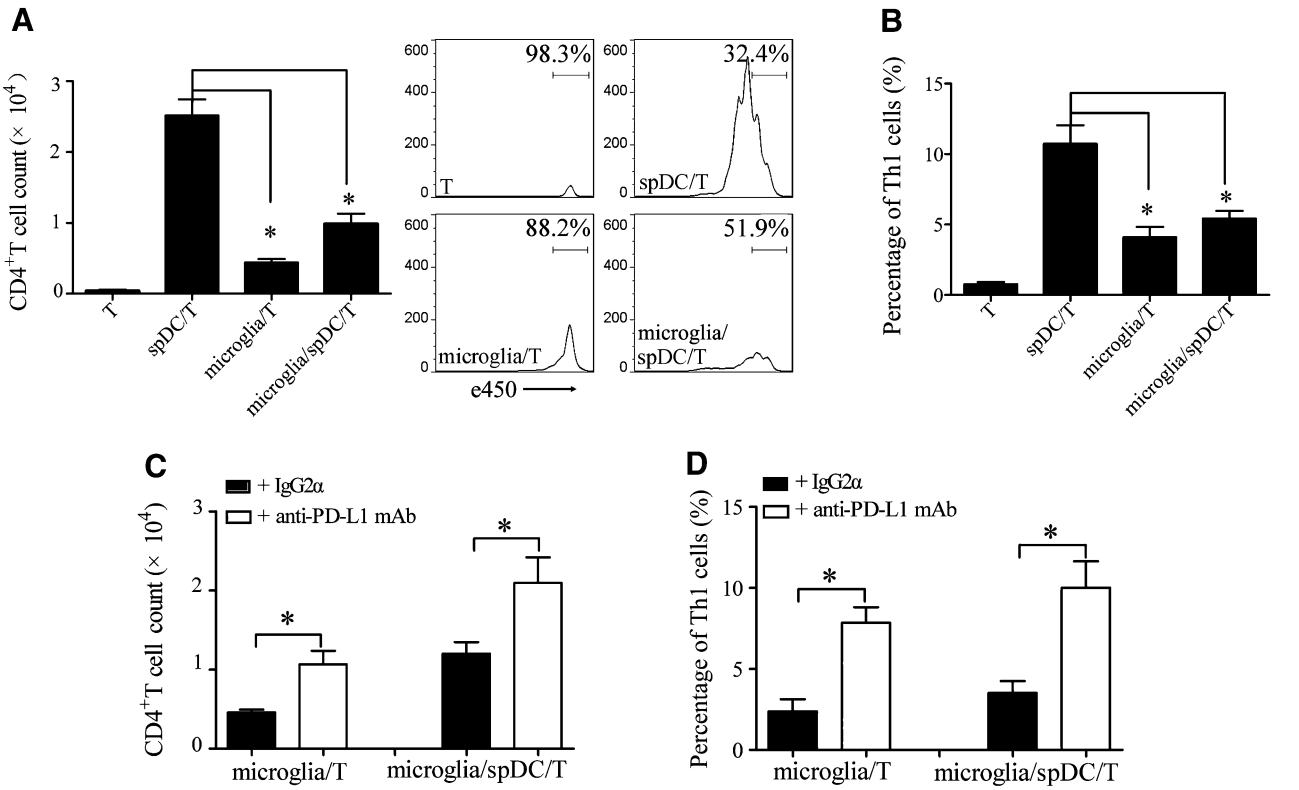


Fig. 3 The proliferation and differentiation of $CD4^+$ T cells were inhibited by PD-L1 expressed on microglia sorted from EAE mice. **A** eFlur450-labeled OT-II $CD4^+$ T-cells (1×10^5 cells/well) were co-cultured with spDCs (2×10^4 cells/well) from naïve mice, or microglia (2×10^4/well) from mice at the peak phase of EAE, or microglia plus spDCs, in the presence of $OVA_{323–339}$ (10 μg/mL) for 72 h *in vitro*. The dilution of eFlur-450 in $CD4^+$ T cells was analyzed. The histogram shows the number of $CD4^+$ T cells counted by flow cytometry ($n = 3$). **B** After additional stimulation by adding anti-CD3 (10 μg/mL) and anti-CD28 mAbs (2 μg/mL) into the co-culture system and incubation for 5 h, the percentage of $IFN\gamma^+CD4^+$ T cells was analyzed by flow cytometry ($n = 3$). **C–D** In blocking experiments, anti-PD-L1 mAb (10 μg/mL) or matched isotype control was added into different co-culture systems ($n = 3$). $CD4^+$ T cell proliferation (**C**) and Th1 cell differentiation (**D**) were analyzed by flow cytometry. Data are presented as mean ± SD (*$P < 0.05$). The results shown above are representative of at least three independent experiments.

system or injection of L-NAME into the peritoneal cavity of mice from 14 to 22 dpi significantly restored the suppressed $CD4^+$ T cell proliferation and Th1 differentiation mediated by microglia *ex vivo* (Fig. 4D). Moreover, more IFN-γ^+CD4^+ Th1 cells were observed in the CNS of EAE mice once NO production had been blocked (Fig. 4E). These data demonstrated that NO contributes to Th1 cell suppression in EAE.

PD-L1 Regulates NO Production by Microglia

To determine whether $CD4^+$ T cells also act as producers of NO, we simultaneously sorted microglia and $CD4^+$ T cells from mice at the peak phase of EAE and analyzed iNOS expression by qPCR and flow cytometry. iNOS expression was detected in microglia, but not in $CD4^+$ T cells (Fig. 5A, B). Similar results were also obtained in cells from the co-culture system (data not shown). To further reveal the relationship between PD-L1 and NO expressed on microglia, blocking experiments were performed in the co-culture system. We found that blocking PD-L1 led to lower levels of NO in the microglia/OT II T cell co-culture with or without spDCs (Fig. 5C), indicating the regulation of NO by PD-L1 in microglia. Flow cytometry showed that microglia activated during EAE upregulated PD1 expression in a manner similar to PD-L1 (Fig. 5D), consistent with previous reports that inflammation and infection upregulate both PD-L1 and PD1 in microglia [20–22]. Therefore, we speculated that activated microglia not only interact with PD1-expressing $CD4^+$ T cells, but also crosstalk with nearby microglia *via* PD-L1-PD1 interaction, thus promoting NO production by microglia.

Discussion

As an animal model for T cell-mediated autoimmune disease, EAE has pathological hallmarks similar to MS, including white matter demyelination, inflammation, axonal damage, and blood-brain barrier disruption.

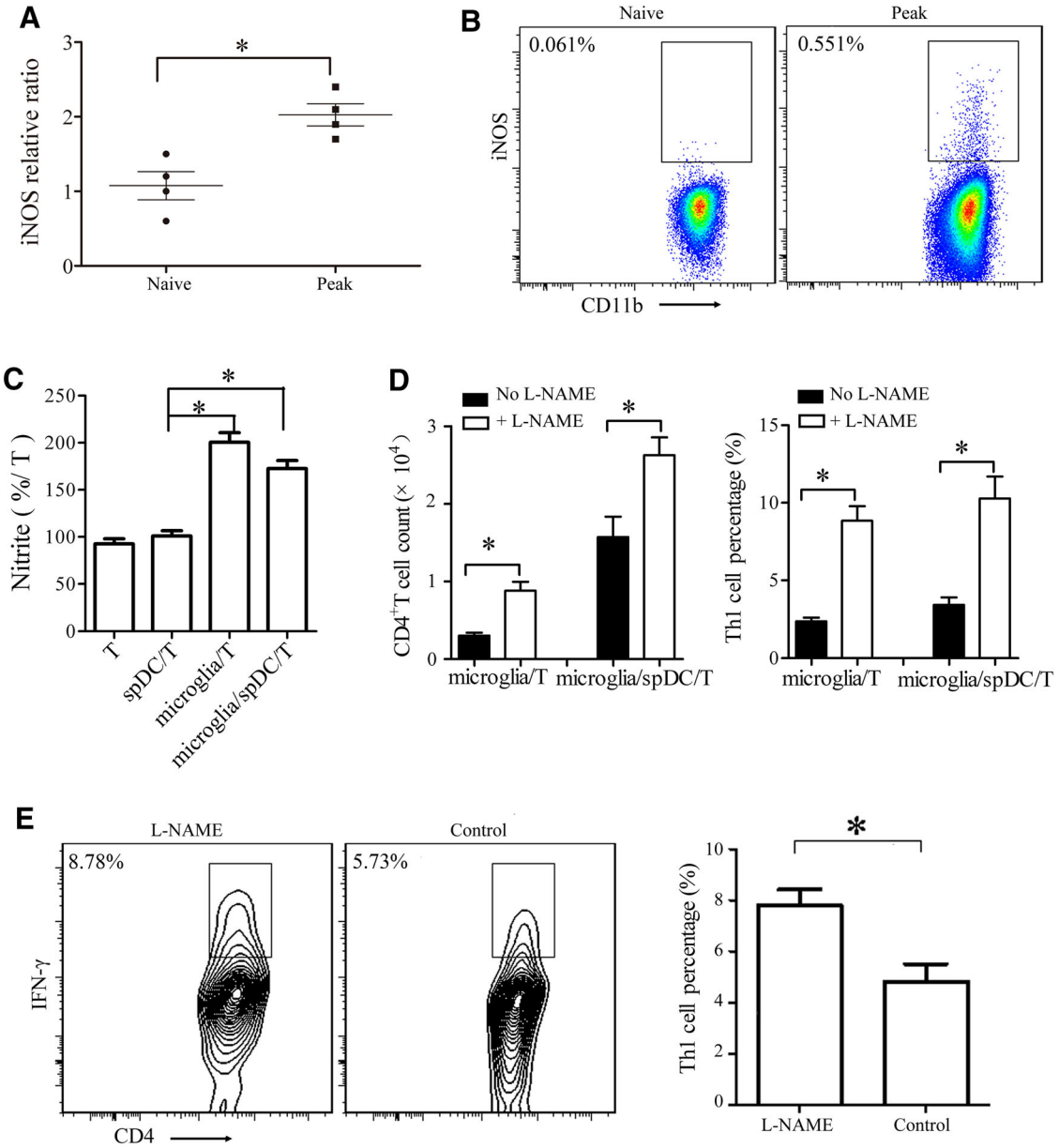


Fig. 4 Th1 cell differentiation was suppressed by microglia sorted from EAE mice *via* NO. **A** Microglia sorted from mice at the peak phase of EAE and naive mice were assessed for the expression of iNOS mRNA by qPCR ($n = 4$). **B** Mononuclear cells in the CNS from EAE mice at the peak phase and naive mice were purified and stained with anti-CD45, anti-CD11b, and anti-iNOS mAbs for flow cytometric analysis. Microglia were analyzed for iNOS expression ($n = 5$). **C** Supernatant of the co-culture that included CNS-sorted microglia form EAE mice at the peak phase was prepared to analyze NO by Griess assay; values are expressed as the percentage of nitrite relative to T cells alone ($n = 3$). **D** Using the same co-culture system, an iNOS inhibitor (L-NAME, 0.5 mmol/L) was used to block NO production, and then $CD4^+$ T cell proliferation (*left*) as well as Th1 cell differentiation (*right*) were assessed ($n = 3$). **E** L-NAME (125 mg/kg) or saline was injected intraperitoneally into mice ($n = 8$) twice a day from day 14 to day 22 after immunization. The day after the last injection, mononuclear cells from the CNS were prepared and stimulated by anti-CD3 (10 μg/mL) and anti-CD28 mAbs (2 μg/mL). Five hours later, cells were stained with anti-CD4 and anti-IFN-γ mAbs for flow cytometric analysis. $^*P < 0.05$. Data are presented as mean ± SD of triplicate samples.

Interestingly, MOG-induced EAE is characterized by monophasic paralysis, which implies the involvement of negative immunoregulation. Previous studies have shown that peripheral $CD4^+$ $Foxp3^+$ Treg cells participate in the control of neuroinflammation [43, 44]. However, little is known about the contribution of CNS-resident cells to the control of EAE. In the present study, we described the dynamic changes of microglia and Th1 cells in EAE mice *in vivo*, and found that microglia inhibited $CD4^+$ T cell proliferation and Th1 cell differentiation *ex vivo*. Further,

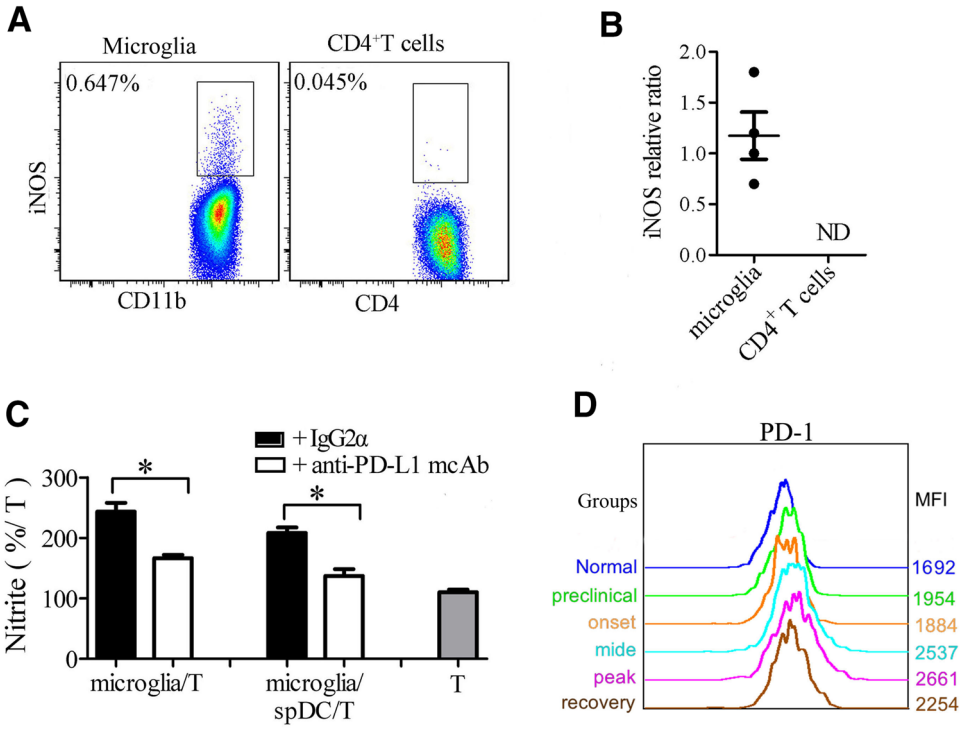


Fig. 5 Expression of PD-L1 was required for NO production in microglia during EAE. **A** Mononuclear cells were prepared from the CNS of EAE mice at the peak phase and stained with anti-CD45, anti-CD11b, anti-CD4, or anti-iNOS mAbs for flow cytometric analysis. Microglia and $CD4^+$ T cells were gated to analyze iNOS expression ($n = 5$). **B** Microglia and $CD4^+$ T cells were sorted from the CNS of mice at the peak phase of EAE and assessed for the expression of iNOS mRNA by qPCR. ND indicates no detection ($n = 4$). **C** To determine the effect of PD-L1 on NO production, anti-PD-L1 mAb (10 μg/mL) or isotype antibody was added into co-culture systems that included CNS-sorted microglia from mice at the peak phase of EAE. Culture supernatant was prepared for NO analysis by Griess assay; values are expressed as the percentage of nitrite relative to T cells alone ($n = 3$). **D** CNS mononuclear cells were prepared from mice at different phases of EAE ($n = 5$). $CD45^{low}$ $CD11b^+$ microglia were gated to evaluate the expressions of PD1 by flow cytometry $^*P < 0.05$. Data are presented as mean ± SD of triplicate samples.

PD-L1 expressed on microglia regulated Th1 cell differentiation *via* NO, suggesting a role of microglia in regulating autoimmune neuroinflammation.

Microglia, originating from the yolk sac [45], represent ~5–20% of total glial cells in rodents. As the CNS-resident immune cells, microglia play an important role in both physiological and pathological conditions. Although the physiological functions of microglia are only partially understood, their key role in certain neurodegenerative disorders is well established [9, 13, 46]. Furthermore, increasing evidence supports the concept that microglia function as a bridge between the CNS and immune systems in autoimmune neuroinflammation, such as in MS and EAE [18, 46–48]. Despite the infiltration of peripheral leukocytes into the CNS, the resident microglia become activated and are required for autoimmune responses. It is noteworthy that modulation of microglial activation can change the outcome of EAE [8–11]. Altered production of neurotoxic or neuroprotective molecules by microglia may explain this phenomenon. Moreover, the regulation of $CD4^+$ T cells by microglia should also be considered.

As an APC, the intrinsic function of microglia in adaptive immunity is their capability of antigen presentation. It is now clear that microglia scarcely present antigens to stimulate T cells based on the negligible expression of MHC class II molecules in the healthy CNS [11, 18]. During inflammation, there is marked upregulation of MHC class II and co-stimulatory molecules on microglia, indicating an enhanced ability to trigger $CD4^+$ T cells. Nevertheless, the expression of antigen presentation-associated molecules on microglia declines when EAE recovery begins, suggesting less efficient APCs at this time [16]. Our *ex vivo* results indicated that microglia at the peak phase of EAE have a limited stimulatory ability compared with spDCs, consistent with previous reports [37, 49]. Furthermore, microglia sorted from EAE mice inhibited spDC-triggered antigen-specific $CD4^+$ T cell proliferation and Th1 cell differentiation *ex vivo*. These results suggested that microglia negatively regulate the $CD4^+$ T cell response in EAE.

To further investigate the mechanism of microglia-mediated immunoregulation, we investigated changes in

the surface expression of molecules on microglia during EAE. PD-L1, a member of the co-stimulatory molecule family, was upregulated on microglia from the increasing phase of EAE in this study, similar to a previous report [19]. Furthermore, PD-L1 deficiency or PD-L1 blockade leads to aggravated symptoms of EAE, demonstrating the important regulatory role of PD-L1 in neuroinflammation [35, 36, 50]. The receptor for PD-L1, PD1 was also upregulated on CNS-infiltrating $CD4^+$ T cells, suggesting an interaction between microglia and $CD4^+$ T cells. We thus designed suppression-associated experiments as in a previous report [51], and found that neutralization of PD-L1 on microglia partially restored the $CD4^+$ T cell proliferation and Th1 cell differentiation. This finding indicated that microglia suppresses Th1 cell differentiation in a PD-L1-dependent manner, which is consistent with the previous report that PD-L1 blockade impairs the stimulation of activated $PD1^+$ T cells by microglia *ex vivo* [37].

The PD-L1/PD1 pathway has been shown to deliver inhibitory signals that regulate both central and peripheral immune tolerance. In the thymus, signals through PD1 regulate the signaling threshold during positive selection and participate in negative selection [52, 53]. In peripheral T-cell tolerance, the PD-L1/PD1 pathway regulates the T cell response in several ways. First, it limits the initial phase of activation and expansion of naive self-reactive T cells, and/or inhibits their differentiation into effector T cells. Second, the PD-L1/PD1 pathway can negatively regulate the reactivation, expansion, and/or functions of effector T cells [54, 55]. Notably, this pathway controls the development, maintenance, and function of induced Treg cells. In EAE, how do microglia suppress $CD4^+$ T cell proliferation and Th1 cell differentiation through the PD-L1/PD1 pathway? Apart from direct suppression of Th1 cells *via* cell-cell contact, we speculate that microglia exert suppression by the PD-L1-dependent production of certain molecules.

Previous reports have shown that PD-L1 expression is associated with the ability of macrophages to produce NO [23]. As the resident macrophages in the brain, microglia also upregulate PD-L1 and NO upon LPS/IFN-γ stimulation [24]. So, it is worthwhile to investigate whether NO acts as an effector molecule in microglia-mediated suppression. NO has long been considered as an important pro-inflammatory cytotoxic mediator to defend against various pathogens by inactivating and destroying infectious agents [56]. Further studies in NOS2/iNOS KO mice revealed that NO suppresses Th1 cells [25–27]. The underlying mechanisms are considered to be associated with the modulation of APC function and maturation. For example, NO inhibits IL-12p70 production by IFN-γ/LPS-treated mouse bone marrow-derived DCs [57] and LPS-treated human monocyte-derived DCs [58]; and autocrine NO affects the maturation of inflammatory DCs and the DC programming of T cells [59]. Our data showed that blocking NO resulted in partial recovery of the $CD4^+$ T cell proliferation and Th1 cell differentiation suppressed by microglia, suggesting the involvement of NO in microglia-mediated suppression. By intraperitoneal administration of an iNOS inhibitor, we also found increased Th1 cells in the CNS of the EAE model, which was consistent with the results from the co-culture system. However, how PD-L1 affects NO production by microglia is not well known and needs further study.

As NO is known to be involved in the microglia-mediated suppression of $CD4^+$ T cells, it is of interest to evaluate the relationship between PD-L1 and NO. Our results showed that neutralization of PD-L1 led to weak production of NO by microglia but not by $CD4^+$ T cells, suggesting the regulation of NO synthesis by PD-L1 signaling. Given the upregulation of PD1 on activated microglia, we suggest that the crosstalk among activated microglia *via* PD-L1-PD1 interaction leads to their production of NO, which subsequently suppresses $CD4^+$ T cells. Our next project is to study the key signal pathways involved in NO-mediated Th1 cell suppression.

Taken together, our findings indicate that microglia from EAE mice suppress $CD4^+$ T cell proliferation and Th1 cell differentiation. The inhibitory mechanisms of microglia are associated with the PD-L1/PD1 pathway which promotes NO production. These results deepen understanding of the role of microglia in EAE pathogenesis, and provide a theoretical and experimental basis for better treatment of MS patients in the future. However, we still cannot exclude the possibility that T cells themselves might interact with nearby T cells through the PD-L1/PD1 pathway to regulate the $CD4^+$T cell response, as PD-L1 is constitutively expressed on $CD4^+$ T cells and is further upregulated upon their activation [60]. To test this hypothesis, mice characterized by $CD4^+$ T cell-specific knockout of PD-L1 by the Cre-Loxp hybrid technique are required. Moreover, we also found dynamic changes of Th17 and Treg cells in the course of EAE (data not shown), but whether microglia affect these cells needs further investigation.

Acknowledgements This work was supported by the National Natural Science Foundation of China (81070961, 81273212, 81202308, 81302604, 31300730, 81172882, and 81241052) and the Natural Science Foundation of Shandong Province (ZR2011CM037). We thank Qiuling Zhang for assistance with the maintenance of mice; and Yalin Li and Yingping Xu for critical reading of the manuscript.

References

1. Stromnes IM, Goverman JM. Active induction of experimental allergic encephalomyelitis. Nat Protoc 2006, 1: 1810–1819.
2. Domingues HS, Mues M, Lassmann H, Wekerle H, Krishnamoorthy G. Functional and pathogenic differences of Th1 and

Th17 cells in experimental autoimmune encephalomyelitis. PLoS One 2010, 5: e15531.
3. Grifka-Walk HM, Giles DA, Segal BM. IL-12-polarized Th1 cells produce GM-CSF and induce EAE independent of IL-23. Eur J Immunol 2015, 45: 2780–2786.
4. O'Connor RA, Prendergast CT, Sabatos CA, Lau CW, Leech MD, Wraith DC, *et al.* Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. J Immunol 2008, 181: 3750–3754.
5. Ishigami T, White CA, Pender MP. Soluble antigen therapy induces apoptosis of autoreactive T cells preferentially in the target organ rather than in the peripheral lymphoid organs. Eur J Immunol 1998, 28: 1626–1635.
6. Seger J, Zorzella-Pezavento SF, Pelizon AC, Martins DR, Domingues A, Sartori A. Decreased production of TNF-alpha by lymph node cells indicates experimental autoimmune encephalomyelitis remission in Lewis rats. Mem Inst Oswaldo Cruz 2010, 105: 263–268.
7. Cao L, He C. Polarization of macrophages and microglia in inflammatory demyelination. Neurosci Bull 2013, 29: 189–198.
8. Bhasin M, Wu M, Tsirka SE. Modulation of microglial/macrophage activation by macrophage inhibitory factor (TKP) or tuftsin (TKPR) attenuates the disease course of experimental autoimmune encephalomyelitis. BMC Immunol 2007, 8: 10.
9. Ponomarev ED, Shriver LP, Maresz K, Dittel BN. Microglial cell activation and proliferation precedes the onset of CNS autoimmunity. J Neurosci Res 2005, 81: 374–389.
10. Starossom SC, Mascanfroni ID, Imitola J, Cao L, Raddassi K, Hernandez SF, *et al.* Galectin-1 deactivates classically activated microglia and protects from inflammation-induced neurodegeneration. Immunity 2012, 37: 249–263.
11. Xiao Y, Jin J, Chang M, Chang JH, Hu H, Zhou X, *et al.* Peli1 promotes microglia-mediated CNS inflammation by regulating Traf3 degradation. Nat Med 2013, 19: 595–602.
12. Liu B, Hong JS. Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. J Pharmacol Exp Ther 2003, 304: 1–7.
13. Lull ME, Block ML. Microglial activation and chronic neurodegeneration. Neurotherapeutics 2010, 7: 354–365.
14. Neumann H, Kotter MR, Franklin RJ. Debris clearance by microglia: an essential link between degeneration and regeneration. Brain 2009, 132: 288–295.
15. Napoli I, Neumann H. Protective effects of microglia in multiple sclerosis. Exp Neurol 2010, 225: 24–28.
16. Almolda B, Gonzalez B, Castellano B. Activated microglial cells acquire an immature dendritic cell phenotype and may terminate the immune response in an acute model of EAE. J Neuroimmunol 2010, 223: 39–54.
17. Monsonego A, Imitola J, Zota V, Oida T, Weiner HL. Microglia-mediated nitric oxide cytotoxicity of T cells following amyloid beta-peptide presentation to Th1 cells. J Immunol 2003, 171: 2216–2224.
18. Saijo K, Glass CK. Microglial cell origin and phenotypes in health and disease. Nat Rev Immunol 2011, 11: 775–787.
19. Magnus T, Schreiner B, Korn T, Jack C, Guo H, Antel J, *et al.* Microglial expression of the B7 family member B7 homolog 1 confers strong immune inhibition: implications for immune responses and autoimmunity in the CNS. J Neurosci 2005, 25: 2537–2546.
20. Jin YH, Hou W, Kang HS, Koh CS, Kim BS. The role of interleukin-6 in the expression of PD-1 and PDL-1 on central nervous system cells following infection with Theiler's murine encephalomyelitis virus. J Virol 2013, 87: 11538–11551.
21. Ren X, Akiyoshi K, Vandenbark AA, Hurn PD, Offner H. Programmed death-1 pathway limits central nervous system inflammation and neurologic deficits in murine experimental stroke. Stroke 2011, 42: 2578–2583.
22. Yao A, Liu F, Chen K, Tang L, Liu L, Zhang K, *et al.* Programmed death 1 deficiency induces the polarization of macrophages/microglia to the M1 phenotype after spinal cord injury in mice. Neurotherapeutics 2014, 11: 636–650.
23. Loke P, Allison JP. PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells. Proc Natl Acad Sci USA 2003, 100: 5336–5341.
24. Zhang XM, Lund H, Mia S, Parsa R, Harris RA. Adoptive transfer of cytokine-induced immunomodulatory adult microglia attenuates experimental autoimmune encephalomyelitis in DBA/1 mice. Glia 2014, 62: 804–817.
25. Bogdan C. Nitric oxide and the immune response. Nat Immunol 2001, 2: 907–916.
26. MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M, *et al.* Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. Cell 1995, 81: 641–650.
27. Wei XQ, Charles IG, Smith A, Ure J, Feng GJ, Huang FP, *et al.* Altered immune responses in mice lacking inducible nitric oxide synthase. Nature 1995, 375: 408–411.
28. Ponomarev ED, Veremeyko T, Barteneva N, Krichevsky AM, Weiner HL. MicroRNA-124 promotes microglia quiescence and suppresses EAE by deactivating macrophages via the C/EBP-alpha-PU.1 pathway. Nat Med 2011, 17: 64–70.
29. Tang H, Guo Z, Zhang M, Wang J, Chen G, Cao X. Endothelial stroma programs hematopoietic stem cells to differentiate into regulatory dendritic cells through IL-10. Blood 2006, 108: 1189–1197.
30. Getts DR, Terry RL, Getts MT, Muller M, Rana S, Shrestha B, *et al.* Ly6c+ "inflammatory monocytes" are microglial precursors recruited in a pathogenic manner in West Nile virus encephalitis. J Exp Med 2008, 205: 2319–2337.
31. D'Agostino PM, Kwak C, Vecchiarelli HA, Toth JG, Miller JM, Masheeb Z, *et al.* Viral-induced encephalitis initiates distinct and functional CD103+ CD11b+ brain dendritic cell populations within the olfactory bulb. Proc Natl Acad Sci USA 2012, 109: 6175–6180.
32. Hesske L, Vincenzetti C, Heikenwalder M, Prinz M, Reith W, Fontana A, *et al.* Induction of inhibitory central nervous system-derived and stimulatory blood-derived dendritic cells suggests a dual role for granulocyte-macrophage colony-stimulating factor in central nervous system inflammation. Brain 2010, 133: 1637–1654.
33. Davalos D, Ryu JK, Merlini M, Baeten KM, Le Moan N, Petersen MA, *et al.* Fibrinogen-induced perivascular microglial clustering is required for the development of axonal damage in neuroinflammation. Nat Commun 2012, 3: 1227.
34. Zhu B, Guleria I, Khosroshahi A, Chitnis T, Imitola J, Azuma M, *et al.* Differential role of programmed death-ligand 1 and programmed death-ligand 2 in regulating the susceptibility and chronic progression of experimental autoimmune encephalomyelitis. J Immunol 2006, 176: 3480–3489.
35. Carter LL, Leach MW, Azoitei ML, Cui J, Pelker JW, Jussif J, *et al.* PD-1/PD-L1, but not PD-1/PD-L2, interactions regulate the severity of experimental autoimmune encephalomyelitis. J Neuroimmunol 2007, 182: 124–134.
36. Ortler S, Leder C, Mittelbronn M, Zozulya AL, Knolle PA, Chen L, *et al.* B7-H1 restricts neuroantigen-specific T cell responses and confines inflammatory CNS damage: implications for the lesion pathogenesis of multiple sclerosis. Eur J Immunol 2008, 38: 1734–1744.

37. Schreiner B, Bailey SL, Shin T, Chen L, Miller SD. PD-1 ligands expressed on myeloid-derived APC in the CNS regulate T-cell responses in EAE. Eur J Immunol 2008, 38: 2706–2717.
38. McAlees JW, Lajoie S, Dienger K, Sproles AA, Richgels PK, Yang Y, *et al.* Differential control of CD4(+) T-cell subsets by the PD-1/PD-L1 axis in a mouse model of allergic asthma. Eur J Immunol 2015, 45: 1019–1029.
39. Li J, Jie HB, Lei Y, Gildener–Leapman N, Trivedi S, Green T, *et al.* PD-1/SHP-2 inhibits Tc1/Th1 phenotypic responses and the activation of T cells in the tumor microenvironment. Cancer Res 2015, 75: 508–518.
40. Gonzalez-Clemente JM, Martinez-Osaba MJ, Minarro A, Delgado MP, Mauricio D, Ribera F. [Hypovitaminosis D: its high prevalence in elderly outpatients in Barcelona. Associated factors]. Med Clin (Barc) 1999, 113: 641–645.
41. Sahrbacher UC, Lechner F, Eugster HP, Frei K, Lassmann H, Fontana A. Mice with an inactivation of the inducible nitric oxide synthase gene are susceptible to experimental autoimmune encephalomyelitis. Eur J Immunol 1998, 28: 1332–1338.
42. Fenyk-Melody JE, Garrison AE, Brunnert SR, Weidner JR, Shen F, Shelton BA, *et al.* Experimental autoimmune encephalomyelitis is exacerbated in mice lacking the NOS2 gene. J Immunol 1998, 160: 2940–2946.
43. Velez de Mendizabal N, Carneiro J, Sole RV, Goni J, Bragard J, Martinez-Forero I, *et al.* Modeling the effector - regulatory T cell cross-regulation reveals the intrinsic character of relapses in Multiple Sclerosis. BMC Syst Biol 2011, 5: 114.
44. Caridade M, Graca L, Ribeiro RM. Mechanisms Underlying CD4+ Treg Immune Regulation in the Adult: From Experiments to Models. Front Immunol 2013, 4: 378.
45. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, *et al.* Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science 2010, 330: 841–845.
46. Appel SH, Beers DR, Henkel JS. T cell-microglial dialogue in Parkinson's disease and amyotrophic lateral sclerosis: are we listening? Trends Immunol 2010, 31: 7–17.
47. Murphy AC, Lalor SJ, Lynch MA, Mills KH. Infiltration of Th1 and Th17 cells and activation of microglia in the CNS during the course of experimental autoimmune encephalomyelitis. Brain Behav Immun 2010, 24: 641–651.
48. Nayak D, Roth TL, McGavern DB. Microglia development and function. Annu Rev Immunol 2014, 32: 367–402.
49. Bailey SL, Schreiner B, McMahon EJ, Miller SD. CNS myeloid DCs presenting endogenous myelin peptides 'preferentially' polarize $CD4^+$ T(H)-17 cells in relapsing EAE. Nat Immunol 2007, 8: 172–180.
50. Yogev N, Frommer F, Lukas D, Kautz-Neu K, Karram K, Ielo D, *et al.* Dendritic cells ameliorate autoimmunity in the CNS by controlling the homeostasis of PD-1 receptor(+) regulatory T cells. Immunity 2012, 37: 264–275.
51. Zhang M, Tang H, Guo Z, An H, Zhu X, Song W, *et al.* Splenic stroma drives mature dendritic cells to differentiate into regulatory dendritic cells. Nat Immunol 2004, 5: 1124–1133.
52. Blank C, Brown I, Marks R, Nishimura H, Honjo T, Gajewski TF. Absence of programmed death receptor 1 alters thymic development and enhances generation of CD4/CD8 double-negative TCR-transgenic T cells. J Immunol 2003, 171: 4574–4581.
53. Keir ME, Freeman GJ, Sharpe AH. PD-1 regulates self-reactive $CD8^+$ T cell responses to antigen in lymph nodes and tissues. J Immunol 2007, 179: 5064–5070.
54. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. Immunol Rev 2010, 236: 219–242.
55. Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK, *et al.* PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. J Exp Med 2009, 206: 3015–3029.
56. Wei XM, Yang W, Liu LX, Qi WX. Effects of L-arginine and N(omega)-nitro-L-arginine methylester on learning and memory and alpha7 nAChR expression in the prefrontal cortex and hippocampus of rats. Neurosci Bull 2013, 29: 303–310.
57. Xiong H, Zhu C, Li F, Hegazi R, He K, Babyatsky M, *et al.* Inhibition of interleukin-12 p40 transcription and NF-kappaB activation by nitric oxide in murine macrophages and dendritic cells. J Biol Chem 2004, 279: 10776–10783.
58. Giordano D, Magaletti DM, Clark EA. Nitric oxide and cGMP protein kinase (cGK) regulate dendritic-cell migration toward the lymph-node-directing chemokine CCL19. Blood 2006, 107: 1537–1545.
59. Giordano D, Li C, Suthar MS, Draves KE, Ma DY, Gale M, Jr., *et al.* Nitric oxide controls an inflammatory-like Ly_{6}-C(hi)$PDCA1^+$ DC subset that regulates Th1 immune responses. J Leukoc Biol 2011, 89: 443–455.
60. Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, *et al.* Expression of programmed death 1 ligands by murine T cells and APC. J Immunol 2002, 169: 5538–5545.