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NFIL3 deficiency alleviates EAE through regulating different immune cell subsets

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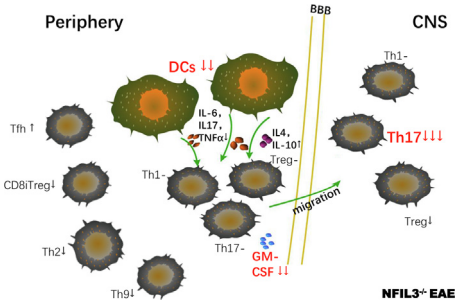
HIGHLIGHTS

- NFIL3 deficiency alleviated MOG35-55 induced EAE along with lower clinical scores, milder neuroinflammation and demyelination.
- NFIL3 deficiency decreased Th17 cells within the CNS of EAE.
- Th17, Th1 and Treg cells did not change obviously in the spleens and lymph nodes in NFIL3-/- EAE.
- In the periphery, the expressions of PD-1 and ICOS on CD4+T cells increased, whereas GM-CSF+CD4+T cells, Th2 cells, Th9 cells and CD8+CD103+T cells decreased in NFIL3-/- EAE.
- NFIL3 deficiency affected CD11c+ dendritic cells both in vivo and in vitro. The anti-inflammatory capacity of NFIL3 knock out CD11c+ DCs increased while their pro-inflammatory capacity decreased.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: The transcription factor NFIL3 exerts comprehensive effects on the immune system. Previous studies revealed that NFIL3 is related to the function and development of different immune cell subsets. Experimental autoimmune encephalomyelitis (EAE) is mediated by immune cells which results in inflammatory demyelination in the central nervous system (CNS). However, how NFIL3 affects EAE has not been thoroughly studied.

Objectives: The current study aimed to investigate how NFIL3 affects EAE, especially the changes of T cells and dendritic cells as well as the crosstalk between them.

Methods: We used NFIL3$^{-/-}$ mice and C57BL/6J mice (wildtype) to establish MOG35-55-induced EAE. The clinical scores were recorded daily. The immune cells within and outside the CNS of EAE mice were

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T cells
Dendritic cells
Autoimmunity

analyzed by flow cytometry. Histology was used to evaluated the neuroinflammation and demyelination in the CNS. Besides, CD11c$^+$ dendritic cells (DCs) were cocultured with T cells and the interplay was measured.

Results: At the peak of EAE, Th17 cells decreased within the CNS accompanying with lower clinical scores and milder neuroinflammation and demyelination in NFIL3 knockout EAE mice. Outside the CNS, PD-1 and ICOS on CD4$^+$T cells increased, whereas Th2, Th9, CD8$^+$CD103$^+$T cells and GM-CSF$^+$CD4$^+$T cells decreased. Besides, the pro-inflammatory capacity of NFIL3$^{-/-}$ CD11c$^+$ dendritic cells was impaired while the anti-inflammatory capacity was promoted.

Conclusions: This study suggests that NFIL3 deficiency could alleviate MOG35-55-induced EAE through regulating different immune cell subsets, which is not only related with adaptive immunity and innate immunity, but also related with the cross-talk between them, especially CD4$^+$ T cells and CD11c$^+$ dendritic cells.

Introduction

Multiple sclerosis (MS) is an autoimmune disease characterized by inflammation and demyelination in the central nervous system (CNS). Experimental autoimmune encephalomyelitis (EAE) is a widely used animal model for studying MS [1]. According to previous studies, autoreactive T cells, especially Th1 cells (IFN-γ^+), Th17 cells (IL-17$^+$), and regulatory T cells (Tregs), are important in the pathogenesis of MS and EAE. Besides, innate immune cells, like dendritic cells, are also involved [2–3]. However, a more accurate pathogenesis remains to be elucidated.

NFIL3 (nuclear factor interleukin-3) is one of the mammalian basic leucine zipper transcription factors. In human T cells, it could *trans*-activate IL-3 promoter [4–5]. Studies have revealed that NFIL3 is related to many physiological and biochemical processes [6]. It involves in regulating the circadian clock [7–8] as well as the growth and survival of neurons [9–10]. In addition, it plays comprehensive roles in both innate and adaptive immunity [11–13]. A number of solid evidences have confirmed that NFIL3 is critical for natural killer cells (NK). Knockout of NFIL3 would lead to deficiency of mature NK cells in mice [14–17]. NFIL3 also has crucial functions in dendritic cells (DCs) [18–19], B cells [5] and macrophages [20]. Moreover, NFIL3 has participated in the polarization and cytokine secretions of CD4$^+$T cells [21–23].

NFIL3 is related with immune-mediated diseases, like systemic lupus erythematosus (SLE) [24–25], arthritis [26] and Crohn's disease [20]. MS is also an autoimmune disease, but how NFIL3 affects MS/EAE has not been thoroughly studied. Since NFIL3 has extensive effects on immunologic system, we hypothesize that NFIL3 might regulate EAE in different ways. In fact, previous research has revealed that NFIL3 deficiency could prevent EAE from remission because of declined expression of IL-10 [21], while another study has showed that this effect was induced by disinhibition on Th17 cells [27]. However, the general changes of immune cells within and outside the CNS during EAE have not been thoroughly studied under the condition of NFIL3 deficiency. In this study, we used NFIL3 knock-out mice to establish EAE. Alterations within the CNS and periphery were investigated. Our results demonstrated that NFIL3 could affect EAE not only by regulating adaptive immunity and innate immunity, but also by regulating the cross-talk between CD11c$^+$ DCs and CD4$^+$ T cells. NFIL3 might contribute to EAE through regulating different immune cell subsets.

Materials and methods

Animals

NFIL3$^{+/-}$ mice (C57BL/6J background) were kind gifts from Prof. Tak W. Mak (University of Toronto) and Prof. A Thomas Look (Harvard Medical School), and NFIL3$^{-/-}$ mice were bred in specific pathogen-free condition. Wild-type C57BL/6J mice and BALB/c mice were obtained from Guangdong Medical Laboratory Animal Center. All mice were housed in the Center of Experimental Animals of Sun Yat-sen University (Guangzhou, China).

Ethics statement

All experimental protocols were approved by the Animal Care and Use Committee of Guangdong Medical Laboratory Animal Center (Approval no. C2021067). And the experiments were carried out according to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Active immunization model of EAE

Wild-type C57BL/6J and NFIL3$^{-/-}$ females (8–10 weeks old, n = 8 for each group) were used to establish EAE model. Three independent experiments were conducted. Briefly, mice were immunized subcutaneously at 3 sites on the back with 300 ug MOG_{35-55} peptide (GL Biochem Ltd., Shanghai) emulsified in complete Freund's adjuvant which contained 400 μg of Mycobacterium tuberculosis (Difco Laboratories, USA). At the time of immunization (Day 0) and 48 h later (Day 2), the mice were intraperitoneally injected with 250 ng pertussis toxin (PTX, Sigma-Aldrich, USA). On Day 7, the second immunization with 200ug MOG_{35-55} peptide in bilateral inguinal skin was injected subcutaneously and 250 ng PTX was injected intraperitoneally. Clinical manifestations were evaluated from Day 0 as follows: 0.0, no symptoms; 0.5, partial paralysis of the tail; 1, total paralysis of the tail; 2.0, hind limbs weakness resulting in a wobbly gait or ataxia; 3.0, paralysis of one hind limb; 3.5, paralysis of both hind limbs; 4.0, paralysis of one forelimb and both hind-limbs; 5.0, moribund state.

Tissue sampling and isolation of immune cells from EAE mice

At day 25, the EAE mice were sacrificed. Firstly, cold phosphate-buffered saline (PBS) was used for transcardiac perfusion in anesthetized EAE mice. Then spleens, lymph nodes, brains and spinal cords were dissected for postprocessing. Briefly, for spleens and lymph nodes, they were put in cell strainers (70 μm) and grinded with syringe plungers, then washed with PBS. For the spleens, red blood cells were lysed additionally. For brains and spinal cords, they were first cut into pieces and then digested by collagenase IV (Sigma Aldrich) at 37℃ with shaking for 60 min. After digestion, the tissues were pressed through cell strainers (70 μm), and then separated in 30%/70% Percoll solution (GE) through centrifugation. Flow cytometric analysis (FACS) were performed to analyze cells at the interface later.

Flow cytometric analysis and antibodies

Isolated cells of EAE mice were stained with anti-NK1.1/CD3/CD4/CD8/CD126/CD103/PD-1/ICOS/CD11c/CD80/CD86/CD69/MHC-II antibodies (Biolegend). As for intracellular staining, cells were first stimulated with PMA and ionomycin (Sigma-Aldrich, USA) for 4 h. After that, anti-IL-17A/IL-10/ IFN-γ/IL-4/IL-9/GM-CSF/Foxp3 antibodies were used to stain. FACS was performed on a BD FACS Fortessa Flow Cytometer. The results of FACS were analyzed by FlowJo software (version 10.0). The gating strategies were shown in Figure S1. The information of FACS antibodies was shown in Table S1.

Bone marrow derived dendritic cells induction and apoptosis analysis

As described in a previous study [28], bone marrow cells were obtained from wild-type C57BL/6 and NFIL3$^{-/-}$ female mice. ACK buffer was used to lyse red blood cells. To induce dendritic cells, the residual cells were cultured in RPMI 1640 with 50 ng/ml rmGM-CSF and 2.5 ng/ml rm-IL-4 (Peprotech), containing 10% heat-inactivated fetal bovine serum (HyClone), 1% HEPES, 1% sodium pyruvate (Corning) and 100 IU/ml penicillin (Gibco). After 6 days incubation (37 °C and 5% CO2), DCs stained with anti-CD11c antibody were detected. Next, BMDCs were stimulated with LPS (2.5 μg/ml) for 24 h and then were stained with anti-CD80/CD86/CD69/MHC-II antibodies (Biolegend) and measured by FACS. Besides, apoptosis of BMDCs was analyzed by using CaspGLOW kit according to the standard protocol.

DC-T cell coculture experiments

Firstly, BMDCs from wild-type C57BL/6 and NFIL3$^{-/-}$ mice were obtained as described above. And CD4^{+}T cells were separated from BALB/c background mice by AutoMACS (Miltenyi). Next, BMDCs were pre-stimulated with LPS (10 ng/ml) and protein transport inhibitor Brefeldin A (1×/ml) for 24 h. Then BMDCs were cocultured with CD4^{+}T cells in 1:1 ratio for 72 h (Total 1 million cells/-hole in 96-well plates). Later, the mixed supernatants from this BMDC-T coculture system were collected for cytokines' analysis.

Cytokines and soluble CD126 analysis

The concentration of sCD126 in mouse serum was measured with an ELISA kit following a standard protocol. As for BMDCs, concentrations of IFN-γ/TNFα/IL-2/IL-4/IL-6/IL-17A in cell culture supernatants were measured by CBA kit (BD Biosciences) or ELISA kit (Life Invitrogen).

Histology

The sections of spinal cords from the EAE mice were dissected and stained with HE and Luxol Fast Blue according to standard protocols. The neuroinflammation was scored as: 0, no infiltration of inflammatory cells; 1, a few scattered inflammatory cells; 2, inflammatory cells infiltrate around blood vessels; 3, infiltrated inflammatory cells formed extensive perivascular cuffing extending to adjacent parenchyma, or inflammatory cells infiltrated into parenchymal without obvious cuffing. The demyelination was scored 1 ∼ 5 as previously described [29].

Rt-PCR

The mRNA expressions of PD-1, B7-H2, B7-H3, B7-H4, IL-1, IL-4, IL-10 and TGF-β in BMDCs were quantified by a SYBP$^{©}$R Premix Ex TaqTM II Kit (TAKARA). The information of primers was shown in Table S1.

Statistical analysis

Statistical analyses were performed in GraphPad Prism software. All data were presented as means ± standard deviation (SD) or standard error of the mean (SEM). *t*-test was used when only two groups were compared. The EAE scores were analyzed by a two tailed Mann–Whitney *U* test. A p *value* < 0.05 was considered statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Results

NFIL3 deficiency alleviated MOG_{35-55}-induced EAE

According to previous study [13], NFIL3 affects both innate immunity and adaptive immunity. Firstly, we confirmed the successful establishment of NFIL3 knockout strain. Consistent with previous publications [14–15], there was no significant difference in frequencies of Tregs, total CD4^{+} T cells and CD8^{+} T cells between normal NFIL3$^{-/-}$ mice and WT mice, while CD3^{-}NK1.1^{+} natural killer cells almost disappeared after knock out of NFIL3 (**Fig. S2, S3**). To evaluate whether NFIL3 deficiency would affect the pathogenesis of EAE, an active immunization model was established with MOG_{35-55} peptide.

Surprisingly, we found that NFIL3 deficiency alleviated EAE rather than exacerbated it (Fig. 1). At the onset of EAE, the clinical scores were similar. However, the disease severity of KO mice was milder than WT mice at the peak phase (Fig. 1**A**). Besides, the incidence of EAE in KO mice was lower (Fig. 1**B**). The EAE mice were sacrificed at day 25. In spinal cords of KO EAE mice, HE staining showed there was less lymphocyte infiltration while Luxol Fast Blue staining showed milder demyelination, which was in accordance with the clinical symptoms (Fig. 1**C, D**). Collectively, these results suggested that NFIL3 deficiency could ameliorate EAE.

NFIL3 deficiency decreased Th17 cells within the CNS

To investigate how NFIL3 deficiency affected the initiation and progression of EAE, we next measured CD4^{+}T cells in the brains and spinal cords, including IFN-γ secreting Th1 cells, IL-17A secreting Th17 cells, IL-10 secreting CD4^{+}T cells, and regulatory T cells (Tregs). As expected, the proinflammatory Th17 cells (CD4^{+}IL17-A^{+}) significantly decreased within the CNS of NFIL3$^{-/-}$ mice when compared with WT mice (Fig. 2**A**) (Brain: p = 0.0088, Spinal cord: p = 0.0155). Meanwhile, Th1 cells (CD4^{+}IFN-γ^{+}) tended to increase though there was no statistical significance (Fig. 2B) (Brain: p = 0.0708, Spinal cord: p = 0.1557). As revealed in previous study [2], Tregs (CD4^{+}Foxp3^{+}) and Type 1 regulatory cells (Tr1, IL-10^{+}Foxp3^{-}) could suppress EAE through regulating anti-inflammatory cytokine IL-10. Interestingly, both Foxp3^{+} Tregs and IL-10^{+}CD4^{+} T cells decreased in spinal cords of NFIL3$^{-/-}$ mice but showed no changes in brains in this study (Fig. 2**C, D**) (Treg: Brain: p = 0.8206, Spinal cord: p = 0.0239; IL-10^{+}CD4^{+} T: Brain: p = 0.2843, Spinal cord: p = 0.0057). Additionally, Foxp3^{-}IL-10^{+}Tr1 cells did not change within the CNS (Tr1: Brain: p = 0.6429, Spinal cord: p = 0.9668), while Foxp3^{+}IL-10^{+} Tregs decreased in spinal cords (Fig. 2**D**) (p = 0.0057). Therefore, our results suggested that NFIL3 deficiency resulting in decrease of Th17 cells within the CNS could alleviate EAE.

NFIL3 deficiency affected the immune response outside the CNS

The immune response outside the CNS had also been evaluated. Th17, Th1, Treg, Tr1 and CD4^{+}IL-10^{+}T cells were detected in spleens and lymph nodes of EAE mice. However, the percentages of these T cells were similar between WT and KO mice (Fig. 3**A-D**). In order to

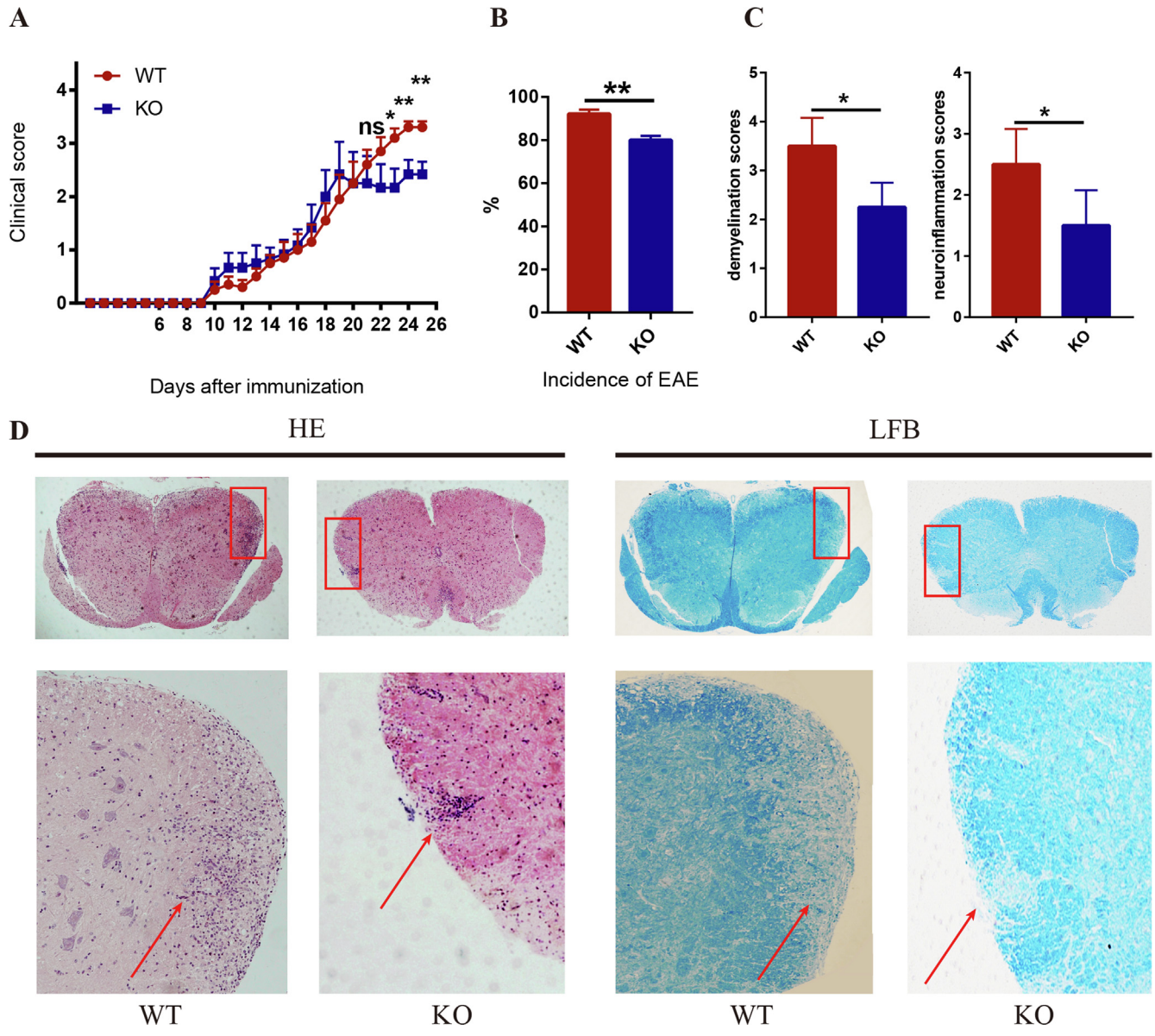

Fig. 1. NFIL3 deficiency alleviated MOG35-55 induced EAE. Wild-type C57BL/6J mice (WT) and NFIL3 knock out mice (KO) were immunized at day 0 with MOG_{35-55} in complete Freund's adjuvant. From the day of immunization (day 0), the condition of the mice was assessed and the clinical scores of the mice were graded and recorded daily. (A) The EAE clinical scores. (B) The incidence of EAE model. (C) The scores of neuroinflammation and demyelination in EAE. (D) HE and LFB staining for the spinal cords of EAE mice. The mice were age and sex matched, n = 8 for each group. All the results are displayed from three independent experiments. *t*-test was used when only two groups were compared. The EAE scores were analyzed by a two tailed Mann–Whitney *U* test. Data are shown as mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns, not significant.

further investigate the changes of immune cells outside the CNS, several other immune parameters had also been examined. Th2 ($CD4^+IL\text{-}4^+$) cells, Th9 ($CD4^+IL\text{-}9^+$) cells and GM-CSF^+CD4^+T cells decreased **(**Fig. 4**A-C)** in $NFIL3^{-/-}$ EAE mice (Th2: spleen: $p = 0.0480$, lymph nodes: $p = 0.5631$; Th9: spleen: $p = 0.0057$, lymph nodes: $p = 0.0353$; GM-CSF^+CD4^+T: spleen: $p = 0.0026$, lymph nodes: $p = 0.0346$), while PD-1 and ICOS on the $CD4^+$T cells increased **(**Fig. 4**D, E)** (PD1: spleen: $p = 0.0019$, lymph nodes: $p = 0.0492$; ICOS: spleen: $p = 0.0016$, lymph nodes: $p = 0.0027$). Besides, the subset of $CD3^+CD8^+CD103^+$T cells decreased obviously in spleens, lymph nodes and blood of $NFIL3^{-/-}$mice **(**Fig. 4**G) (**$p = 0.0027, 0.0221, 0.0373$**).** Moreover, we found that membrane CD126 decreased in the splenocytes of $NFIL3^{-/-}$ EAE mice **(**Fig. 4**F)**, while the titre of soluble CD126 in the serum was elevated **(**Fig. 4**H)** (spleen: $p = 0.0113$, lymph nodes: $p = 0.3879$, serum: $p = 0.0286$). In general, these data suggested NFIL3 dificiency could affect the immune reactions outside the CNS.

NFIL3 deficiency affected $CD11c^+$ dendritic cells both in vivo and in vitro

DCs are the most powerful and professional APCs. $CD11c^+$ DCs were found to be impaired in spleens and lymph nodes of $NFIL3^{-/-}$ EAE mice. The MFI suggested that the expression of CD11c, CD80 and CD86 declined simultaneously, while MCH II increased in $NFIL3^{-/-}$ EAE mice compared with WT group **(**Fig. 5**A)** (CD11c: lymph nodes: $p = 0.0267$, spleen: $p = 0.0033$; CD80: lymph nodes: $p = 0.0006$, spleen: $p = 0.0581$; CD86: lymph nodes: $p = 0.0044$, spleen: $p = 0.05438$; MHC II: lymph nodes: $p = 0.1226$, spleen: $p = 0.0106$). For further investigating the impact of NFIL3 on $CD11c^+$ DCs, we induced the BMDCs in vitro.

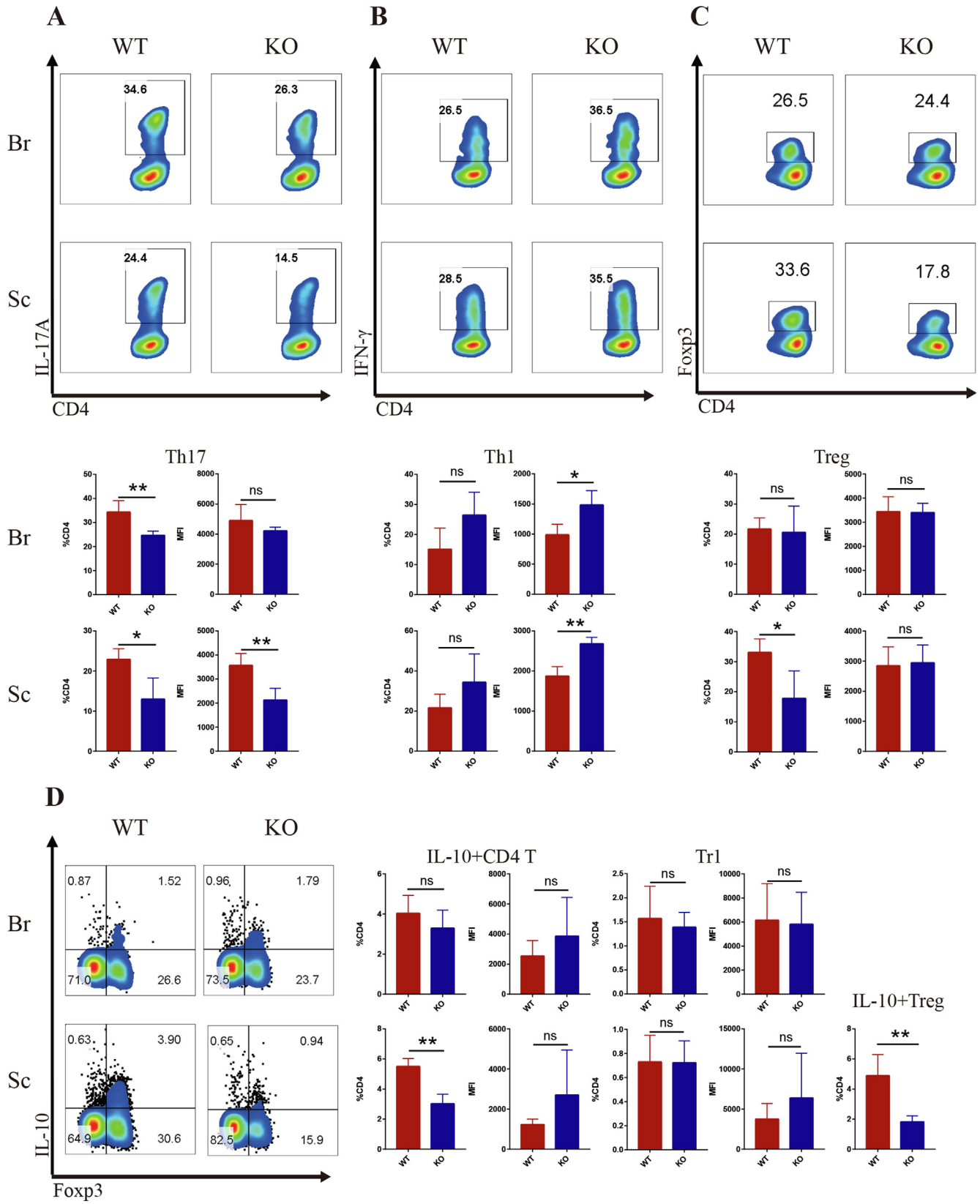

Fig. 2. NFIL3 deficiency decreased Th17 cells within the CNS. EAE mice were sacrificed at Day 25. Different subsets of $CD4^+$ T cells were isolated from brains (Br) and spinal cords (Sc) of EAE mice and were measured by flow cytometry. Th17 cells (A), Th1 cells (B), Tregs (C) and IL-10 secreting $CD4^+$ T cells (D) from WT or $NFIL3^{-/-}$ EAE mice. Flow charts gated from $CD4^+$ subsets. All the results are displayed from three independent experiments. Data are shown as mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns, not significant.

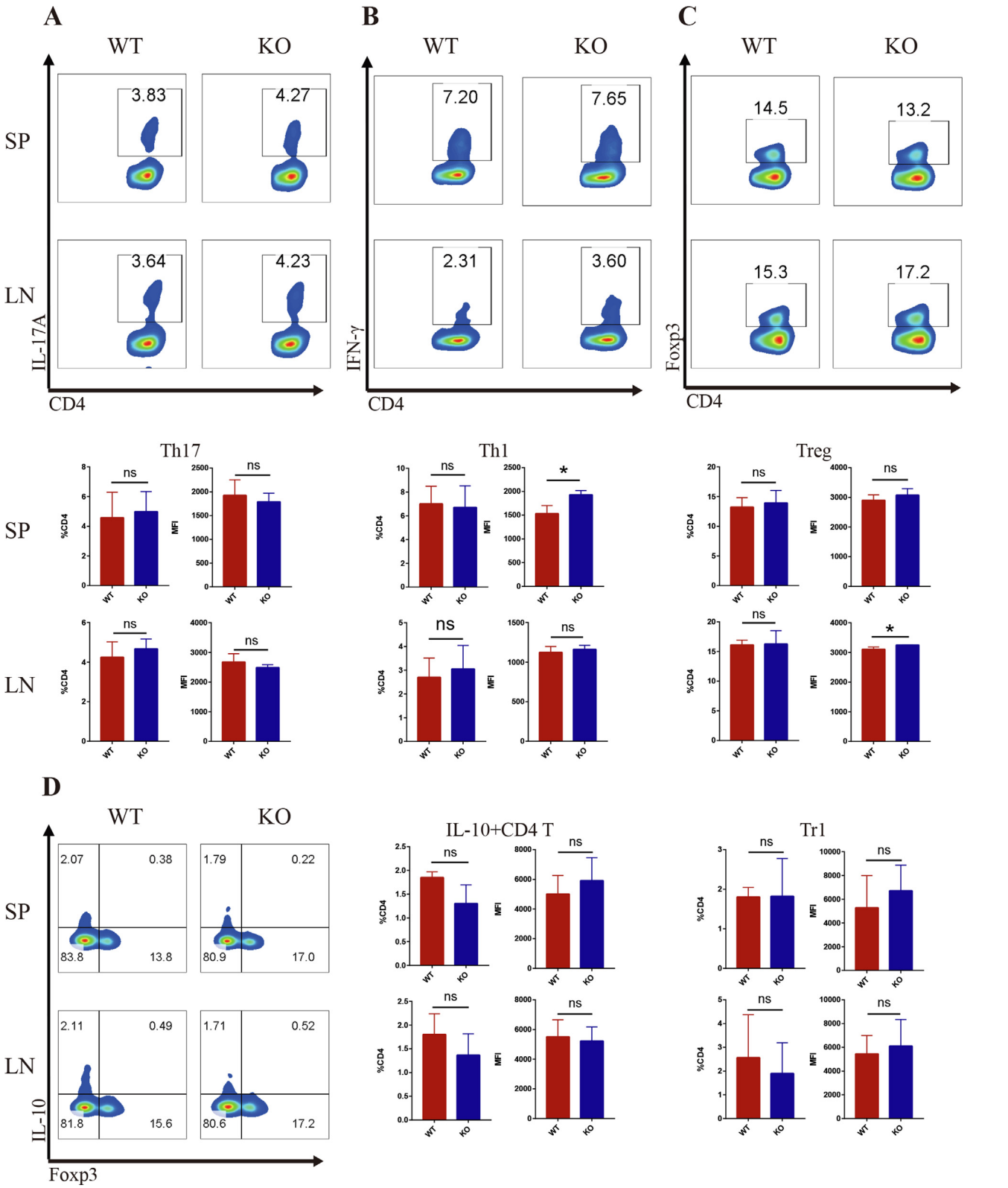

Fig. 3. NFIL3 deficiency affected the immune cells response outside the CNS. Immune cells in spleens (SP) and lymph nodes (LN) were measured by flow cytometry when the EAE mice were sacrificed. (A-D) Th17 cells, Th1 cells, Tregs, IL-10 secreting $CD4^+$ T cells and $Foxp3^-IL\text{-}10^+$ Tr1 cells of spleens and lymph nodes from EAE mice. Flow charts gated from $CD4^+$ subsets. All the results are displayed from three independent experiments. Data are shown as mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns, not significant.

In accordance to the in vivo result, we found that NFIL3 deficiency led to a decline in BMDCs induction **(**Fig. 5**B) (**$p = 0.0189$). Besides, the expressions of CD80, CD86 and CD69 decreased as compared to WT BMDCs **(**Fig. 5**C)** (CD80, CD86, CD69: $p <$ 0.0001). In addition, lower level of apoptosis was observed in $NFIL3^{-/-}$ BMDCs **(**Fig. 5**D)** ($p = 0.0430$). Then we examined the cytokine secretion capacity of $NFIL3^{-/-}$ BMDCs in vitro. Interestingly, the secretion of pro-inflammatory cytokines, like IFN-γ, TNF-α, IL-2,

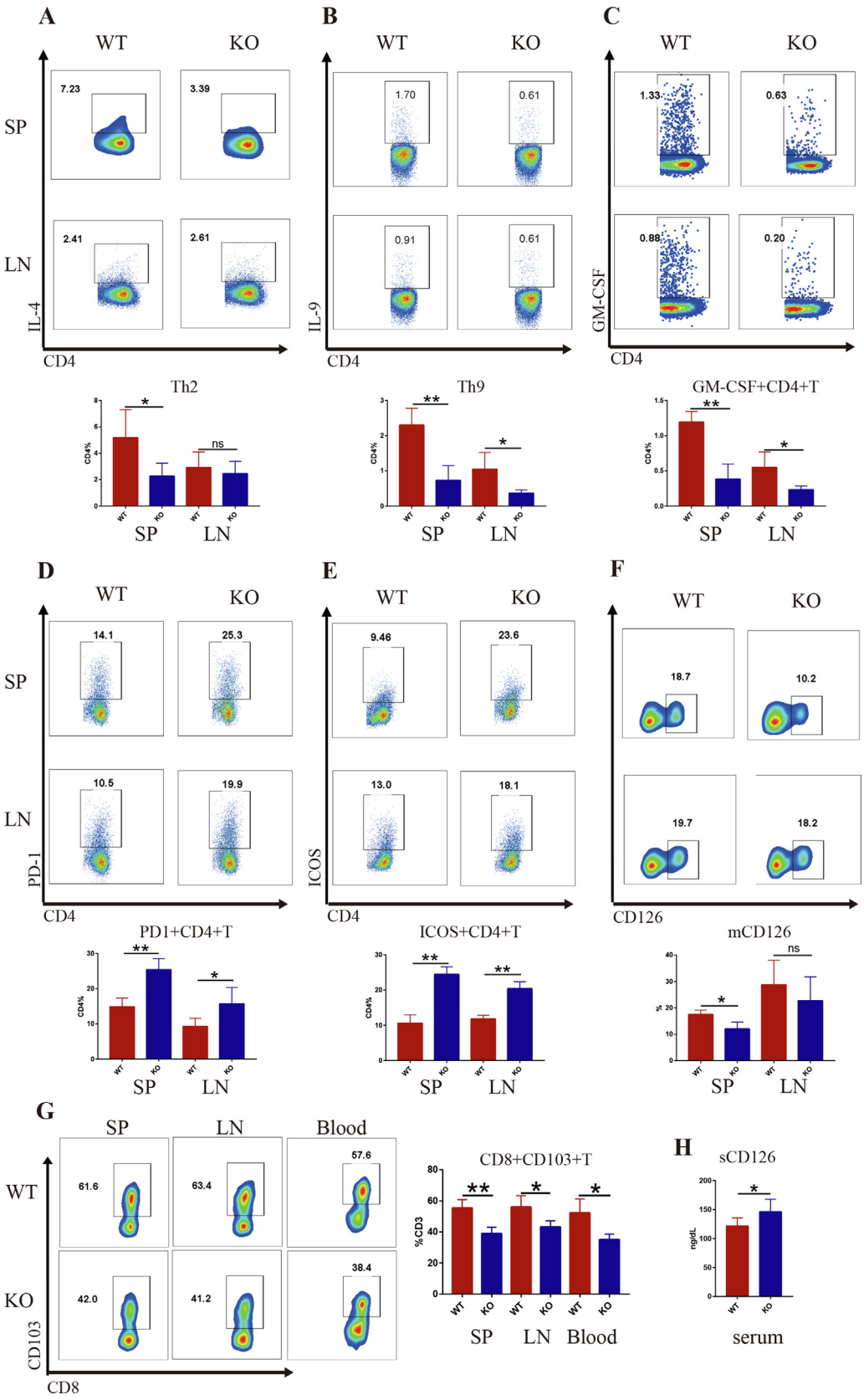

Fig. 4. NFIL3 deficiency affected other immune cells outside the CNS. (A, B) Th2 cells, Th9 cells of spleens and lymph nodes from EAE mice. (C-F) GM-CSF, PD-1, ICOS and CD126 of spleens and lymph nodes from EAE mice. (G) $CD8^+CD103^+$ T cells of spleens and lymph nodes from EAE mice. Gated on $CD3^+$ T cells. (H) Soluble CD126 in sera from EAE mice. All the results are displayed from three independent experiments. Data are shown as mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns, not significant.

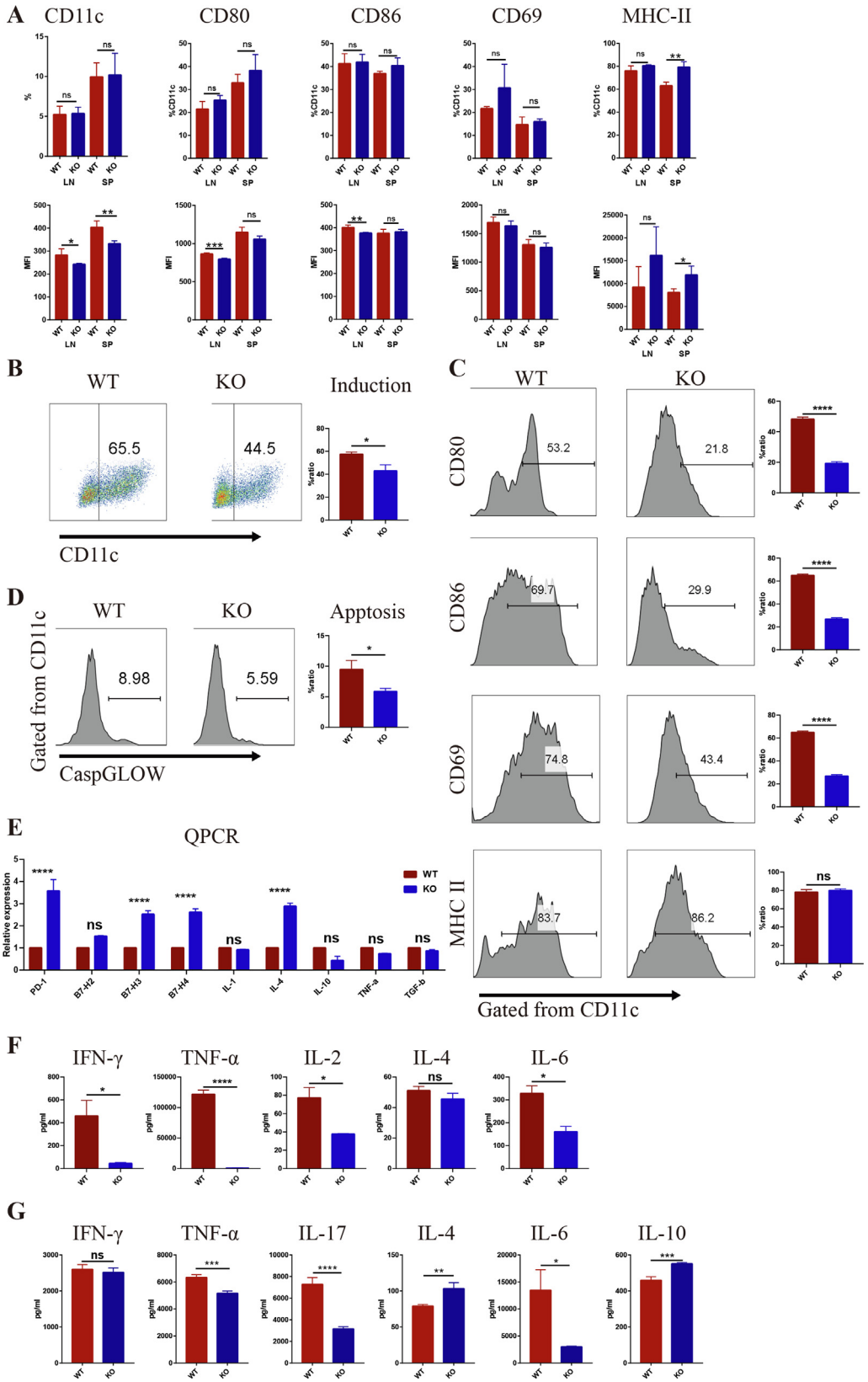

Fig. 5. NFIL3 deficiency affected CD11c^{+} dendritic cells both in vivo and in vitro. (A) The MFI level of CD11c, CD80, CD86, CD69 and MHC II in spleens and lymph nodes from EAE model mice. BMDCs ratio(B), the expression of CD80, CD86, CD69 and MHC II on WT or NFIL3 deficiency BMDCs (C) and BMDCs apoptosis(D). (E) QPCR array of relative molecules from WT or NFIL3 deficiency BMDCs. (F) Cytokines secretion from WT or NFIL3 deficiency BMDCs. (G) Cytokines secretion from T cells after the stimulation of WT or NFIL3 deficiency BMDCs. All the results are displayed from three independent experiments. Data are shown as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns, not significant.

and IL-6, were reduced in NFIL3$^{-/-}$ BMDCs, while there were no significant changes of IL-4 **(**Fig. 5**F)** (IFNγ: p = 0.0384, TNF-α: $p <$ 0.0001, IL-2: p = 0.0237, IL-6: p = 0.0232, IL-4: p = 0.2921). Meanwhile, the mRNA level of PD-1, B7-H3, B7-H4, IL-4 increased in NFIL3$^{-/-}$ BMDCs, while B7-H2, IL-1, IL10, TNFα and TGF-β didn't change when compared to WT BMDCs **(**Fig. 5**E).** Next, we explored whether the decline of DCs' pro-inflammatory function has effect on CD4$^+$ T cells. We cocultured the BMDCs with CD4$^+$ T cells and then detected the cytokines in the coculture medium. As shown in Fig. 5**G**, the secretion of IL-17, TNFα, and IL-6 decreased while IL-4 and IL-10 increased in NFIL3$^{-/-}$ group (IL-17: $p < 0.0001$, TNFα: p = 0.0007, IL-6: p = 0.0229, IL-4: p = 0.0084, IL-10: p = 0.0001). Taken these results together, it suggested that NFIL3 deficiency might alleviate EAE through promoting anti-inflammatory capacity and impairing pro-inflammatory capacity of CD11c$^+$ DCs.

Discussion

In this study, we used wildtype C57BL/6J mice and NFIL3$^{-/-}$ mice to establish MOG_{35-55}-induced EAE. Although the total numbers of CD4$^+$ T cells and CD8$^+$ T cells were similar in normal NFIL3$^{-/-}$ mice and WT mice, T cell subsets such as Th1/Th2/Th17/Tregs/CD8$^+$-CD103$^+$ T cells were different between NFIL3$^{-/-}$ and WT EAE mice. The NFIL3$^{-/-}$ EAE mice showed lower clinical scores, milder neuroinflammation and demyelination, as well as decreased Th17 cells within the CNS. Besides, the anti-inflammatory capacity was promoted while the pro-inflammatory capacity was impaired in NFIL3$^{-/-}$CD11c$^+$ DCs. Our results suggest that knockout of NFIL3 could alter the immune response in EAE.

Autoreactive T cells, especially Th17 cells, are critical in the initiation and progression of EAE [30], while Tregs play a role in controlling autoimmune cells and maintaining homeostasis of micro circumstance [31]. The imbalance between Th17 cells and Tregs would result in neuroinflammation [32]. Unexpectedly, our data showed that Th17 cells and Tregs decreased simultaneously within the CNS of NFIL3$^{-/-}$ EAE mice. This controversy might be due to the characteristics of Tregs during EAE. Tregs were unable to control the autoimmune reaction within the CNS at the peak phase [33], but act as a pivotal role in regulating recovery [34]. Therefore, the alleviation of NFIL3$^{-/-}$ EAE might be mainly due to the reduction of Th17 cells within the CNS.

Meanwhile, a complex immune reaction was also observed in the periphery in NFIL3$^{-/-}$ EAE. PD-1 and ICOS on CD4$^+$ T cells increased in NFIL3$^{-/-}$ mice, which were consistent with Wang's research [24], suggesting the function of T follicular helper cells might be enhanced. Besides, the reduction of IL-4-secreting Th2 cells was as reported before [23]. Previous research [35] had revealed controversial roles of Th9 cells in EAE, and in this study IL-9 decreased in NFIL3$^{-/-}$ EAE. In addition, CD103 had been reported to help distinguishing CD8$^+$ Treg from non-Tregs [36]. CD8$^+$CD103$^+$ Tregs induced *ex vivo* (CD8$^+$iTreg) could act as immunosuppressor regardless of Foxp3 expression [37]. In the current study, however, CD8$^+$CD103$^+$ T cells decreased simultaneously in spleens, lymph nodes and blood of NFIL3$^{-/-}$ mice, which were more likely to be CD8$^+$ iTregs instead of tissue-resident memory CD8$^+$ T cells referring to previous research [38]. But the exact role of this subset in EAE is still unknown. In addition, the upregulation of soluble CD126 in serum but downregulation of membrane CD126 in spleens and lymph nodes might be another reason for the unapparent increase of Th17 cells in the periphery.

The CNS is not routinely surveyed by immune cells due to the existence of blood brain barrier (BBB) and blood-CSF barrier. An "outside-in hypothesis" believes that the initiation of CNS inflammation begins with autoreactive CD4$^+$ T cells activated in the periphery. These cells would be recruited into the CNS and result in neuroinflammation [32]. Accumulating evidences had indicated that GM-CSF is indispensable in commanding CNS invasion [39–40]. In this study, a reduction of GM-CSF on CD4$^+$ T cells was observed in the periphery of NFIL3$^{-/-}$ EAE mice. NFIL3 was capable of *trans*-activating IL-3 promoter [4], while IL-3 could promote the influx of T cells into the brain and promote EAE [41]. Therefore, the recruitment of T cells into the CNS might be impaired in NFIL3$^{-/-}$ EAE mice. Besides, it was believed that encephalitogenic CD4$^+$ T cells were primed in the periphery by APCs such as DCs, which were able to shape the fate of naïve CD4$^+$ T cells through secreting cytokines directly [42–43]. In this study, the capacity of NFIL3$^{-/-}$ CD11c$^+$ DCs was changed. Along with the increase of PD-1, B7-H3, B7-H4 and IL-4, the decrease of IFN-γ, TNFα, and IL-6, the pro-inflammatory capacity was promoted while anti-inflammatory capacity was impaired in NFIL3$^{-/-}$ BMDCs. In fact, DCs are discovered as therapeutic targets in neuroinflammation [42,44–45]. To the best of our knowledge, the most obvious change in NFIL3$^{-/-}$ mice is the deficiency of NK cells. Although previous research [46] about the effect of NK cells in EAE were controversial, it seems to act as a proinflammatory subset in our study.

Nevertheless, as mentioned in the Introduction, our data is different from two previous studies by Motomura and Farez [21,27]. It's a pity that both studies did not show the alteration of immune cells within the CNS. Considering such a controversy, we had conducted the experiment of EAE induction for three times independently, and the clinical manifestations were similar. In our study, knockout of NFIL3 did show a capacity to alleviate EAE. Some explanations might be as follows. Firstly, Motomura's study mainly focused on IL-10 secretion. In fact, in our study, a decrease of IL-10 was also observed within the CNS in NFIL3$^{-/-}$ EAE mice. IL-10 plays an anti-inflammatory role at the stage of remission. The effect of IL-17 reduction might outweigh the reduction of IL-10, leading to a milder neuroinflammation. Secondary, the NFIL3$^{-/-}$ mice were from different labs, and the housing circumstances were different, which might also affect EAE. Thirdly, although research suggested NFIL3 could restrain Th17 cell differentiation by directly repressing transcription factor ROR-γt [22], other studies showed that NFIL3 did not affect Th17 cell differentiation [21,47]. Our results did not mean to challenge the intrinsic effect of NFIL3 on Th17 cells within the CNS, but to show a general effect on EAE after knockout of NFIL3.

There are some limitations in the current study. Firstly, this study only analyzed the immune response at the acute phase at Day 25, a kinetic analysis at different time would be helpful for our better understanding of the role of NFIL3 in EAE. Secondary, the EAE model induced by MOG_{35-55} on C57BL/6 background mice is an acute and monophasic model of MS without remitting. It is CD4$^+$ T cells driven, while CD8$^+$ T cells play little role in it. Although this model is the most classic and widely used EAE model to explore MS, it is limited and unable to imitate every aspect of MS. More direct evidence is needed to verify the exact role of NFIL3 in MS. Moreover, this study mainly focused on the general effect of NFIL3 on EAE. Considering the comprehensive effect of NFIL3 on immune system, this effect might be direct or indirect, and the precise mechanism remains to be elucidated. Besides, there is a lack of data in vivo about how DCs interplay with T cells. In order to find out the underlying mechanism about how NFIL3 affects EAE and MS, further experiments such as kinetics analysis about immune response during EAE and constructing other models of MS, are necessary in the future.

Conclusion

Taken these together, we propose that NFIL3 deficiency could alleviate MOG_{35-55}-induced EAE by regulating both innate and adaptive immunity as well as the cross-talk between them, espe-

cially DCs and T cells. Knockout of NFIL3 might inhibit the recruitment of T cells into the CNS, which eventually resulted in the alleviation in neurologic dysfunction in EAE.

CRediT authorship contribution statement

Zhigang Chen: Methodology, Software, Validation, Formal analysis, Data curation, Investigation, Writing – original draft, Writing – review & editing. **Rong Fan:** Methodology, Software, Formal analysis, Data curation, Investigation, Writing – original draft, Writing – review & editing. **Jie Liang:** Methodology, Software, Formal analysis, Data curation, Investigation, Writing – original draft, Writing – review & editing. **Zexiu Xiao:** Methodology, Investigation, Resources, Data curation, Writing – review & editing. **Junlong Dang:** Methodology, Investigation, Resources, Data curation. **Jun Zhao:** Investigation, Resources. **Ruihui Weng:** Investigation, Resources. **Cansheng Zhu:** Supervision, Project administration, Funding acquisition. **Song Guo Zheng:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration. **Ying Jiang:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

[1] Procaccini C, De Rosa V, Pucino V, Formisano L, Matarese G. Animal models of Multiple Sclerosis. Eur J Pharmacol 2015;759:182–91. doi: https://doi.org/10.1016/j.ejphar.2015.03.042.
[2] Hemmer B, Kerschensteiner M, Korn T. Role of the innate and adaptive immune responses in the course of multiple sclerosis. Lancet Neurol 2015;14(4):406–19. doi: https://doi.org/10.1016/S1474-4422(14)70305-9.
[3] Thompson AJ, Baranzini SE, Geurts J, Hemmer B, Ciccarelli O. Multiple sclerosis. Lancet 2018;391(10130):1622–36. doi: https://doi.org/10.1016/S0140-6736(18)30481-1.
[4] Zhang W, Zhang J, Kornuc M, Kwan K, Frank R, Nimer SD. Molecular cloning and characterization of NF-IL3A, a transcriptional activator of the human interleukin-3 promoter. Mol Cell Biol. 1995;15(11):6055–63. doi: https://doi.org/10.1128/MCB.15.11.6055.
[5] Ikushima S, Inukai T, Inaba T, Nimer SD, Cleveland JL, Look AT. Pivotal role for the NFIL3/E4BP4 transcription factor in interleukin 3-mediated survival of pro-B lymphocytes. Proc Natl Acad Sci U S A. 1997;94(6):2609–14. doi: https://doi.org/10.1073/pnas.94.6.2609.
[6] Cowell IG. E4BP4/NFIL3, a PAR-related bZIP factor with many roles. BioEssays 2002;24(11):1023–9. doi: https://doi.org/10.1002/bies.10176.
[7] Mitsui S, Yamaguchi S, Matsuo T, Ishida Y, Okamura H. Antagonistic role of E4BP4 and PAR proteins in the circadian oscillatory mechanism. Genes Dev. 2001;15(8):995–1006. doi: https://doi.org/10.1101/gad.873501.
[8] Doi M, Okano T, Yujnovsky I, Sassone-Corsi P, Fukada Y. Negative control of circadian clock regulator E4BP4 by casein kinase Iepsilon-mediated phosphorylation. Curr Biol. 2004;14(11):975–80. doi: https://doi.org/10.1016/j.cub.2004.05.043.
[9] Junghans D, Chauvet S, Buhler E, Dudley K, Sykes T, Henderson CE. The CES-2-related transcription factor E4BP4 is an intrinsic regulator of motoneuron growth and survival. Development. 2004;131(18):4425–34. doi: https://doi.org/10.1242/dev.01313.
[10] MacGillavry HD, Cornelis J, van der Kallen LR, Sassen MM, Verhaagen J, Smit AB, et al. Genome-wide gene expression and promoter binding analysis identifies NFIL3 as a repressor of C/EBP target genes in neuronal outgrowth. Mol Cell Neurosci. 2011;46(2):460–8. doi: https://doi.org/10.1016/j.mcn.2010.11.011.
[11] Male V, Nisoli I, Gascoyne DM, Brady HJM. E4BP4: an unexpected player in the immune response. Trends Immunol. 2012;33(2):98–102. doi: https://doi.org/10.1016/j.it.2011.10.002.
[12] Seillet C, Rankin LC, Groom JR, Mielke LA, Tellier J, Chopin M, et al. Nfil3 is required for the development of all innate lymphoid cell subsets. J Exp Med 2014;211(9):1733–40. doi: https://doi.org/10.1084/jem.20140145.
[13] Yin J, Zhang J, Lu Q. The role of basic leucine zipper transcription factor E4BP4 in the immune system and immune-mediated diseases. Clin Immunol. 2017;180:5–10. doi: https://doi.org/10.1016/j.clim.2017.03.013.
[14] Gascoyne DM, Long E, Veiga-Fernandes H, de Boer J, Williams O, Seddon B, et al. The basic leucine zipper transcription factor E4BP4 is essential for natural killer cell development. Nat Immunol. 2009;10(10):1118–24. doi: https://doi.org/10.1038/ni.1787.
[15] Kamizono S, Duncan GS, Seidel MG, Morimoto A, Hamada K, Grosveld G, et al. Nfil3/E4bp4 is required for the development and maturation of NK cells in vivo. J Exp Med 2009;206(13):2977–86. doi: https://doi.org/10.1084/jem.20092176.
[16] Kostrzewski T, Borg AJ, Meng Y, Filipovic I, Male V, Wack A, et al. Multiple Levels of Control Determine How E4bp4/Nfil3 Regulates NK. J Immunol . 2018;200(4):1370–81. doi: https://doi.org/10.4049/jimmunol.1700981.
[17] Seillet C, Huntington ND, Gangatirkar P, Axelsson E, Minnich M, Brady HJM, et al. Differential requirement for Nfil3 during NK cell development. J Immunol. 2014;192(6):2667–76. doi: https://doi.org/10.4049/jimmunol.1302605.
[18] Kashiwada M, Pham NL, Pewe LL, Harty JT, Rothman PB. NFIL3/E4BP4 is a key transcription factor for CD8alpha(+) dendritic cell development. Blood 2011;117(23):6193–7. doi: https://doi.org/10.1182/blood-2010-07-295873.
[19] Seillet C, Jackson JT, Markey KA, Brady HJ, Hill GR, Macdonald KP, et al. CD8alpha+ DCs can be induced in the absence of transcription factors Id2, Nfil3, and Batf3. Blood 2013;121(9):1574–83. doi: https://doi.org/10.1182/blood-2012-07-445650.
[20] Kobayashi T, Matsuoka K, Sheikh SZ, Elloumi HZ, Kamada N, Hisamatsu T, et al. NFIL3 is a regulator of IL-12 p40 in macrophages and mucosal immunity. J Immunol. 2011;186(8):4649–55. doi: https://doi.org/10.4049/jimmunol.1003888.
[21] Motomura Y, Kitamura H, Hijikata A, Matsunaga Y, Matsumoto K, Inoue H, et al. The transcription factor E4BP4 regulates the production of IL-10 and IL-13 in CD4+ T cells. Nat Immunol. 2011;12(5):450–9. doi: https://doi.org/10.1038/ni.2020.
[22] Yu X, Rollins D, Ruhn KA, Stubblefield JJ, Green CB, Kashiwada M, et al. TH17 cell differentiation is regulated by the circadian clock. Science 2013;342(6159):727–30. doi: https://doi.org/10.1126/science.1243884.
[23] Kashiwada M, Cassel SL, Colgan JD, Rothman PB. NFIL3/E4BP4 controls type 2 T helper cell cytokine expression. EMBO J. 2011;30(10):2071–82. doi: https://doi.org/10.1038/emboj.2011.111.
[24] Wang Z, Zhao M, Yin J, Liu L, Hu L, Huang Y, et al. E4BP4-mediated inhibition of T follicular helper cell differentiation is compromised in autoimmune diseases. J Clin Invest 2020;130(7):3717–33. doi: https://doi.org/10.1172/JCI129018.
[25] Zhao M, Liu Q, Liang G, Wang L, Luo S, Tang Q, et al. E4BP4 overexpression: a protective mechanism in CD4+ T cells from SLE patients. J Autoimmun. 2013;41:152–60. doi: https://doi.org/10.1016/j.jaut.2013.01.004.
[26] Schlenner S, Pasciuto E, Lagou V, Burton O, Prezzemolo T, Junius S, et al. NFIL3 mutations alter immune homeostasis and sensitise for arthritis pathology. Ann Rheum Dis 2019;78(3):342–9. doi: https://doi.org/10.1136/annrheumdis-2018-213764.
[27] Farez M, Mascanfroni I, Méndez-Huergo S, Yeste A, Murugaiyan G, Garo L, et al. Melatonin Contributes to the Seasonality of Multiple Sclerosis Relapses. Cell 2015;162(6):1338–52. doi: https://doi.org/10.1016/j.cell.2015.08.025.
[28] Xiao ZX, Hu X, Zhang X, Chen Z, Wang J, Jin Ke, et al. High salt diet accelerates the progression of murine lupus through dendritic cells via the p38 MAPK and STAT1 signaling pathways. Signal Transduct Target Ther 2020;5(1). doi: https://doi.org/10.1038/s41392-020-0139-5.
[29] Zappia E, Casazza S, Pedemonte E, Benvenuto F, Bonanni I, Gerdoni E, et al. Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. Blood 2005;106(5):1755–61. doi: https://doi.org/10.1182/blood-2005-04-1496.
[30] Segal B. The Diversity of Encephalitogenic CD4+ T Cells in Multiple Sclerosis and Its Animal Models. J Clin Med 2019;8(1):120. doi: https://doi.org/10.3390/jcm8010120.
[31] Vasileiadis GK, Dardiotis E, Mavropoulos A, Tsouris Z, Tsimourtou V, Bogdanos DP, et al. Regulatory B and T lymphocytes in multiple sclerosis: friends or foes? Auto Immun Highlights 2018;9(1):9. doi: https://doi.org/10.1007/s13317-018-0109-x.
[32] Korn T, Kallies A. T cell responses in the central nervous system. Nat Rev Immunol 2017;17(3):179–94. doi: https://doi.org/10.1038/nri.2016.144.
[33] Korn T, Reddy J, Gao W, Bettelli E, Awasthi A, Petersen TR, et al. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune

inflammation. Nat Med. 2007;13(4):423–31. doi: https://doi.org/10.1038/nm1564.
[34] Koutrolos M, Berer K, Kawakami N, Wekerle H, Krishnamoorthy G. Treg cells mediate recovery from EAE by controlling effector T cell proliferation and motility in the CNS. Acta Neuropathol Commun 2014;2:163. doi: https://doi.org/10.1186/s40478-014-0163-1.
[35] Elyaman W, Khoury SJ. Th9 cells in the pathogenesis of EAE and multiple sclerosis. Semin Immunopathol 2017;39(1):79–87. doi: https://doi.org/10.1007/s00281-016-0604-y.
[36] Keino H, Masli S, Sasaki S, Streilein JW, Stein-Streilein J. CD8+ T regulatory cells use a novel genetic program that includes CD103 to suppress Th1 immunity in eye-derived tolerance. Invest Ophthalmol Vis Sci 2006;47(4):1533–42. doi: https://doi.org/10.1167/iovs.04-1454.
[37] Liu Y, Lan Q, Lu L, Chen M, Xia Z, Ma J, et al. Phenotypic and functional characteristic of a newly identified CD8+ Foxp3- CD103+ regulatory T cells. J Mol Cell Biol 2014;6(1):81–92. doi: https://doi.org/10.1093/jmcb/mjt026.
[38] Wu H, Liao W, Li Q, Long H, Yin H, Zhao M, et al. Pathogenic role of tissue-resident memory T cells in autoimmune diseases. Autoimmun Rev. 2018;17(9):906–11. doi: https://doi.org/10.1016/j.autrev.2018.03.014.
[39] Croxford AL, Spath S, Becher B. GM-CSF in Neuroinflammation: Licensing Myeloid Cells for Tissue Damage. Trends Immunol. 2015;36(10):651–62. doi: https://doi.org/10.1016/j.it.2015.08.004.
[40] Zhao J, Sun L, Li X. Commanding CNS Invasion: GM-CSF. Immunity 2017;46(2):165–7. doi: https://doi.org/10.1016/j.immuni.2017.02.003.
[41] Renner K, Hellerbrand S, Hermann F, Riedhammer C, Talke Y, Schiechl G, et al. IL-3 promotes the development of experimental autoimmune encephalitis. JCI Insight 2016;1(16):. doi: https://doi.org/10.1172/jci.insight.87157e87157.
[42] Comabella M, Montalban X, Münz C, Lünemann JD. Targeting dendritic cells to treat multiple sclerosis. Nature reviews Neurology. 2010;6(9):499–507. doi: https://doi.org/10.1038/nrneurol.2010.112.
[43] Yang C, Lai W, Zhou J, Zheng X, Cai Y, Yang W, et al. Betaine Ameliorates Experimental Autoimmune Encephalomyelitis by Inhibiting Dendritic Cell-Derived IL-6 Production and Th17 Differentiation. J Immunol. 2018;200(4):1316–24. doi: https://doi.org/10.4049/jimmunol.1700920.
[44] Giles D, Duncker P, Wilkinson N, Washnock-Schmid J, Segal B. CNS-resident classical DCs play a critical role in CNS autoimmune disease. J Clin Invest 2018;128(12):5322–34. doi: https://doi.org/10.1172/JCI123708.
[45] Lüssi F, Zipp F, Witsch E. Dendritic cells as therapeutic targets in neuroinflammation. Cell Mol Life Sci 2016;73(13):2425–50. doi: https://doi.org/10.1007/s00018-016-2170-9.
[46] Liu Q, Sanai N, Jin W-N, La Cava A, Van Kaer L, Shi F-D. Neural stem cells sustain natural killer cells that dictate recovery from brain inflammation. Nat Neurosci 2016;19(2):243–52. doi: https://doi.org/10.1038/nn.4211.
[47] Ciofani M, Madar A, Galan C, Sellars MacLean, Mace K, Pauli F, et al. A validated regulatory network for Th17 cell specification. Cell 2012;151(2):289–303. doi: https://doi.org/10.1016/j.cell.2012.09.016.