

Microglia and Dendritic Cells as a Source of IL-6 in a Mouse Model of Multiple Sclerosis

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Abstract—Multiple sclerosis (MS) is a complex autoimmune disease of central nervous system (CNS) characterized by the myelin sheath destruction and compromised nerve signal transmission. Understanding molecular mechanisms driving MS development is critical due to its early onset, chronic course, and therapeutic approaches based only on symptomatic treatment. Cytokines are known to play a pivotal role in the MS pathogenesis with interleukin-6 (IL-6) being one of the key mediators. This study investigates contribution of IL-6 produced by microglia and dendritic cells to the development of experimental autoimmune encephalomyelitis (EAE), a widely used mouse model of MS. Mice with conditional inactivation of IL-6 in the CX₃CR1⁺ cells, including microglia, or CD11c⁺ dendritic cells, displayed less severe symptoms as compared to their wild-type counterparts. Mice with microglial IL-6 deletion exhibited an elevated proportion of regulatory T cells and reduced percentage of pathogenic IFN γ -producing CD4⁺ T cells, accompanied by the decrease in pro-inflammatory monocytes in the CNS at the peak of EAE. At the same time, deletion of IL-6 from microglia resulted in the increase of CCR6⁺ T cells and GM-CSF-producing T cells. Conversely, mice with IL-6 deficiency in the dendritic cells showed not only the previously described increase in the proportion of regulatory T cells and decrease in the proportion of T_H17 cells, but also reduction in the production of GM-CSF and IFN γ in the secondary lymphoid organs. In summary, IL-6 functions during EAE depend on both the source and localization of immune response: the microglial IL-6 exerts both pathogenic and protective functions specifically in the CNS, whereas the dendritic cell-derived IL-6, in addition to being critically involved in the balance of regulatory T cells and T_H17 cells, may stimulate production of cytokines associated with pathogenic functions of T cells.

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INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune disease that affects the central nervous system (CNS) and causes severe consequences, including visual disturbances, motor and cognitive impairments. In recent years, there has been an increasing trend in the

number of people diagnosed with MS, however, only symptomatic treatments aimed at modulating the symptoms are currently available [1]. Therefore, the search for new therapeutic targets remains a very important task. One of the most widely used experimental models of MS in mice is experimental autoimmune encephalomyelitis (EAE) [2]. Pathogenesis of

Abbreviations: Δ DC, gene deletion only in dendritic cells; Δ MG, gene deletion only in microglia; BBB, blood-brain barrier; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; IL, interleukin; MS, multiple sclerosis; T_H, T helper cells; T_{reg}, regulatory T cells.

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EAE primarily involves the activation of CD4⁺ T cells, followed by their differentiation, and infiltration into the CNS in response to immunization with antigens from the myelin sheath of neurons in complete Freund's adjuvant [3]. Cytokines, including IL-6, play a crucial role in EAE pathogenesis. In particular, it has been shown that pharmacological and genetic inactivation of IL-6 in the mouse model of multiple sclerosis leads to a reduction in disease severity [4] or absolute resistance [5].

It has been established that the pathogenic role of IL-6 is realized via various mechanisms involved in the regulation of blood-brain barrier (BBB) permeability and the development of CD4⁺ T cell subsets. Immunization of wild-type mice with the myelin peptide MOG₃₅₋₅₅ was shown to increase the expression of adhesion molecules such as VCAM-1 and ICAM-1 on the surface of endothelial cells in the BBB as compared to IL-6-deficient mice [5]. It is known that activation of two subunits of the IL-6 receptor complex, IL-6R α and gp130, is required for IL-6 signal transduction. It has been shown that *trans*-presentation of IL-6 (transduction of an intracellular signal by interaction of IL-6R–IL-6 on the surface of one cell with gp130 on the surface of another) is necessary for the polarization of pathogenic T helper cells producing IL-17A (T_H17 cells) in the EAE model [6]. This has been confirmed by the fact that deletion of gp130 from the surface of T cells suppresses the differentiation of CD4⁺ T cells into FoxP3⁺ regulatory T cells (T_{reg}), thus facilitating the development of ROR γ ⁺ T_H17 cells [7]. Remarkably, there is a positive feedback between IL-6 and IL-17A produced by pathogenic T_H17 cells: IL-17A stimulates IL-6 production by astrocytes, which further facilitates T_H17 polarization [8, 9].

The main sources of IL-6 in the CNS are various cells of non-immune origin, in particular, neurons, astrocytes, and endothelial cells [10], but in the EAE model the main source of IL-6 are myeloid cells [6], including dendritic cells and microglia (resident macrophages of the CNS), in particular. Although there is a large amount of data supporting various functions of IL-6 in the EAE model, the characteristics of cytokine production by T cells over the course of disease pathogenesis with respect to the specific subpopulations of myeloid cells have not been investigated in sufficient detail, which was the aim of this study.

MATERIALS AND METHODS

Mice. *Cd11c*^{Cre} \times *Il6*^{fl/fl} (mice with *Il6* deletion predominantly in dendritic cells) [11] and *Cx3cr1*^{CreER} \times *Il6*^{fl/fl} (mice with *Il6* deletion in CX₃CR1⁺ myeloid cells, including microglia). *Il6*^{fl/fl} mice [12] were crossed to *Cx3cr1*^{CreER} mice [13] to generate mice with *Il6* deletion in CX₃CR1⁺

myeloid cells. Both male and female mice aged 9 to 12 weeks were used. Mice were bred and housed in SPF conditions at the Animal Facility of the Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences and at the Animal Facility of the Center for Precision Editing and Genetic Technologies for Biomedicine, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences [EIMB RAS] (under the contract #075-15-2019-1660 from the Ministry of Science and Higher Education of the Russian Federation). All manipulations with animals were carried out in accordance with the protocol approved by the Bioethics Committee of the EIMB RAS (Protocol No. 3 from 21/09/2023).

Tamoxifen treatment. Tamoxifen (Sigma Aldrich, USA) was dissolved in corn oil (Sigma Aldrich) to a concentration of 15 mg/ml by prolonged incubation with continuous shaking on a thermoshaker at 37°C overnight and protected from light. Two ml aliquots of the obtained solution were placed into tubes and stored at 4°C for 5 days. To induce IL-6 deletion in microglia, 8-9-week-old *Cx3cr1*^{CreER} \times *Il6*^{fl/fl} mice and control *Il6*^{fl/fl} mice were injected intraperitoneally daily with 100 μ l of tamoxifen solution at a dose of 75 mg/kg for 5 days.

EAE induction. Mice were subcutaneously immunized with 100 μ g of MOG₃₅₋₅₅-peptide (myelin oligodendrocyte glycoprotein; Anaspec, USA) in complete Freund's adjuvant (Sigma Aldrich) supplemented with 5 mg/ml of inactivated *Mycobacterium tuberculosis* (Difco, USA), followed by intraperitoneal injections of 200 ng Pertussis toxin (Sigma Aldrich) on days 0 and 2 to increase BBB permeability. EAE clinical scores were defined as follows: 0 – no disease; 0.5 – partial tail paralysis; 1 – complete tail paralysis; 1.5 – partially impaired righting reflex; 2 – impaired righting reflex; 3 – hind limbs paresis; 3.5 – complete paralysis of hind limbs; 4 – forelimbs paresis; 4.5 – complete paralysis of forelimbs.

Cell isolation. For isolation of blood mononuclear cells, peripheral blood was collected in tubes with heparin and centrifuged (without acceleration or brakes) in a Ficoll density gradient for 30 min (1.077 g/cm³, PanEko, Russia). Single cell suspensions of spleen and lymph nodes were prepared by mechanical dissociation through 70 μ m filter (NEST, China) in PBS supplemented with 2% FBS. Red blood cells were lysed in ACK buffer (1.5 M NH₄Cl; 100 mM KHCO₃; 10 mM EDTA-2Na in distilled water, pH 7.2). Immune cells from the CNS were isolated according to the protocol [14]. Briefly, mice were anesthetized and transcardially perfused using 0.9% NaCl solution. Spinal cord and brain were removed, mechanically dissociated, and digested enzymatically in DPBS containing 1.5 mg/ml collagenase II (Gibco) and 50 μ g/ml DNase I (Roche, Switzerland). Cells were homogenized using

the syringe with 18G × 1.5" needle and washed in PBS supplemented with 2% FBS. Next, fractions of immune cells were separated by centrifugation (500g, 25°C, 40 min, (without acceleration or brakes) in a Percoll density gradient (30/37/70, GE Healthcare, USA).

Flow cytometry analysis. For intracellular cytokine staining, single cell suspensions prepared from spleen or lymph nodes were stimulated with phorbol-12-myristate-13-acetate (PMA; Sigma Aldrich), 500 ng/ml of ionomycin (Sigma Aldrich) for 4 h, 37°C, 5% CO₂ in the presence of 3 mg/ml of Brefeldin A (eBioscience, USA). To prevent non-specific binding of antibodies, single cell suspensions were incubated for 20 min at 4°C with purified anti-mouse CD16/CD32 (clone 2.4G2, in-house DRFZ collection), washed and stained with following antibodies: anti-CD45 (clone 30-F11, BioLegend, USA), anti-CD11b (clone M1/70, BioLegend), anti-Ly6C (clone HK1.4, Invitrogen, USA), anti-MHCII (clone M5/114.15.2, BioLegend), anti-CX₃CR1 (clone SA011F11, BioLegend), anti-CD4 (clone RM4-5, BioLegend), anti-TCRb (clone H57-597, eBioscience), anti-IFN γ (clone XMG1.2, BioLegend), anti-IL-17A (clone eBio17B7, BioLegend), anti-GM-CSF (clone MP1-22E9, BioLegend), anti-FoxP3 (clone FJK-16s, Invitrogen), anti-ROR γ t (clone B2D, Invitrogen). Dead cell exclusion was performed by using a Fixable Viability Dye (eBioscience). Samples were acquired with BD FACSCanto II or BDFACS Aria III (Beckton Dickinson, USA). CD45⁺CD4⁺CX₃CR1⁺ blood monocytes or CD45⁺CD11b⁺CX₃CR1⁺ microglia were sorted using a BDFACS Aria III with a purity of >90%. Data were analyzed using FlowJo Software (Beckton Dickinson).

ELISA. Sorted monocytes and microglia were activated with lipopolysaccharide (LPS form *E. coli* O111:B4, Sigma [100 ng/ml] for 4 h). IL-6 concentration in supernatant was determined using mouse IL-6 ELISA Ready-SET-Go! kit (eBioscience) according to manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 9. All the data were analyzed using unpaired Student's *t*-test or one-way ANOVA. Differences were considered significant when *p*-values were < 0.05.

RESULTS AND DISCUSSION

Myeloid cell-derived IL-6 plays a critical role in the pathogenesis of EAE. To investigate role of IL-6 produced by myeloid cells in EAE development mice with IL-6 deletion either (i) only in dendritic cells (*IL6^{ADC}*), or (ii) only in microglia (*IL6^{AMG}*) were used. Littermate *IL6^{fl/fl}* were used as control mice in all experiments. Mice with IL-6 deletion in dendritic cells have been previously generated and are characterized by constitutive inactivation of IL-6 in the CD11c⁺

cells [11, 15]. Mice with IL-6 deletion in microglia were generated by crossing *Cx3cr1^{CreER}* mice, which express tamoxifen-dependent Cre-recombinase under the control of the CX₃CR1 promoter [13], to mice with the floxed *Il6* gene (*Il6^{fl/fl}*) [12]. The principle of this system is the cell-specific inactivation of the *Il6* gene flanked by loxP-sites (floxed) by Cre-recombinase fused with the mutant form of the estrogen receptor only in the CX₃CR1⁺ cells in response to the administration of tamoxifen (Fig. 1a).

For tissue-specific inactivation of IL-6 exclusively in microglia, *Cx3cr1^{CreER} × Il6^{fl/fl}* and wild type mice (*Il6^{fl/fl}*) were injected with tamoxifen at a dose of 75 μ g/g for 5 days; 14 days after tamoxifen administration, IL-6 deletion occurred both in monocytes (Fig. 1b) and dendritic cells, as well as in microglia [13]. After complete repopulation of CX₃CR1⁺ myeloid cells from the bone marrow, i.e., 28 days after tamoxifen administration, mice retained IL-6 deletion only in the population of long-lived tissue-resident macrophages, including microglia (Fig. 1c).

EAE was induced by immunization with the MOG₃₅₋₅₅-peptide in complete Freund's adjuvant, followed by injection of Pertussis toxin to increase BBB permeability. Clinical signs were assessed starting day 8 after immunization. Mice with IL-6 deficiency only in microglia or only in dendritic cells developed milder disease symptoms as compared to wild-type mice (Fig. 1d). These results are consistent with previous observations indicating that exactly dendritic cells [6] and microglia [16] are critical sources of IL-6 in the EAE model.

Microglia-derived IL-6 regulates immune response in the CNS at the peak of EAE. Given that microglia are tissue-resident macrophages in the CNS, it was hypothesized that the functions mediated by these cells would mainly affect the immune response in the CNS. Therefore, on day 16 after immunization (effector phase of the disease), the frequency of myeloid cells and antigen-specific CD4⁺ T cells in the CNS was assessed by flow cytometry. Mice with IL-6 inactivation in microglia showed a reduced frequency (Fig. 2a) and absolute number (Fig. 2b) of Ly6C^{hi}MHCII⁺ monocytes, which play an exclusively pathogenic role in EAE. Additionally, there was a decrease in the percentage of IFN γ -producing CD4⁺ T cells (Fig. 2c). These results are consistent with each other, as it is precisely IFN γ that supports differentiation of the Ly6C^{hi}-monocytes into MHCII⁺ effector dendritic cells [17]. At the same time, these findings are consistent with the previously published data on the stimulation of T_{reg} development (Fig. 2d) at low concentrations of IFN γ [18].

As shown in the current study, mice with microglial IL-6 deletion develop milder EAE symptoms as compared to wild-type mice, yet, they do not exhibit complete resistance to disease development (Fig. 1d).

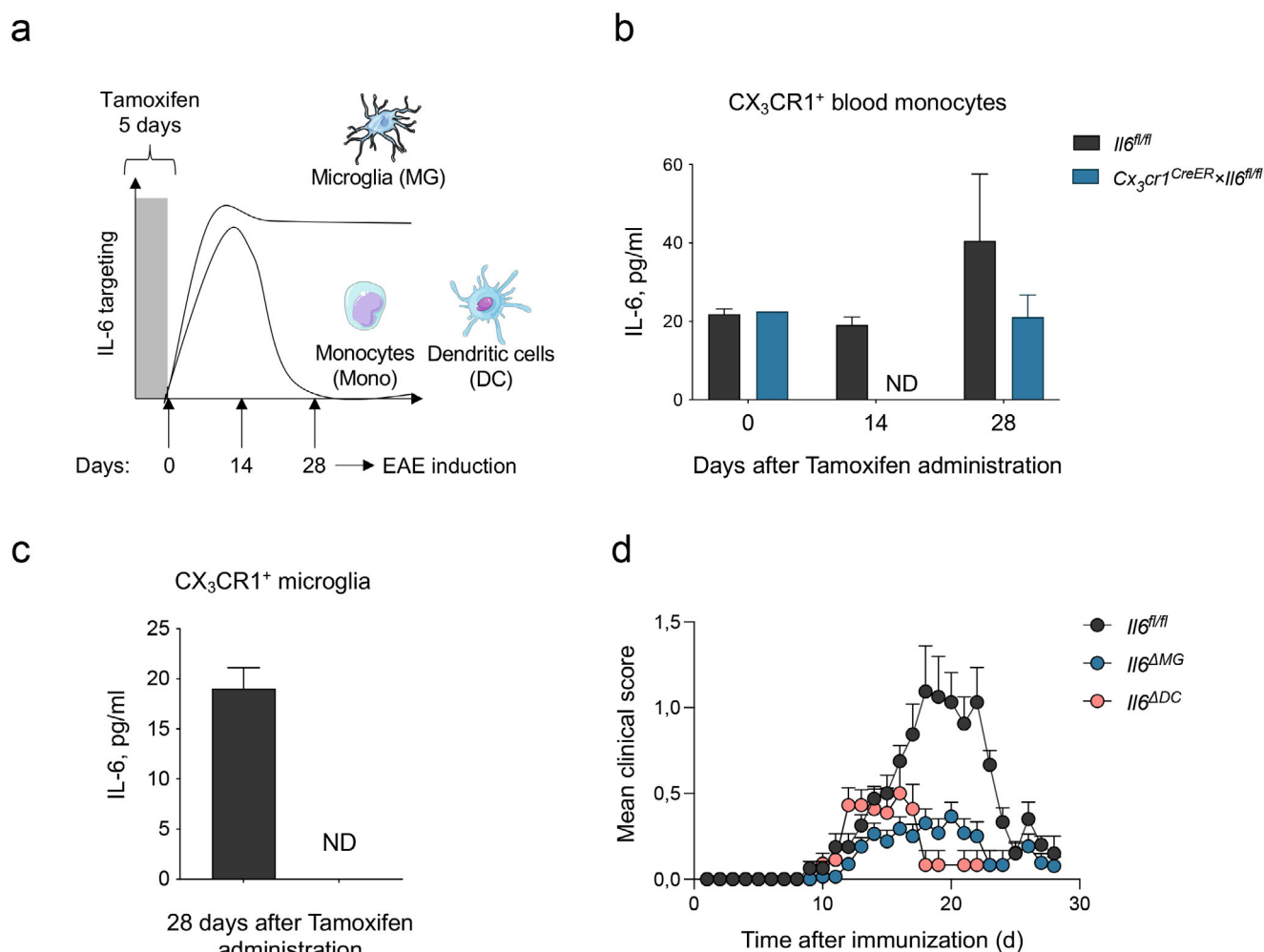


Fig. 1. Mice with tamoxifen-dependent inactivation of IL-6 in CX₃CR1⁺ microglia are hyposusceptible to EAE induction. a) Scheme of experiment. Mice were administered Tamoxifen (75 µg/g) i.p. for 5 consecutive days. At 7-14 days after Tamoxifen injections, IL-6 deletion was detected in both tissue-resident macrophages and monocytes, but after 28 days, replenishment of the monocyte pool from the bone marrow occurred and IL-6 deficiency was observed only in tissue-resident macrophages including microglia; b) concentration of IL-6 in the supernatant of sorted CX₃CR1⁺ monocytes isolated from the peripheral blood of *Cx3cr1^{CreER} × Il6^{fl/fl}* mice on days 0, 14, 28 after Tamoxifen administration and activated with LPS for 4 h; ND – not detected; c) concentration of IL-6 in the supernatant of sorted CX₃CR1⁺ microglia isolated from the CNS of *Cx3cr1^{CreER} × Il6^{fl/fl}* mice on day 28 after Tamoxifen administration and activated with LPS for 4 h; d) Clinical disease course of MOG₃₅₋₅₅ emulsified in complete Freund's adjuvant-induced EAE in wild-type mice (*Il6^{fl/fl}*), mice with IL-6 deletion in microglia (*Il6^{AMG}*), and mice with IL-6 deletion in dendritic cells (*Il6^{ADC}*). Data are shown as mean ± SEM and are representative of three independent experiments with four or more mice per group in each experiment.

Moreover, these mice show an increase in the percentage of T_{reg}, that suppress the development of EAE (Fig. 2d), suggesting the presence of an additional component that contributes to the manifestation of clinical symptoms. We hypothesized that these mice may demonstrate an increase in the infiltration of pathogenic T cells into the CNS. The CCR6/CCL20 axis is crucial for the migration of immune cells into the CNS and is particularly important for the initial wave of T cell infiltration into the CNS [19]. Indeed, mice with IL-6 deletion in microglia exhibited an increase in the frequency of CCR6⁺ T cells in the CNS (Fig. 3a). Furthermore, these mice showed an increased percent-

age of GM-CSF-producing, but not IL-17A-producing, CD4⁺ T cells (Fig. 3b). GM-CSF production by T cells is critically important for the pathogenesis of EAE [20, 21], as GM-CSF promotes the migration of immune cells into the CNS [22]. Thus, the increased presence of CCR6⁺ T cells in the CNS correlates with the enhanced polarization of T cells into GM-CSF-producing cells.

Thus, the reduction in the severity of clinical symptoms of EAE in mice with microglial IL-6 inactivation correlated with a decrease in the percentage and absolute number of inflammatory Ly6C^{hi}MHCII⁺ monocytes in the CNS, as well as with reduced IFN_γ production by T cells and an increase in the proportion

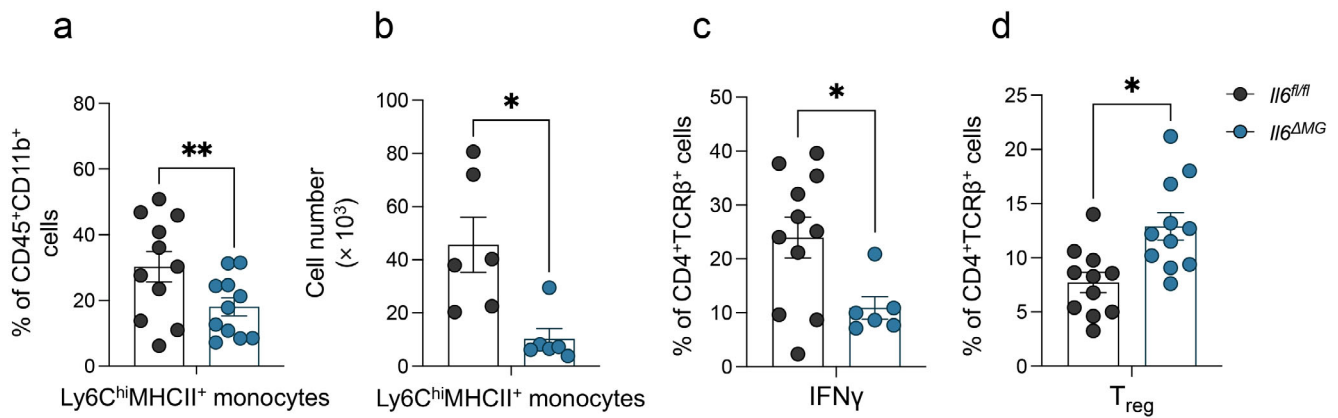


Fig. 2. IL-6 produced by microglia stimulates development of pathogenic monocytes and IFN γ production by T cells in the CNS at the peak of EAE. a) Frequency of Ly6C^{hi}MHCII⁺ cells among CD45⁺CD11b⁺ cells in the CNS at the peak of EAE; b) absolute number of Ly6C^{hi}MHCII⁺ cells in the CNS at the peak of EAE; c) frequency of IFN γ -producing cells restimulated with PMA/ionomycin among CD4⁺TCR β ⁺ cells in the CNS at the peak of EAE; d) frequency of FoxP3⁺ T_{reg} among CD4⁺TCR β ⁺ cells in the CNS at the peak of EAE. Pooled (a, c, and d) or representative (b) data are shown with number of mice for each genotype $n = 5-6$. Data are shown as mean \pm SEM. Student's t -test or Mann-Whitney test; * $p < 0.05$; ** $p < 0.01$.

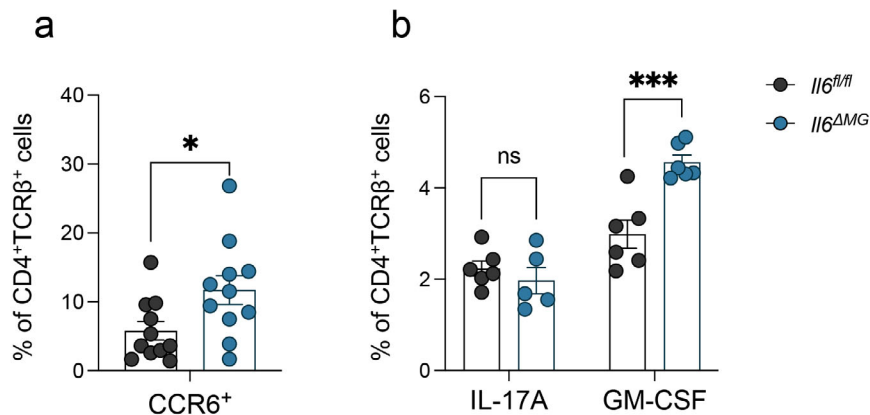


Fig. 3. IL-6 deletion in microglia results in the increased T cell migration and induction of GM-CSF by CD4⁺ T cells in the CNS. a) Frequency of CCR6⁺ cells among CD4⁺TCR β ⁺ cells in the CNS at the peak of EAE. b) Frequency of GM-CSF-producing cells restimulated with PMA/ionomycin among CD4⁺TCR β ⁺-cells in the CNS at the peak of EAE. Pooled (a) or representative (b) data are shown with number of mice for each genotype $n = 5-6$. Data are shown as mean \pm SEM. Student's t -test or Mann-Whitney test; * $p < 0.05$; *** $p < 0.001$; ns, non-significant.

of protective T_{reg} cells. At the same time, partial resistance to EAE in mice with IL-6 inactivation in microglia may be associated with increased of T cell migration into the CNS, coupled with an increase in the percentage of the GM-CSF-producing CD4⁺ T cells.

IL-6 from dendritic cells not only determines the T_H17/T_{reg} ratio, but also affects IFN γ and GM-CSF production in the peripheral lymphoid organs during EAE. It is known that one of the functions of IL-6 in EAE is to inhibit of the transcription factor FoxP3, which, in turn, suppresses development of protective T_{reg} [23]. On the other hand, IL-6 is crucial for the development of T_H17 cells [6]. Indeed, a decrease in the percentage of ROR γ t⁺ T_H17 cells and an increase in the percentage of FoxP3⁺ T_{reg} (Fig. 4a) were observed in the lymph nodes at the peak of EAE in mice with IL-6 deletion in dendritic cells. This is consistent with

previous studies on the pathogenic role of IL-6 produced by dendritic cells.

Next, we addressed cytokine production by T cells isolated from lymph nodes and spleens of *Il6^{fl/fl}* and *Il6^{ΔDC}* mice at the peak of EAE and restimulated with PMA/ionomycin. It was found that IL-6 deletion in dendritic cells does not affect the production of IL-17A by T cells (Fig. 4b), which is consistent with the literature [6]. However, the *Il6^{ΔDC}* mice were characterized by a reduction in GM-CSF (Fig. 4c) and IFN γ (Fig. 4d) production both in lymph nodes and in spleen.

Thus, mice with IL-6 deletion in dendritic cells are characterized by an increase in the proportion of T_{reg} and a decrease in IFN γ and GM-CSF production by T cells in peripheral lymphoid organs, resulting in the development of mild EAE symptoms. Moreover, the results of this study indicate that IL-6 from dendrit-

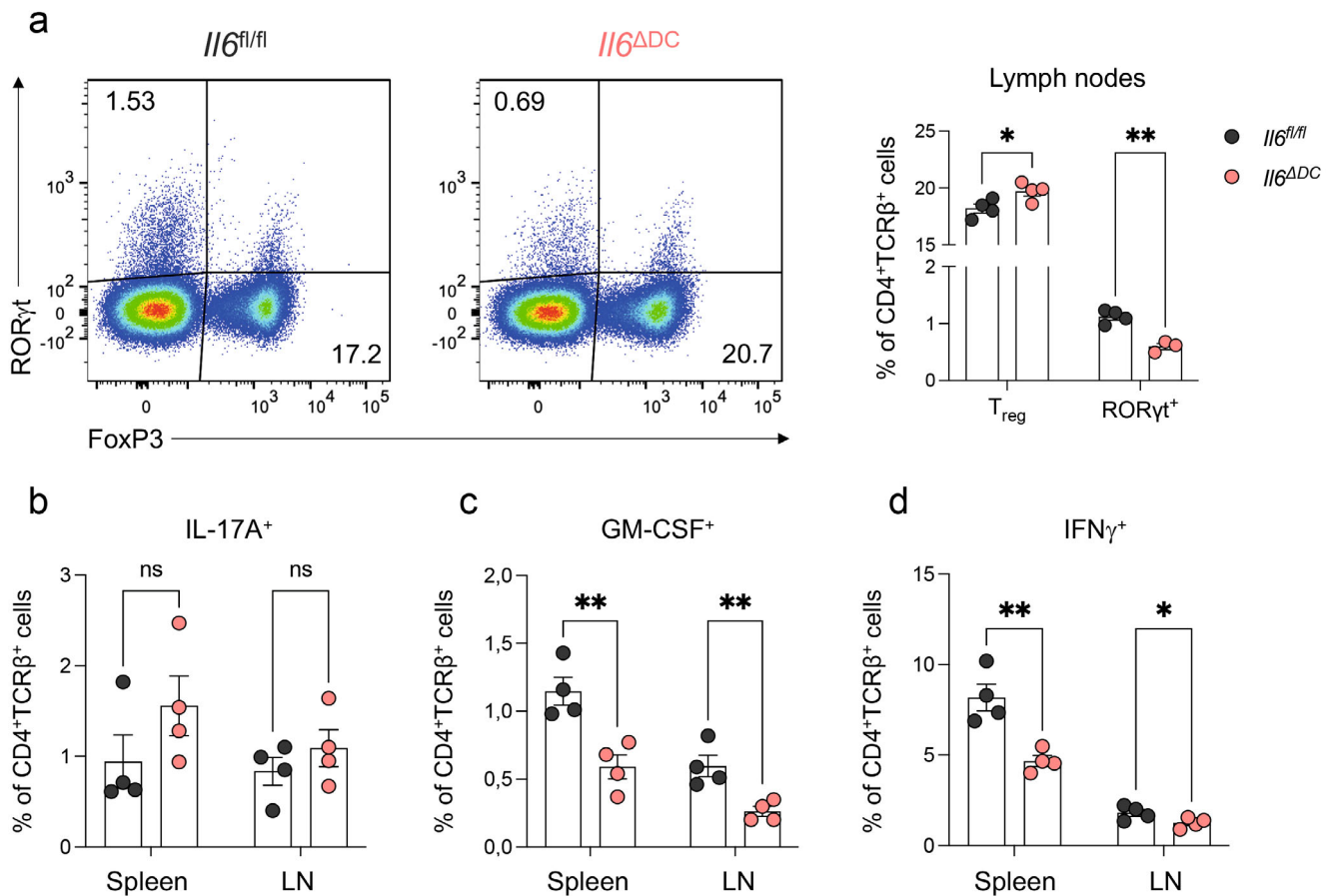


Fig. 4. IL-6 from dendritic cells stimulates production of cytokines by CD4⁺ T cells in the peripheral lymphoid organs at the peak of EAE. **a**) Representative dot plots (left) and percentage (right) of ROR γ t⁺ and FoxP3⁺ CD4⁺ T cells isolated from lymph nodes of *Il6^{fl/fl}* and *Il6^{ΔDC}* mice at the peak of EAE. **b-d**) Frequencies of IL-17A⁺, GM-CSF⁺, and IFN γ -producing CD4⁺ T cells isolated from the spleen and lymph nodes and restimulated with PMA/ionomycin. Data are shown as mean \pm SEM. Student's *t*-test or Mann-Whitney test; * *p* < 0.05; ** *p* < 0.01; ns, non-significant.

ic cells may not only be involved in the induction of pathogenic T_H17 cells [6], but also stimulate GM-CSF and IFN γ production by T cells.

CONCLUSIONS

Cytokines play a key role in the pathogenesis of EAE. IL-6 is one of the few cytokines that is absolutely required for the development of EAE [24, 25]. Although astrocytes being the main source of IL-6 in the CNS [10], it has been shown that myeloid cells are the main source of IL-6 in the EAE model [6]. Interestingly, deletion of IL-6 in LysM⁺ myeloid cells (macrophages and neutrophils) does not affect the course of disease [6, 26]. Microglia and dendritic cells are critically important IL-6 producing populations in the pathogenesis of EAE [6].

Indeed, the results of this study demonstrate that IL-6 deletion in microglia leads to the development of mild EAE symptoms as compared to wild type mice. It is worth noting that in another study, IL-6 deletion

in microglia resulted in a reduction in the severity of clinical symptoms of EAE in female but not in male mice [16], which correlates with the predominance of MS within the female population. In this study, mice with IL-6 deletion in microglia showed a reduction in the number of pro-inflammatory Ly6C^{hi}MHCII⁺ monocytes, which play a central role in the demyelination. This finding is consistent with the literature demonstrating a decrease in spinal cord demyelination upon IL-6 deletion in microglia [16]. On the other hand, this could also indicate one of the degenerative functions of IL-6 since the same phenotype is observed upon IL-6 deletion in various sources in the CNS, namely astrocytes and neurons [16]. Furthermore, IL-6 deletion in microglia resulted in a decrease in IFN γ production by T cells, which, on one hand, explains the reduced number of Ly6C^{hi}MHCII⁺ monocytes [17], and, on the other hand, is consistent with an increase in the percentage of protective T_{reg} in the CNS [18].

In contrast to the decrease in clinical symptoms of EAE, mice with IL-6 deletion in microglia exhibited increased migration of CD4⁺ T cells into the CNS and

increased production of GM-CSF. Indeed, another study demonstrated that the infiltration of T cells does not change upon IL-6 deletion from various cellular sources in the CNS [16]. Furthermore, the results obtained provide evidence for the correlation between the co-expression of CCR6 and GM-CSF in T cells infiltrating the CNS [27]. Taken together, these data suggest that in the context of neuroinflammation, microglia may perform both protective and pathogenic functions.

On the contrary, the results of this study highlight the exclusively pathogenic role of IL-6 from dendritic cells [6]. Specifically, IL-6 from dendritic cells is crucial for maintaining the balance between ROR γ t⁺ T_H17 cells and FoxP3⁺ T_{reg}. A therapeutic approach targeting this signaling pathway by inhibiting STAT3 with a small molecule has been recently proposed [28] for immunomodulation of the clinical symptoms in MS patients. Interestingly, this study also demonstrated that IL-6 from dendritic cells is essential for stimulating production of IFN γ and GM-CSF by T cells, although IL-6 has not traditionally been considered a cytokine required for stimulation of GM-CSF production [21].

Thus, while IL-6 produced by microglia may have both pathogenic and protective functions, IL-6 from dendritic cells appears to play an exclusively pathogenic role.

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Contributions. M.S.D. conceptualized and supervised the study; V.S.G. and Q.C.N. performed experiments, discussed results of the experiments, wrote the original draft of the paper; M.S.D. edited the manuscript.

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Ethics declarations. All experiments with laboratory animals were conducted in accordance with the local legislation and institutional requirements. The authors of this work declare that they have no conflicts of interest.

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