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Cytokine production profiles in chronic relapsing–remitting experimental autoimmune encephalomyelitis: IFN-γ and TNF-α are important participants in the first attack but not in the relapse

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ABSTRACT

Multiple sclerosis (MS) is a chronic demyelinating disease often displaying a relapsing–remitting course of neurological manifestations that is mimicked by experimental autoimmune encephalomyelitis (EAE) in animal models of MS. In particular, NOD mice immunized with myelin oligodendrocyte glycoprotein peptide 35–55 develop chronic relapsing–remitting EAE (CREAE). To elucidate the mechanisms that cause MS relapse, we investigated the histopathology and cytokine production of spleen cells and mRNA expression levels in the central nervous system (CNS) of CREAE mice. During the first attack, inflammatory cell infiltration around small vessels and in the subarachnoid space was observed in the spinal cord. Spleen cell production and mRNA expression in the CNS of several cytokines, including IFN-γ, TNF-α, IL-6, IL-17, and CC chemokine ligand 2 (CCL2), were higher in CREAE mice than in controls. Afterwards, parenchymal infiltration and demyelination were observed histologically in the spinal cord and corresponded with the more severe clinical symptoms of the first and second relapses. IL-17 and CCL2, but not IFN-γ, TNF-α, or IL-6, were also produced by spleen cells during recurrences. Our results suggested that the immune mechanisms in relapses were different from those in the first attack for CREAE. Further investigation of CREAE mechanisms may provide important insights into successful therapies for human relapsing–remitting MS.

1. Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease that is associated with inflammation in the white matter of the central nervous system (CNS). While the etiology of MS remains to be determined, autoimmune reactions against myelin are thought to contribute to the pathological mechanisms of this disease [1]. MS is clinically categorized as either relapsing–remitting MS (RRMS), which is the most common type of MS (80–90%), or primary progressive MS (PPMS) [2,3]. Experimental autoimmune encephalomyelitis (EAE) is induced by immunization with myelin antigens, such as myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and their derived peptides [4,5]. Although EAE is regarded as an animal model of MS because of the resemblance of their clinical manifestations and pathological findings, the clinical course of most forms of EAE does not display a chronic relapsing–remitting pattern [5]. In this context, chronic relapsing–remitting EAE (CREAE) is considered to be the most useful animal model for elucidating the pathophysiological mechanisms of MS.

NOD mice naturally develop type 1 diabetes and are receptive to autoimmune disease induction [6]. In particular, this strain develops MS-like chronic relapsing–remitting demyelinating disease when administered a single dose of encephalitogenic MOG peptide 35–55 ($MOG_{35–55}$). The clinical course and pathology of demyelinating lesions observed in the CNS of NOD mice with CREAE induced by $MOG_{35–55}$ are very similar to those of human disease MS [5].

In the present study, we induced CREAE in NOD mice by immunization with $MOG_{35–55}$ and then assessed histopathological findings and cytokine production profiles during the first attack, first relapse, and second relapse to elucidate the mechanisms responsible for CREAE onset and recurrence. Our results may shed light on the pathological and immunological mechanisms involved in CREAE and RRMS.

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2. Materials and methods

2.1. Animals and reagents

Five-week-old female NOD/Shi mice were purchased from Japan CLEA (Tokyo, Japan). Mice were housed and cared for in a nationally approved facility in accordance with National Institutes of Health guidelines. Synthetic mouse $MOG_{35–55}$ (Met-Glu-Val-Gly-Trp-Tyr-Arg-Ser-Pro-Phe-Ser-Arg-Val-Val-His-Leu-Try-Arg-Asn-Gly-Lys) that was obtained from Operon Biotechnology (Tokyo, Japan) was synthesized using standard F-moc chemistry. $MOG_{35–55}$ purity (>90%) was confirmed using reverse phase HPLC.

2.2. Immunization and grading of symptoms

NOD/Shi mice were subcutaneously injected into both flanks with 100 μg of $MOG_{35–55}$ peptide that was dissolved in saline, emulsified with an equal volume of complete Freund's adjuvant (CFA), and supplemented with 4 mg/mL of *Mycobacterium tuberculosis* (Difco, H37Ra), as previously described [7]. Immediately before and 2 days after injection of the emulsion, the mice received an intravenous injection of 300 ng of pertussis vaccine in 100 μL of phosphate-buffered saline (PBS). Control mice received CFA without the MOG peptide along with the pertussis vaccine. Mice were assessed daily for clinical signs of disease. Neurological dysfunction was graded using the following system: normal, 0; limp tail, 1; hind leg weakness, 2; hind leg paralysis, 3; foreleg paralysis, 4; and moribund state or death, 5. Batches of the peptide and adjuvant used were identical throughout the study.

2.3. Histological examination

Mice were anesthetized with ether inhalation and exsanguinated by transcardial perfusion with a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Whole CNS tissue samples, including the entire spinal cord, were carefully dissected free from the cranium and spinal canal and immersed overnight in the same fixative solution at room temperature. The CNS was divided into coronal sections along the rostrocaudal axis. Tissue slices were dehydrated in a graded series of ethanol, cleared in Hemo-D, and embedded in paraffin. Serial sections of 5-mm-thickness were mounted on silane-coated glass slides for observation using hematoxylin–eosin (HE) and Klüver–Barrera staining.

2.4. Cytokine assay

Mononuclear cells (MNCs) were obtained from the spleen. Aliquots of 1×10^6 cells were cultured in 24-well flat-bottom microculture plates (Falcon, Becton-Dickinson, Franklin Lakes, NJ, USA) with RPMI-1640 medium and 10% fetal calf serum (FCS). Triplicate cultures of $MOG_{35–55}$-immunized cells as well as control cells were cultured with $MOG_{35–55}$ (1 μg/mL) or nothing at 37 °C in a 5% CO_2 incubator for 72 h, after which culture supernatants were collected and cell-free samples were stored at −30 °C until cytokine assaying. Concentrations of TNF-α, IFN-γ, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, and CCL2 were measured using a cytometric bead array (CBA) with a series of anti-cytokine mAb-coated beads and PE-conjugated anti-cytokine mAbs followed by Epics XL flow cytometric analysis (Beckman-Coulter Electronics, Fullerton, CA, USA) using the CBA kit (BD Bioscience, San Diego, CA, USA) and software (BD Bioscience).

2.5. Real-time PCR analysis

Quantification of the mRNA expression levels of TNF-α, IFN-γ, IL-6, and CCL2 was performed using real-time PCR for spinal cord tissue that was obtained from $MOG_{35–55}$-immunized CREAE mice and controls. Total RNA was isolated using the QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA, USA). First-strand cDNA was synthesized from approximately 1 μg of RNA using a random primer and Moloney murine leukemia virus reverse transcriptase. A total of 5 μL of the reverse transcription reaction mixture was used for quantitative PCR. The primers and probe mixture that were adopted to amplify the target and housekeeping (glyceraldehyde-3-phosphate dehydrogenase; *GAPDH*) genes were purchased from Applied Biosystems (Foster City, CA, USA).

The reaction solution for real-time PCR was prepared by mixing 5 μL of the synthesized cDNA solution with 25 μL of TaqMan Universal PCR Master Mix (Applied Biosystems) and 2.5 μL of reaction mixture. Real-time PCR was carried out using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) at 50 °C for 2 min, 95 °C for 10 min, 50 cycles at 95 °C for 15 s, and 60 °C for 1 min. Each assay was performed in triplicate. The expression of *GAPDH* was used to normalize that of the target genes. Before using the comparative threshold cycle (Ct) method for relative quantification, a validation experiment was performed according to the manufacturer's user bulletin to verify that the efficiencies of target and *GAPDH* genes were approximately equal. The abundance of target genes relative to that of *GAPDH* was calculated as $\Delta\Delta Ct = (\Delta Ct \text{ of target genes}) - (\Delta Ct \text{ of } GAPDH)$. The ratio was calculated as $2^{-\Delta\Delta Ct}$ to evaluate alterations in target gene expression level in CNS tissue.

2.6. Statistical analysis

Clinical scores, supernatant cytokine levels, and mRNA expression levels were analyzed using the Mann–Whitney U-test. For all values, $p < 0.05$ (two-tailed test) was taken as indicating significance. All results are expressed as mean ± SEM.

3. Results

3.1. Clinical symptoms of mice immunized with $MOG_{35–55}$

NOD mice immunized with $MOG_{35–55}$ exhibited the typical disease course of MOG-peptide-induced MS-like disease, namely, chronic relapsing–remitting neurological impairment. Three representative disease courses are shown in Fig. 1. The neurological symptoms in the first attack, which included weight loss, limp tail, and hind limb paralysis, were relatively mild and occurred at 11–14 days postimmunization (first attack group, n = 6). After complete recovery from the first attack, the mice experienced a more severe first relapse at 28–60 days postimmunization (first relapse group, n = 6). Lastly, following complete improvement of these symptoms, the mice exhibited an increasingly severe second relapse at 82–108 days postimmunization (second relapse group, n = 4). The incidence of the first attack was

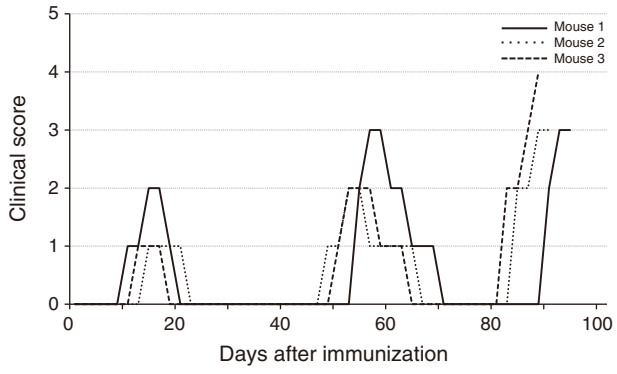

Fig. 1. Representative disease courses of CREAE mice immunized with $MOG_{35–55}$. Mild neurological symptoms typically occurred at first (first attack) and became completely resolved in approximately 10 days. More severe paralytic symptoms later relapsed (first relapse) and then completely improved again. The severity of neurological symptoms worsened ever further during the second relapse.

Table 1
Clinical symptoms of $MOG_{35–55}$-immunized NOD mice with CREAE.

Group	n	Day of onset	Peak clinical score
First attack	6	12.7 ± 0.5	1.33 ± 0.21
First relapse	6	50.0 ± 4.9	2.33 ± 0.21*
Second relapse	4	86.0 ± 11.7	3.25 ± 0.25*

Day of onset and peak clinical score are expressed as mean ± SEM.
* $p < 0.05$ compared with the peak clinical score of the first attack.

78.1% of all immunized mice, that of the first relapse was 72.7% of mice experiencing a first attack, and that of the second relapse was 75.1% of mice exhibiting a first relapse (Table 1). The mean postimmunization onset time until the first attack, first relapse, and second relapse was 12.7 ± 0.5, 50.0 ± 4.9, and 86.0 ± 11.7 days, respectively. The peak clinical scores of both the first relapse group (2.33 ± 0.21) and the second relapse group (3.25 ± 0.25) were significantly higher than those of the first attack group (1.33 ± 0.21) ($p < 0.05$ for each).

3.2. Histology

Representative $MOG_{35–55}$–immunized mice were sacrificed for histological examination at 17, 56, and 86 days postimmunization, which corresponded to the first attack, first relapse, and second relapse, respectively. HE-stained sections of the lumbar spinal cord obtained during the first attack showed moderate inflammatory cell infiltration of lymphocytes, macrophages, and sometimes neutrophils around small vessels and in the subarachnoid space (Fig. 2). In contrast, HE-stained sections taken from mice during the first relapse (Fig. 3) and second relapse (Fig. 4) revealed a remarkable number of mononuclear cells infiltrating the parenchyma of the white matter. Demyelination changes in the spinal cord were identified by Klüver–Barrera staining during the first and second relapses, but not in the first attack (data not shown). Inflammatory lesions with mononuclear cell infiltration in the lumbar spinal cord were consistently observed in all $MOG_{35–55}$-immunized mice at every disease stage.

3.3. Cytokine production

Spleen cells were harvested from mice at 12–14 days postimmunization during the first attack, 30–62 days postimmunization during the first relapse, and 85–110 days postimmunization during the second relapse for comparisons of cytokine production profiles using a CBA assay. All cytokine levels in the supernatants of mononuclear cells cultured without $MOG_{35–55}$ re-stimulation were below detection limits. The IFN-γ levels of the first attack group (1540.4 ± 753.1 pg/mL) were significantly higher than those of the control group (0.4 ± 0.4 pg/mL), which were similar to those of the first (29.2 ± 14.5 pg/mL) and second (3.8 ± 1.4 pg/mL) relapse groups (Fig. 5A). The TNF-α levels of the first attack group (291.4 ± 87.1 pg/mL) were also significantly higher than those of the control group (21.2 ± 5.8 pg/mL), which were comparable to those of the first (40.3 ± 7.4 pg/mL) and second (29.0 ± 6.7 pg/mL) relapse groups (Fig. 5B). Similarly, the IL-6 levels of the first attack group (37.6 ± 11.2 pg/mL) were significantly higher than those of the control group (0.0 ± 0.0 pg/mL), which were identical to those of the first (0.0 ± 0.0 pg/mL) and second (0.0 ± 0.0 pg/mL) relapse groups (Fig. 5C). Meanwhile, the CCL2 levels of the first attack group (1448.4 ± 420.7 pg/mL), first relapse group (1092.6 ± 350.5 pg/mL), and second relapse group (429.7 ± 97.3 pg/mL) were all significantly higher than those of the control group (25.6 ± 9.1 pg/mL) (Fig. 5D). The IL-17 levels of the first attack group (12.7 ± 2.9 pg/mL), first relapse group (11.4 ± 2.6 pg/mL), and second relapse group (8.2 ± 2.1 pg/mL) were significantly higher than those of the control group (2.3 ± 0.4 pg/mL) as well (Fig. 5E). The levels of IL-4, IL-5, IL-10, and IL-12 were below detection limits in all assays (data not shown). Our results showed that spleen cells in $MOG_{35–55}$-immunized NOD mice with CREAE had potent abilities to produce IFN-γ, TNF-α, IL-6, IL-17, and CCL2 when re-stimulated with $MOG_{35–55}$ in vitro during the first attack, whereas significant levels of IL-17 and CCL2, but not IFN-γ, TNF-α, or IL-6, were produced during the first and second relapses.

3.4. Expression of cytokine mRNA

Since histological examination of the $MOG_{35–55}$-immunized CREAE NOD mice revealed remarkable inflammatory cell infiltration in the spinal cord, we investigated the mRNA expression levels of IFN-γ, TNF-α, IL-6, and CCL2 in these tissues using real-time PCR. The IFN-γ mRNA expression levels of the first attack group (7136.6 ± 4176.8) were significantly higher than those of the control group (1.0 ± 1.0), which were comparable to those of the first (197.8 ± 82.7) and second (63.9 ± 38.4) relapse groups (Fig. 6A). The TNF-α mRNA expression levels of the first attack group (81.8 ± 19.2) were also significantly higher than those of the control group (1.0 ± 0.1), which were comparable to those of the first (9.7 ± 4.2) and second (11.5 ± 2.6) relapse groups (Fig. 6B). The IL-6 mRNA expression levels of the first attack group (24.1 ± 8.4) were significantly higher than those of the control group (1.0 ± 0.2) (Fig. 6C). IL-6 expression of the first (14.5 ± 11.3) and second (5.0 ± 1.5) relapse groups tended to be elevated ($p = 0.157$ and $p = 0.051$ vs. controls, respectively). Lastly, the CCL2 mRNA expression levels of the first attack group (281.8 ± 155.9), first relapse group (25.1 ± 10.7), and second relapse group (17.9 ± 1.9) were all significantly higher than those of the control group (1.0 ± 0.6) (Fig. 6D). These results were consistent with the cytokine production findings in spleen cells re-stimulated with $MOG_{35–55}$ in vitro and provided direct evidence that whereas IFN-γ, TNF-α, IL-6,

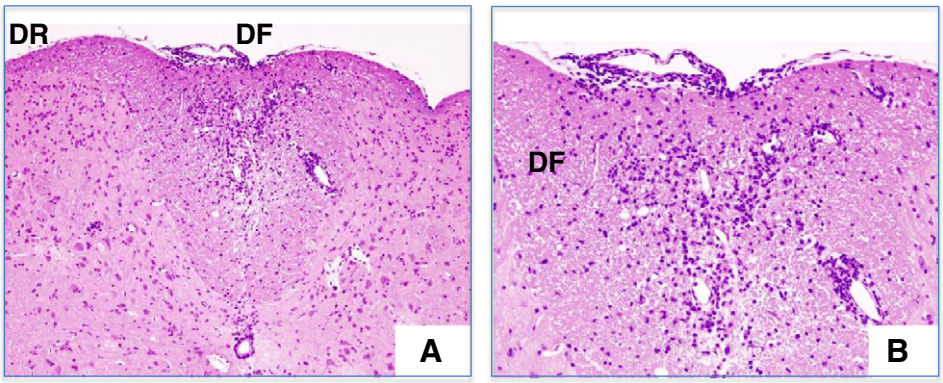

Fig. 2. Histological findings of lumbar spinal cord sections obtained from NOD mice during the first attack of CREAE at 17 days after $MOG_{35–55}$ immunization (A: 80×; B: 160×). A number of inflammatory cells were observed in the subarachnoid space and around small blood vessels. Some mononuclear cells were seen in the white matter of the spinal cord. DF: dorsal fasciculus; DR: dorsal root.

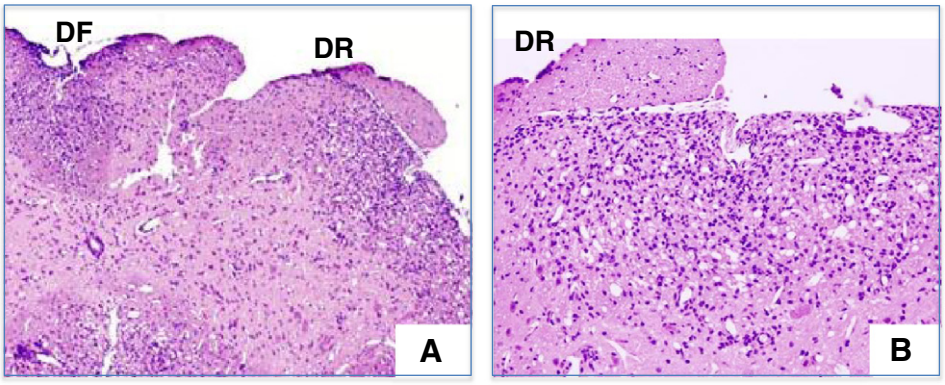

Fig. 3. Histological findings of lumbar spinal cord sections obtained from NOD mice during the first relapse of CREAE at 56 days after MOG_{35-55} immunization (A: 80×; B: 160×). Numerous inflammatory cells were observed in the white matter around the dorsal root and lateral fasciculus as well as the anterior root and dorsal fasciculus. DF: dorsal fasciculus; DR: dorsal root.

and CCL2 played an important role in the first attack of CREAE, CCL2 and possibly IL-17 were implicated during the first and second relapses.

4. Discussion

MOG is a member of the immunoglobulin superfamily that is expressed in the outermost lamella of the CNS myelin sheath and on the surface of oligodendrocytes. While its function is unknown, a number of studies have shown that MOG and its encephalitogenic peptide, MOG_{35-55}, are able to generate a paralytic neurological disease with extensive demyelination in several strains of mice [8–10]. NOD mice spontaneously develop insulitis and type 1 diabetes, which are autoimmune conditions characterized by T-cell-mediated destruction of insulin-producing β cells in the pancreas [6]. A single injection of MOG_{35-55} in NOD mice produces CREAE that is very similar to the clinical manifestations and pathology of MS in humans [5]. Our MOG_{35-55}-immunized NOD mice demonstrated typical CREAE first attack symptoms, which included weight loss, limp tail, and hind leg paralysis, at 12.7 ± 0.5 days postimmunization. Following apparently complete recovery, the first and second recurrences of neurological symptoms developed at 50.0 ± 4.9 and 86.0 ± 11.7 days postimmunization, respectively.

Our results uncovered several interesting findings with regard to cytokine profiles at different stages of CREAE progression. Cytokines play an important role in both establishing and maintaining EAE and MS [11]. IL-12 and IL-12-derived IFN-γ-producing Th1 cells have long been known as key contributors to the induction and maintenance of EAE and autoimmune diseases [12]. However, IFN-γ −/− mice were highly susceptible to EAE and exhibited impaired recovery in one study, while administration of anti-IFN-γ mAbs caused EAE exacerbation in another [13,14]. Balabanov et al. [15] have also reported that IFN-γ–oligodendrocyte interactions have important regulatory roles in EAE. Meanwhile, as a pleiotropic cytokine, TNF-α is involved in the effector arm of cellular immune responses and may be pervasive throughout the inflammation process. TNF-α also has suspected immunoregulatory activities [16,17]. Reports on TNF-α −/− mice with EAE have shown conflicting results: whereas Liu et al. [18] reported more severe EAE in TNF-α −/− C57BL/6 mice immunized with MOG, Körner et al. [19] observed a delayed first attack of EAE using the same mice and antigen. In the present study, we focused on cytokine production to elucidate the mechanisms of relapse in CREAE mice immunized with MOG_{35-55} and witnessed that IFN-γ and TNF-α production by spleen cells and mRNA expression in the CNS were elevated only during the first attack. Thus, these cytokines may be essential in the initial stage of CREAE, but not in ensuing relapses. There was a possibility that epitope spread had occurred during recurrences. However, we presumed that this possibility was slight because of the low mRNA expression of these cytokines at the times of relapse.

Our findings revealed perivascular inflammatory cell infiltration in the CNS during the first attack in CREAE animals. As disease severity was mild, it appeared that IFN-γ and TNF-α did not correlate with disability stage. Sun et al. [20] reported that local injection of IFN-γ or TNF-α into the brain of animals in the prodromal phase of EAE significantly enhanced inflammation in the CNS and caused widespread distribution of perivascular cuffing by T lymphocytes. Thus, IFN-γ and TNF-α may be associated with perivascular infiltration, but not

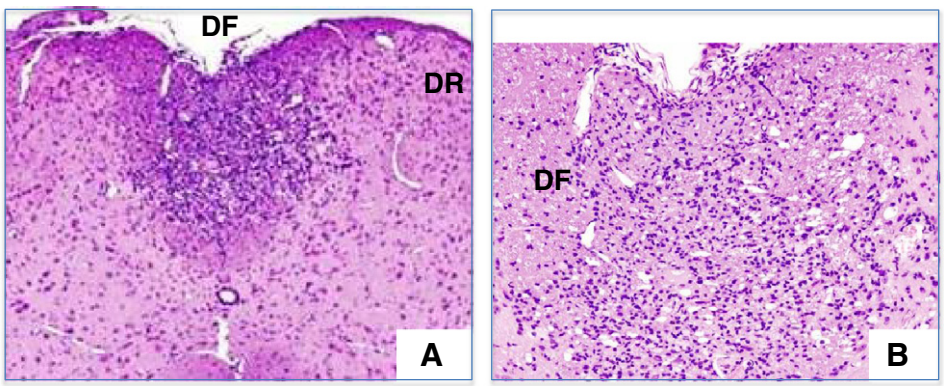

Fig. 4. Histological findings of lumbar spinal cord sections obtained from NOD mice during the second relapse of CREAE at 86 days after MOG_{35-55} immunization (A: 80×; B: 160×). A large number of inflammatory cells were observed in the white matter around the dorsal root and lateral fasciculus as well as the anterior root and dorsal fasciculus. DF: dorsal fasciculus; DR: dorsal root.

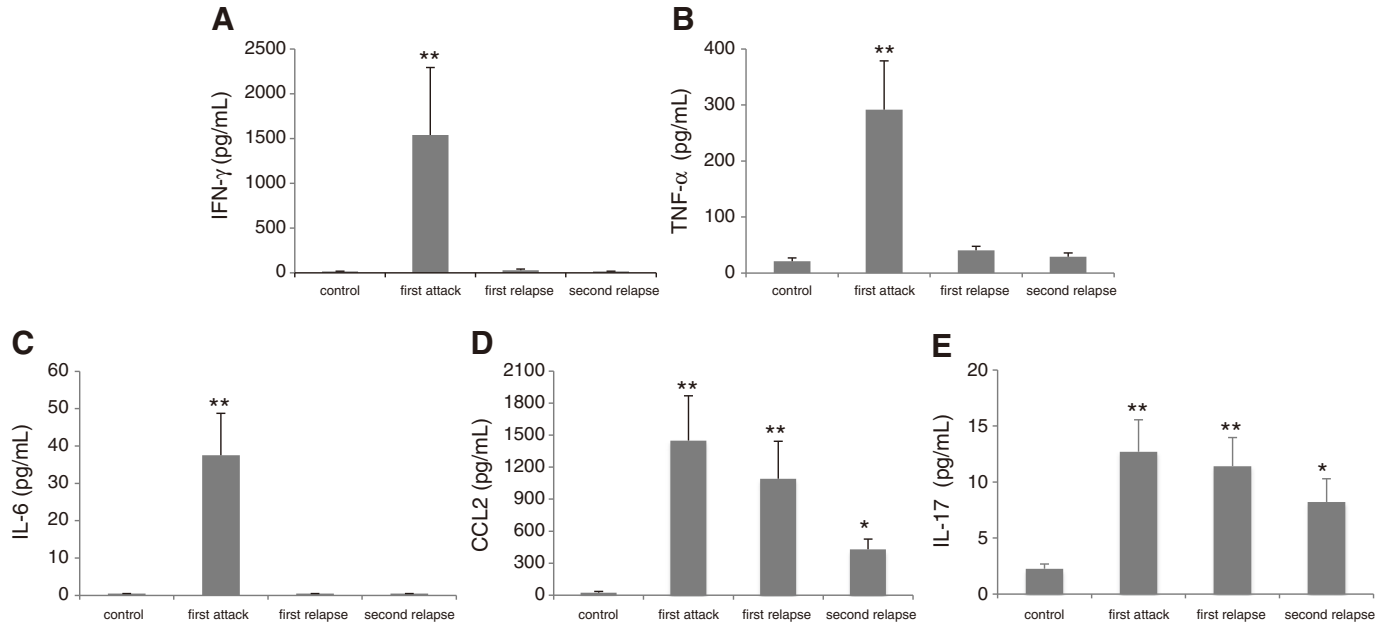

Fig. 5. Cytokine production in MOG_{35-55}-immunized CREAE NOD mice. Spleen cells were harvested during the first attack, first relapse, and second relapse, and then cultured with MOG_{35-55} for 72 h. Culture supernatants were collected and analyzed with a CBA assay. The IFN-γ (A), TNF-α (B), and IL-6 (C) production levels of the first attack group were higher than those of the control group, whose levels were comparable to those of the first and second relapse groups. CCL2 (D) and IL-17 (E) production levels of the first attack, first relapse, and second relapse groups were higher than those of the control group. $^{*}p < 0.05$; $^{**}p < 0.01$.

with parenchymal infiltration, of inflammatory cells in the CNS in CREAE.

IL-6 is a plurifunctional cytokine that is necessary for inducing cerebrovascular adhesion molecules, such as VCAM-1, which are essential for leukocyte trafficking to the CNS during EAE [21]. Other studies on EAE have demonstrated that the formation of $CD4^{+}$ Th17 cells is driven by IL-6 together with TGF-β [22]. Furthermore, IL-6-deficient mice are resistant to EAE induction, and decreased IL-6 expression in the CNS has been associated with attenuated EAE [23,24]. Such reports confirm the importance of this cytokine in CNS pathology. In the present study, we demonstrated that IL-6 production by spleen cells and IL-6 mRNA in the CNS of CREAE mice were elevated during the first attack only, which, along with IFN-γ and TNF-α, implied that different mechanisms were involved in disease development and recurrence.

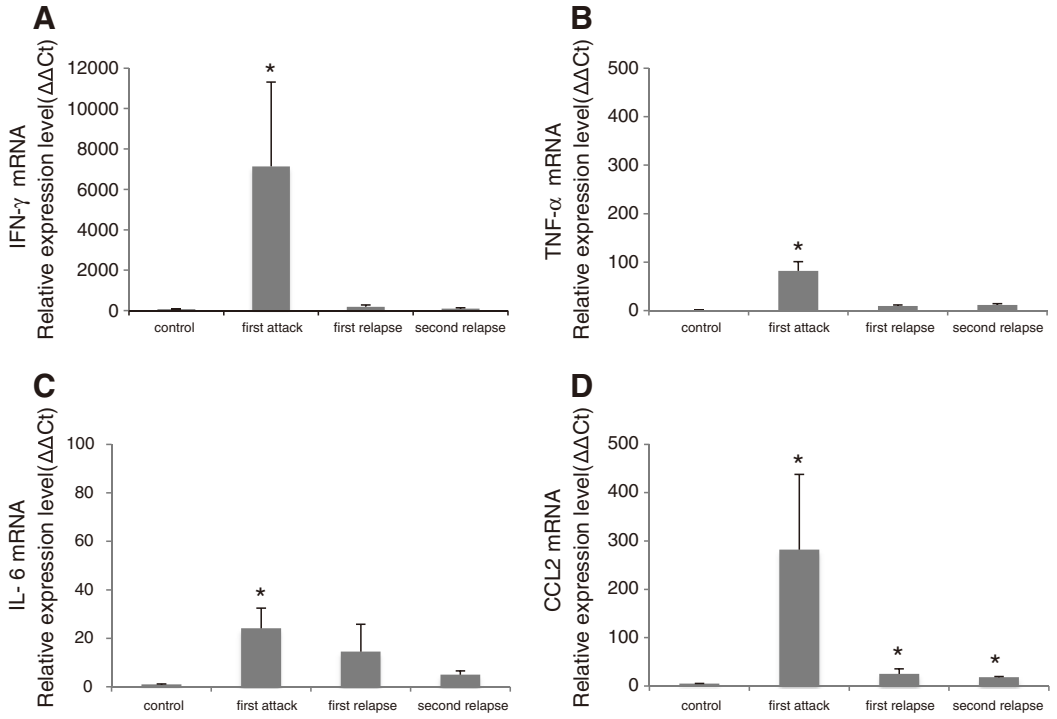

Fig. 6. Expression of mRNA in the CNS of MOG_{35-55}-immunized CREAE NOD mice. Spinal cord samples were obtained during the first attack, first relapse, and second relapse for analysis using real-time PCR. The IFN-γ (A), TNF-α (B), and IL-6 (C) mRNA expression levels of the first attack group were higher than those of the control group, whose levels were comparable to those of the first and second relapse groups. The CCL2 (D) mRNA expression levels of the first attack, first relapse, and second relapse groups were higher than those of the control group. $^{*}p < 0.05$.

Meanwhile, we found that IL-17 production by spleen cells was increased during the first attack and in both relapses. IL-17 is strongly believed to contribute to CREAE relapse. In support of this, Li et al. [25] reported an increase in CD4+Th17 cells in MS patients.

Our histopathological findings in MOG_{35-55}-immunized mice showed marked inflammatory cell infiltration in the perivascular and subarachnoid space at the first attack followed by infiltration into the white matter and demyelination during relapses. The later, more severe, histological findings were consistent with the increasing severity of the clinical symptoms. To our knowledge, this is the first report to analyze CNS pathology in CREAE. Although both Th1- and Th17-cell cytokines appeared to play an important role in the initial attack of CREAE, Th17-cell cytokines alone seemed to be critical for the occurrence of relapses. These cytokine production profiles might have also influenced CNS pathology in CREAE.

Lastly, chemokines govern the transmigration of monocytes and T cells and play a pivotal role in the pathogenesis of EAE [26,27]. CCL2 was one of the first chemokines to be associated with pathological inflammation [28], and tissue sections from MS patients have shown abundant CCL2 immunoreactivity in both active and chronic active lesions [29,30]. CCL2 mRNA expression in CNS lesions was seen to be up-regulated in murine EAE and neutralized with anti-CCL2 antibody, while DNA vaccination before immunization protected mice against EAE [31,32]. Furthermore, administration of a mutated CCL2 protein, which could perturb with chemotactic properties, inhibited cell recruitment and protected against demyelination and axonal loss in CNS lesions of EAE mice [33]. Our findings clearly showed that CCL2 was involved in both disease onset and recurrence. However, although CCL2 and IL-17 were both considered to contribute to the relapse of CREAE, their elevated concentrations during recurrences may have been residual of those produced at the first attack. Further analysis of recovery stages would be informative.

In conclusion, we have demonstrated that IFN-γ, TNF-α, IL-6, and CCL2 production and mRNA expression are increased during the first attack of CREAE. These findings are consistent with the histopathological observations of inflammatory cell infiltration into the perivascular space. On the other hand, CCL2 and possibly IL-17 appear to play a prominent role in the first and second relapses in CREAE mice, when more severe histopathological findings, such as parenchymal infiltration and demyelination, are evident. Thus, the mechanisms responsible for relapses of CREAE may be different from those initiating the first attack. Further investigation of CREAE mechanisms may provide important insights into future therapies for human RRMS.

Authors' contributions

YH observed the symptoms of EAE animals and conducted the cytokine production and mRNA expression study. YI and MIc directed the study and wrote the article. KM participated in the real-time PCR experiments. MIt participated in the analysis of histopathology. TK, YN, and C-SK participated in technical discussions and helped to draft the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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