Chronological Changes of CD4$^+$ and CD8$^+$ T Cell Subsets in the Experimental Autoimmune Encephalomyelitis, a Mouse Model of Multiple Sclerosis

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Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS). Although the etiology of MS remains unclear, T cells specific for myelin components such as myelin basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG) are thought to play a critical role in MS pathophysiology (Bitsch et al. 2000; Nosewothy et al. 2000). The development of MS is related to major histocompatibility complex
(MHC) class II allele expression, suggesting a large pathogenic role for CD4+ T cells (Sawcer et al. 1996; The Multiple Sclerosis Genetics Group 1996). Experimental autoimmune encephalomyelitis (EAE) is used as an animal model of MS and can be induced by immunization with myelin peptides such as MOG and MBP. T helper type 1 (Th1) cells play an essential role for the development and progression of EAE through the production of Th1 cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) (Ichikawa et al. 2000). Th17 cells also play an important role in the development of EAE (Komiya et al. 2006). However, myelin antigen specific CD8+ T cells are also seen in EAE mice and patients with MS (Biddison et al. 1998; Huesby et al. 2001; Sun et al. 2001; Zang et al. 2004; Crawford et al. 2004; Ford and Evavold 2005). Adoptive transfer of MBP or MOG specific CD8+ T cells induces EAE in recipient mice (Huesby et al. 2001; Ford and Evavold 2005), and CD8+ T cells isolated from MS patients specifically damage COS cells expressing MBP ex vivo (Zang et al. 2004). Therefore, CD8+ T cells may play a predominant role in the development of MS or EAE than previously expected. We wished to examine the development and cytokine profile of CD8+ T cells in both the periphery and CNS of EAE mice.

The number of T cells within the CNS is very low, and most studies of EAE have analyzed the immunological function and phenotype of cells in peripheral lymphoid tissues such as draining lymph nodes and the spleen. In patients with MS, peripheral blood mononuclear cells are most frequently examined. Though Brabb et al. (2000) showed that naïve-MBP specific T cells do not trigger autoimmunity, little is known about the activated MOG-reactive T cell populations within the CNS. Similarly, the possible relationship between CNS T cell populations and those in peripheral lymphoid tissues are unknown. Therefore, to better understand the pathophysiological mechanism of EAE, we examined the mRNA expression for Th1 cytokines (IFN-γ and TNF-α), Th2 cytokines (IL-10 and IL-4), and IL-17 in MOG35-55-specific CD4+ and CD8+ T cells in the spleen and CNS following immunization with MOG35-55 using real time RT-PCR.

**MATERIALS AND METHODS**

**Animals and reagents**

C57BL/6J mice were purchased from Japan SLC (Hamamatsu). The protocols for animal experiments were approved by the Animal Experiment Committee of Research Institute of Environmental Medicine, Nagoya University. MOG35-55 peptide (MEVGWYRSPFSRVVHLYRNGK) was synthesized and purified by Operon Biotechnologies (Tokyo). Incomplete Freund’s adjuvant (IFA) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Heat-killed Mycobacterium tuberculosis H37Ra was obtained from Difco (Detroit, MI, USA). Pertussis toxin (PTX) was obtained from List Biological Laboratories (Campbell, CA, USA).

**Induction of EAE**

EAE was induced as described previously (Kato et al. 2004; Takeuchi et al. 2006). Briefly, C57BL/6J mice aged 8 weeks were immunized subcutaneously at the base of the tail with 0.2 ml of emulsion containing 200 μg MOG 35-55 in phosphate buffered saline (PBS) combined with an equal volume of IFA containing 300 μg heat-killed Mycobacterium Tuberculosis H37Ra. Mice were injected with PTX intraperitoneally on the day of immunization and two days after immunization (200 ng/mouse). Mice were assessed daily for clinical signs of EAE according to the following scale: 0-normal; 1-limp tail or mild hind limb weakness; 2-moderate hind limb weakness or mild ataxia; 3-moderate to severe hind limb weakness; 4-severe hind limb weakness; 5-paraplegia with moderate forelimb weakness; 6-paraplegia with severe forelimb weakness, severe ataxia, or moribund. All the mice (4 mice per time frame) immunized developed EAE as shown in Fig. 1. The CNS tissue and spleen samples were collected from mice at days 0, 6, 12, 14-16 (the onset of illness), 17-19 (the peak of clinical symptoms), and 20-22 (the chronic phase) after immunization with MOG35-55.

**Cell preparation and culture**

The spleen was isolated on a sterile wire sieve over a Petri dish filled with PBS. Single cell suspensions were prepared by lysing red blood cells using Red blood cell lysing buffer (Sigma). Spleen cells were plated in a 24-well culture plate at 3 x 10^6/well in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum.
CD4+ and CD8+ T Cell Subsets During the Course of EAE

Flow cytometric assay

Single cell suspensions were resuspended in fluorescence activated cell sorting (FACS) buffer consisting of PBS supplemented with 2% FBS and 0.01% Sodium azide. The cells were then incubated with rat anti-mouse monoclonal antibodies for cell surface staining (anti-CD3-FITC, anti-CD4-PerCP, anti-CD8-PE; all from BD Biosciences, San Jose, CA, USA) for 30 min at 4°C.

For the detection of MOG35,55 specific T cells, we used the 5-carboxyfluorescein diacetate succinimidyl ester (CFSE)–based proliferation assay reported by Crawford et al. (2004). This method detects dividing cells through the sequential halving of their green fluorescence. The single cell suspensions obtained from either spleen or CNS were incubated at 37°C for 7 min with 2 μM CFSE. After washing, the cells were cultured with MOG as described above. On the day 4 of culture, after elimination of CD11b+ cells as described above, cells were incubated with antibodies against CD4 (rat anti-mouse CD4 from Serotec, Oxford, UK) followed by labeling with goat anti-rat PE (BD Biosciences) or CD8 (anti-CD8-PE) for 30 min 4°C. The samples were examined with a Cytomics FC500 (Beckman Coulter, Fullerton, CA, USA) and analyzed with CXP Software ver.2.0 (Beckman Coulter). We detected CFSElow cells and calculated the change in proliferating fraction (ΔPF) by subtracting the proportion of non-stimulated CFSElow cells (no antigen) from that of CFSElow cells in response to MOG35,55.

RNA isolation and reverse transcription (RT)

RNA isolation and RT were performed as described previously (Sonobe et al. 2005). In brief, after centrifugation of sorted cell suspensions, total RNA was extracted from the pellet using RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol. After 0.2 μg of total RNA was denatured for 5 min at 65°C, RT was performed by incubating at 42°C for 50 min in 50 μl of reaction solution containing 50 mM Tris-HCl (pH8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 0.5 mM dNTPs, 0.5 μg oligo (dT) (12-18) primer, 40 U RNase inhibitor and 200 U Superscript II RNase H-Reverse transcriptase (Invitrogen) followed by heating at 70°C for 15 min.

Fig. 1. The course of EAE in MOG immunized mice. EAE was induced by immunizing C57BL/6J mice with MOG35,55 and i.p. injections of PTX on days 0 and 2 after immunization. Mice were scored daily as described in Materials and Methods. A representative disease severity curve is shown (n = 4).

(FBS) (JRH Biosciences, Lenexa, KS, USA), 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Invitrogen, San Diego, CA, USA) with or without 20 μg/ml MOG35,55.

The CNS cells were prepared from mice intracardially perfused with PBS from the left ventricle. The brain and spinal cord were then removed and passed through a nylon mesh (NB 60; NBC, Tokyo). The CNS infiltrating mononuclear cells were enriched using a 30% percoll gradient. The cells were stimulated with 20 μg/ml MOG35,55 with 50 μg/ml mitomycin C (Wako, Osaka)-treated spleen cells (1 : 3) from normal mice as antigen presenting cells in a round bottomed 96-well culture plate at 4 × 10^5/well in RPMI 1640 supplemented as described above.

Unless indicated, isolated cells were incubated for four days with MOG35,55. After incubation, they were collected and subjected to flow cytometric assay and cell sorting using MACS beads as below.

CD4+ and CD8+ T cell sorting using magnetic beads

Cells were first incubated with magnetic beads conjugated to anti-CD11b (Miltenyi Biotec, Bergisch Gladbach, Germany) antibody for 15 min at 4°C to deplete CD4+ macrophages or microglia, washed with MACSTM buffer (0.5% FBS in PBS) and sorted according to the manufacturer’s protocol. The negatively selected fraction was then incubated with anti-CD4 or anti-CD8 conjugated magnetic beads for 15 min at 4°C and sorted. The purity of sorted cells was > 98%.

CD4+ and CD8+ T cell subsets during the course of EAE
Real time polymerase chain reaction (PCR)

Real time PCR was performed using an ABI PRISM® as described previously (Sonobe et al. 2005). In brief, the primers were designed on different exons using Primer Express® software version 2.0 (Applied Biosystems, Piscataway, NJ, USA) and each PCR product was confirmed as a single band by agarose gel electrophoresis analysis. Then, 4 μl cDNA from the Superscript II reaction described above was mixed with a final concentration of 1 x SYBR® Green PCR Master Mix (Applied Biosystems), 500 nM forward primer, 500 nM reverse primer, and deionized water in a total volume of 50 μl. Thermal cycling parameters for primer optimization were as follows: activation at 95°C for 10 min followed by 50 cycles 95°C for 15 sec and 60°C for 1 min. The mRNA level for each sample was normalized against GAPDH mRNA. Specific primers are shown in Table 1.

Statistical analyses

Significant differences were examined using one-way ANOVA. p-values < 0.05 were considered significant.

RESULTS

The number of CD4+ and CD8+ T cells in the spleen and CNS of EAE

Mice immunized with MOG35-55 peptide in CFA developed EAE between days 14 and 16, and the clinical scores peaked (score 4 or score 5) at days 17 to 20. Thereafter, the clinical score was diminished and maintained at score 3 or 4 (the chronic phase) (Fig. 1). At various time points after immunization (day 0, day 6, day12, onset, peak and chronic phase), spleen and CNS cells were isolated from immunized mice, and the number of CD4+ and CD8+ T cells was determined (Fig. 2). In the spleen, the number of CD4+ T cells peaked at day 12, just before the onset of disease. Then, they decreased as EAE progression, whereas the number of CD8+ T cell increased only slightly. In contrast, in the CNS, the number of CD4+ T cells remained unchanged until day 12, and then they began to rapidly increase to a maximum at the peak of disease severity; CD4+ T cell numbers declined slightly during the chronic disease phase (Fig. 2B, CNS). CD8+ T cell numbers followed a similar pattern, to the lesser extent. At all time points examined in both the spleen and CNS, the number of CD4+ T cells were much more than CD8+ T cells.

MOG35-55 specific CD4+ T cells and CD8+ T cells in the spleen and CNS

We next examined the presence of MOG35-55 specific CD4+ and CD8+ T cells in both the spleen and CNS at various time points after EAE induction using a CFSE-based proliferation assay (Fig. 3). MOG35-55 specific CD4+ and CD8+ T cells proliferated in the spleen at all time points examined (Fig. 3B, Spleen). In contrast, in the CNS, MOG-reactive T cells first appeared at day 12 just before the disease onset and dramatically increased to reach a maximum at the peak of EAE severity (Fig. 3B, CNS). CD4+ and CD8+ T cell reactivity to MOG35-55 was virtually the same at all time points after day 12 in the CNS. Taken together, MOG-reactive T cells in the CNS play roles in the development of EAE.

Functional differences in CD4+ and CD8+ T cells specific for MOG35.55 at various time points in the spleen and the CNS

To clarify the functional differences between

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<tr>
<th>Molecule</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>GAPDH</td>
<td>TGTGTCCGTCGTGGATCTGA</td>
<td>CCTGCTTCACCCACCTTCTTTGA</td>
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<tr>
<td>IFN-γ</td>
<td>TGGCATAGATGGGAAGAAAGAG</td>
<td>TGCAGGAATTTITCATGTCCCAT</td>
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<tr>
<td>IL-10</td>
<td>GAGAAGCATGCCAGAATACT</td>
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<td>IL-4</td>
<td>TGTGCCAAGCTCCITCACA</td>
<td>GCACCCTGGGAAGCCCTACAG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>GACCCCTCACACTCAGATCATCTTCT</td>
<td>CCACCTGGTGGTTTGGCTACAG</td>
</tr>
<tr>
<td>IL-17</td>
<td>TCACTGTGTCTCTGGATGCTTGTG</td>
<td>TCGCTGCTTCCACTGT</td>
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| Table 1. Primer sets for real time PCR. |
MOG-specific CD4$^{+}$ and CD8$^{+}$ T cells in the spleen and CNS at various time points after EAE induction, we used real-time RT-PCR to assess mRNA expression for Th1 and Th2 cytokines (Figs. 4 and 5). In the spleen, IFN-$\gamma$ mRNA expression was strongly induced in CD4$^{+}$ T cells at the disease onset, and its level remained relatively high during the disease course (Fig. 4). In contrast, IFN-$\gamma$ mRNA expression in CD8$^{+}$ T cells was only slightly elevated at the peak of disease severity. TNF-$\alpha$ mRNA expression followed a similar course as IFN-$\gamma$. IL-4 mRNA expression in CD4$^{+}$ T cells was rapidly induced on day 6, then it returned to baseline levels. A mild early and stable increase in IL-4 mRNA expression was seen in CD8$^{+}$ T cells. In contrast, IL-10 mRNA expression dropped before the disease onset in both CD4$^{+}$ and CD8$^{+}$ T cells. IL-17 mRNA expression was highly induced in CD4$^{+}$ T cells, but not CD8$^{+}$ T cells, on day 6 and peaked at the disease onset.

In the CNS, IFN-$\gamma$ mRNA expression reached a maximum in CD4$^{+}$ T cells at the disease onset (Fig. 5). In contrast, IFN-$\gamma$ mRNA expres-
Fig. 3. Detection of MOG35-55 specific CD4+ and CD8+ T cells. CFSE-labeled spleen cells and CNS cells were cultured for four days in the presence of MOG35-55. After exclusion of CD11b+ cells using MACS beads, remaining cells were incubated with Abs against CD4 followed by labeling with goat anti-rat PE conjugated Abs or stained with PE-labeled anti-CD8 Abs, then analyzed by flow cytometry. (A) Representative data of the spleen cells at the peak of disease severity. (B) ΔPF was calculated by subtracting the background proliferation from the proliferating fraction in response to MOG35-55 to designate the reactivity to MOG35-55. Black bar indicates CD4+ T cells and white bar shows CD8+ T cells. Results are expressed as the mean ± s.e.m. (n = 3).
expression was only slightly increased at the peak of disease severity. But the degree of expression was much greater in CD4⁺ T cells. They showed similar kinetics to splenic T cells. TNF-α mRNA expression reached a maximum at the peak of disease severity in both CD4⁺ and CD8⁺ T cells. In contrast, no IL-4 mRNA expression was seen throughout the disease course in both T cell subsets, suggesting that neither Th2 nor Tc2 cells developed in the CNS during EAE progression. IL-17 mRNA expression was induced on day 6 in CD4⁺ T cells, and peaked with disease severity, but it remained low in CD8⁺ T cells at all time points examined.

**DISCUSSION**

The expression and production of cytokines are essential to the pathogenesis of EAE, and several studies have examined the role of cytokines in the development of EAE both in the periphery and the CNS (Kennedy et al. 1992; Targoni et al. 2001; Hofstetter et al. 2005a). Kennedy et al.
(1992) examined the expression of six cytokine mRNAs (IL-1α, IL-2, IL-4, IL-6, IL-10, and IFN-γ) in the CNS of EAE mice. Targoni et al. (2001) and Hofstetter et al. (2005b) assessed the protein levels of four cytokines (IFN-γ, IL-2, IL-4, and IL-5) in the periphery and in the CNS. However, these studies did not determine whether CD4+ or CD8+ T cells produced these cytokines. In addition, CD4+ and CD8+ T cell expression of IL-17, and TNF-α during the course of EAE remained unknown. In the present study, we examined the cytokine profile of CD4+ and CD8+ T cells both in the periphery and in the CNS during the course of EAE.

We first evaluated the numbers of both T cell subsets at various time points after MOG35-55 immunization. In the spleen, CD4+ and CD8+ T cells increased in number before EAE onset; the increase in CD4+ T cells was more prominent. In contrast, both T cell subsets in the CNS increased as disease severity increased. These results support the hypothesis that activated/memory T cells...
have an advantage over naïve T cells in crossing the blood brain barrier (Brabb et al. 2000). Consistent with this, we also showed that both splenic and CNS CD4$^+$ and CD8$^+$ T cells were MOG$_{35,55}$ reactive. In the spleen, both CD4$^+$ and CD8$^+$ T cells were MOG-reactive at day 6, while the MOG reactivity of both CD4$^+$ and CD8$^+$ T cells in the CNS increased dramatically during disease progression. These data may suggest that activated CD4$^+$ T cells entering the CNS are re-stimulated by local antigen presenting cells. Alternatively, CD4$^+$ T cell infiltration into the CNS is biphasic. Flügel et al. (2001) reported that a low number of CD4$^+$ T cells first infiltrated into the CNS, but this was followed by a second wave of T cell infiltration. In contrast to the CD4$^+$ T cell data, the number of CD8$^+$ T cells rapidly increased at the EAE onset but subsequently decreased at later time points. However, these CD8$^+$ T cells were highly MOG$_{35,55}$ reactive. It is possible that only highly MOG-reactive CD8$^+$ T cells can infiltrate into the CNS at the later phase of EAE, or MOG-reactive CD8$^+$ T cells may be generated locally. The early infiltration of CD4$^+$ T cells may be important for the development of EAE, and the subsequent presence of highly MOG-reactive CD8$^+$ T cells may promote disease maintenance. We detected low numbers of both T cell subsets in the CNS before the disease onset, and this is consistent with previous study by Wekerle et al. (1986) and Bradl et al. (2005) that activated T cells may exist or patrol in the CNS.

IFN-γ promotes inflammatory demyelination through a variety of mechanisms. It also induces neuronal degeneration by stimulating cytotoxic T cells and microglia (Madana et al. 2001; Takeuchi et al. 2005). Transgenic mice expressing IFN-γ induce CNS demyelination through upregulation of MHC molecules, gliosis and lymphatic infiltration (Horwitz et al. 1997). Both CD4$^+$ and CD8$^+$ T cells can produce IFN-γ, and CD4$^+$ T cells were induced to express IFN-γ mRNA at the onset of EAE, while IFN-γ mRNA expression in CD8$^+$ T cells increased only slightly later during the disease course. These data suggest that the source of IFN-γ in EAE lesions is mostly Th1 cells. Adoptive transfer of CD4$^+$ T cells induces EAE earlier than transfer of CD8$^+$ T cells (Ford and Evavold 2005). Thus, CD4$^+$ T cell-produced IFN-γ is likely related to the induction of EAE, but IFN-γ from CD8$^+$ T cells may play a role in maintenance of EAE later stages. TNF-α may also be involved in neuronal degeneration both directly and indirectly via activation of microglia in the chronic stage of MS (Selmaj et al. 1991; Brück and Stadelmann 2003; Kuno et al. 2005). The expression of TNF-α mRNA by both T cell subsets in the CNS is most strongly induced at the later stage of EAE, and T cell-derived TNF-α may be important in the pathophysiology of the EAE late stage.

Recent studies suggest that IL-17 plays a greater role in EAE induction than IFN-γ (Langrish et al. 2005). The development of EAE is completely suppressed in IL-23p19 deficient mice that are highly defective in IL-17 (Cua et al. 2003). However, as shown here, the kinetics of IL-17 mRNA expression in CD4$^+$ T cells was very similar to those of IFN-γ and TNF-α, but increases in IL-17 mRNA occur slightly earlier. These data suggest that both Th1 and IL-17 producing CD4$^+$ T cells are important for the development of EAE.

IL-10 is produced by CD4$^+$CD25$^+$ and CD8$^+$CD122$^+$ regulatory T cells and can suppress EAE severity (Bettelli et al. 1998; Dieckmann et al. 2001; Endoharti et al. 2005). IL-10 mRNA expression in both CD4$^+$ and CD8$^+$ T cells decreased immediately prior to disease onset in the spleen, but gradually increased in the CNS, suggesting that these regulatory cells might suppress the immunopathology during disease recovery and the chronic phase. IL-10 mRNA expression in CD8$^+$ T cells gradually increased in the late phase of EAE. This is consistent with the report by Kennedy et al. (1992) showing that IL-10 mRNA expression in the spinal cord was low during the early stages of EAE but maximized in the recovery phase. In addition, IL-10 mRNA expression in CD8$^+$ T cells was more higher than inflammatory cytokines mRNA expression. It has been reported that anti-CD8 antibody treatment against EAE mice enhances EAE induction (Najafian et al. 2003; Montero et
al. 2004). From these findings, CD8+ T cells have mainly regulatory role in the later phase of EAE. Kennedy et al. (1992) also reported that IL-4 mRNA was induced in the spinal cord at an early stage of EAE. However, IL-4 mRNA expression in CD4+ and CD8+ T cells remained low in the CNS during the course of EAE, but it was high at day 6 in peripheral CD4+ T cells. IL-4 may be produced by non-T cells as suggested by Hofstetter et al. (2005b), if it is present in the CNS.

In summary, our data suggest that CD4+ T cells are mainly involved in the early induction phase of EAE, and CD8+ T cells may play a regulatory role in the later phase of EAE. Both CD4+ and CD8+ T cells produce the Th1 cytokines IFN-γ and TNF-α in the periphery and CNS, while CD4+ T cells also produce IL-17. The kinetics of Th1 cytokine and IL-17 expression by CD4+ T cells are similar, suggesting that both are important for the induction of EAE. IL-10 mRNA expression is higher than inflammatory cytokine mRNA expression. The complete absence of IL-4 producing T cells in the CNS may also be important for disease onset or severity.

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