

an attractive Alzheimer's disease animal model.<sup>94</sup> Investigators have been employed with rhesus monkeys for studies on brain injuries<sup>95,96</sup> and AD.<sup>97,98</sup> Lemere et al. immunized five aged rhesus monkeys (16–25 yr) with A $\beta$  peptide over 10 months and examined plasma and CSF samples longitudinally. By day 42, immunized monkeys generated plasma A $\beta$  antibodies that labeled plaques in the human AD and model mouse brain. The antibody levels peaked at day 100 (1,677  $\mu$ g/ml in plasma, 2.1  $\mu$ g/ml in CSF) in the immunized animals and dropped to relatively steady levels by Day 301 (452 mg/ml in plasma, 0.2 mg/ml in CSF). Soluble A $\beta$  levels were elevated approximately 2- to 5-fold in plasma and decreased up to 64% in CSF in immunized rhesus monkeys. Insoluble A $\beta$  was decreased by 66% in brain homogenates of the immunized animals compared to controls.<sup>99</sup> Since it is difficult to obtain rhesus monkeys, rhesus monkeys (*Macaca mulatta*) were also examined extensively.<sup>100</sup> Senile plaques appear in variable numbers in animals >23 years but were not seen in monkeys <15 years of age.<sup>101</sup> A $\beta$  deposits were observed in the cerebral cortex of all aged animals.<sup>102</sup> Gandy et al. injected aggregated A $\beta$ 1–42 to rhesus monkeys (*Macaca mulatta*). Immunized monkeys developed anti-A $\beta$  titers exceeding 1:1,000, and their plasma A $\beta$  levels were 5–10-fold higher than the plasma A $\beta$  levels observed in monkeys vaccinated with aggregated amylin.<sup>103</sup>

Recently, we evaluated the effects of DNA vaccines on A $\beta$  reduction in aged rhesus monkeys (15–18 years old) (manuscript in preparation). DNA vaccine and empty vector (control) at a dose of 3 mg were injected 15 times for 6 months ( $n = 3$  in each group). Quantitative analysis revealed a significant reduction of A $\beta$  in treated monkeys without obvious side effects. Previous two studies<sup>99,103</sup> and our examination clearly demonstrated A $\beta$  reduction in monkeys after vaccination. Analysis of oligomeric A $\beta$  and cognitive assessment will be necessary to determine whether such immunotherapies provide beneficial effects on behavioral disorders. Collectively, these findings suggest that DNA vaccines are also effective in A $\beta$  reduction in non-human primates.

## Conclusion

Current drug therapies for AD aim at slowing the cognitive decline and ameliorating the affective and behavioral signs associated with disease progression. Based on the results obtained in animal and human clinical studies, A $\beta$  vaccine therapy is a principal strategy for AD treatment. Active peptide immunotherapy and passive immunotherapy have some problems to be solved before clinical application. Newly developed DNA vaccines were effective in reducing A $\beta$  plaque on model mice and aged monkeys. Oligomeric A $\beta$ , which is most neurotoxic among A $\beta$  species, was also decreased in the mouse brain after vaccination. Although preclinical trials are under way, DNA vaccination will be a promising therapy for Alzheimer's disease in the near future.

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# Characterization of CD8-positive Macrophages Infiltrating the Central Nervous System of Rats With Chronic Autoimmune Encephalomyelitis

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CD8<sup>+</sup> macrophages appear in the central nervous system (CNS) under various pathological conditions such as trauma and ischemia. Furthermore, macrophages expressing CD8 were found in CNS lesions of chronic, but not acute, experimental autoimmune encephalomyelitis (EAE). To further characterize cells with this phenotype, we examined CD8<sup>+</sup> macrophages/monocytes in the CNS and peripheral organs during the course of acute and chronic EAE that had been induced by immunization of rats with myelin basic protein and myelin oligodendrocyte glycoprotein, respectively. Counting CD8<sup>+</sup> macrophages in CNS lesions revealed that their numbers increased reaching about 60% of total infiltrating macrophages in chronic EAE, while CD8<sup>+</sup> macrophages remained less than 5% throughout the course of acute EAE. Unexpectedly, however, higher abundance of CD8<sup>+</sup> monocytes/macrophages in the peripheral blood was found in both acute and chronic EAE. Real-time polymerase chain reaction analysis revealed no significant difference in the levels of chemokines and chemokine receptors of blood CD8<sup>+</sup> monocytes between acute and chronic EAE. mRNA expression of perforin, a cytotoxic substance, was up-regulated in CD8<sup>+</sup> monocytes compared with that of CD8<sup>-</sup> monocytes in both acute and chronic EAE. These findings suggest that activated CD8<sup>+</sup> macrophages may play a cytotoxic role in chronic EAE lesions and that cells other than CD8<sup>+</sup> monocytes/macrophages determined the difference in CNS pathology between acute and chronic EAE. Analysis of CD8<sup>+</sup> monocytes/macrophages may provide useful information to permit further dissect the pathomechanisms of multiple sclerosis and to develop effective immunotherapies against autoimmune diseases in the CNS. © 2008 Wiley-Liss, Inc.

**Key words:** experimental autoimmune encephalomyelitis; chronicity; CD8; macrophage

Multiple sclerosis (MS), which is considered to be an autoimmune disease involving the central nervous system (CNS) (Sospendra and Martin, 2005), is characterized by relapse and remission of the disease (Lublin and Reingold, 1996). In about half of all MS patients,

the clinical course changes from relapsing–remitting to secondary progressive MS after 10 years (Noseworthy et al., 2000). However, little is known with regard to the pathomechanism of disease progression. In experimental settings, it is suggested that encephalitogenic T-cell activation is closely associated with disease relapse and progression because inactivation or elimination of pathogenic T cells prevented further relapse (Chen et al., 1998; Shao et al., 2003; Pryce et al., 2005). In contrast, tolerance induction to T cells failed to halt progression of a certain type of experimental autoimmune encephalomyelitis (EAE), suggesting that non-T immune cells, such as macrophages (MΦ), are involved in disease progression (Pryce et al., 2005).

MΦ are major immunocompetent cells that have multiple functions. As reviewed by Duffield (2003), MΦ in inflammatory lesions either promote immune-mediated damage or act as tissue-healing cells, depending on their subtypes, the stage of inflammation, and other undetermined factors. In vitro studies demonstrated that MΦ phagocytose degraded myelin proteins such as myelin basic protein (MBP), proteolipid protein, and myelin oligodendrocyte glycoprotein (MOG) and released tumor necrosis factor alpha (TNF-α) and nitric oxide, which may further contribute to the overall process of demyelination during MS and EAE (van der Laan et al., 1996; van der Goes et al., 2005). Recently, it was also reported that a large number of CD8<sup>+</sup> MΦ were found in the spinal cord of rats with chronic, but not acute, EAE (Schroeter et al., 2003), suggesting that CD8<sup>+</sup> MΦ play a role in the progression of EAE. Importantly, CD8<sup>+</sup> monocytes (MO)/MΦ were found in the peripheral blood of healthy human subjects and in the target

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organ of patients with autoimmune disease (Nakamura et al., 2004; Baba et al., 2006).

In the present study, we tried to elucidate the pathomechanisms of the disease progression of chronic EAE by examining the role of CD8<sup>+</sup> MΦ that infiltrate CNS lesions. For this purpose, we first performed quantitative and longitudinal examinations of CD8<sup>+</sup> MΦ in injured spinal cord tissues. Then we measured the abundance and the kinetics of this MΦ subpopulation in the peripheral blood and lymphoid organs during EAE. Finally, we determined the levels of chemokine, chemokine receptor, and MΦ-associated factor mRNA of sorted CD8<sup>+</sup> MO in the peripheral organs in comparison with those of the CD8<sup>-</sup> counterpart. The present analysis revealed several interesting features of CD8<sup>+</sup> MΦ and strongly suggested that infiltration of CD8<sup>+</sup> MΦ into the CNS is closely associated with the maintenance of chronic lesions in the CNS.

## MATERIALS AND METHODS

Unless otherwise indicated, all reagents and apparatus were obtained in Tokyo, Japan.

### Animals

LEW.1AV1 rats (RT1<sup>av1</sup> in the LEW background gene) were provided by Dr. R. Gold, Department of Neurology, Würzburg University, Germany, and maintained in our animal facility. Lewis (LEW) rats (RT1<sup>1</sup> in the LEW background gene) were purchased from Japan SLC Inc. (Shizuoka). Rats were 8–12 weeks of age. All animal experiments were approved by the appropriate institutional committee and were performed in accordance with institutional guidelines.

### Reagents

Recombinant rat MOG was prepared as described previously (Sakuma et al., 2004). Briefly, the gene coding the extracellular domain (amino acid 1–125) of MOG was amplified by using primers specific to the corresponding MOG sequence. The polymerase chain reaction (PCR) products were then digested with *SphI* and *HindIII* and subcloned into pQE30 (Qiagen) used for large-scale preparation. Recombinant MOG produced in transformed *Escherichia coli* were isolated under denaturing conditions and purified with Ni-NTA Agarose (Qiagen). Then purified MOG was diluted and refolded in phosphate-buffered saline containing 1 M L-arginine, 2 mM glutathione (reduced form), and 0.2 mM glutathione (oxidized form). The obtained protein contained endotoxins at less than 10 EU/1 mg protein as determined with a Toxinometer ET-2000 (Wako). Guinea pig, bovine, and rat MBP were prepared as described previously (Deibler et al., 1972).

### EAE Induction and Clinical Evaluation

In early experiments in the present study, acute EAE was induced in LEW rats by immunization with 100 μg MBP emulsified in an equal volume of complete Freund's adjuvant (CFA) (*Mycobacterium tuberculosis*, 2.5 mg/ml) in the footpads and chronic EAE was induced in LEW.1AV1 rats by immunization with 250 μg MOG/CFA (*M. tuberculosis* H37RA, 2.5

mg/ml) in the tail base. Later, we learned that LEW.1AV1 rats developed acute monophasic EAE after immunization with MBP/CFA, and we also analyzed this type of EAE. Pertussis toxin (2 μg) was administered intraperitoneally once at a time of MOG immunization. Clinical signs were evaluated daily as the total score of the degree of paresis of each limb and tail (partial paresis, 0.5; complete paresis, 1.0). Therefore, the clinical score of complete paralysis of four limbs plus the tail or moribund conditions was 5.

### Histological and Immunohistochemical Examination

The optic nerve, cerebrum, brain stem, and cerebellum and the cervical, thoracic, and lumbar spinal cord were routinely examined for histology of chronic EAE, while acute EAE was mainly evaluated by examination of the lumbar spinal cord. The tissues were fixed in 4% paraformaldehyde and processed for paraffin embedding. Six-micron sections were cut and stained with hematoxylin and eosin (HE) and with Kruever and Barrera's (KB) method (Luxol fast blue plus cresyl violet). Inflammatory lesions were graded using sections stained with HE into four categories (grade 1, leptomeningeal and adjacent subpial cell infiltration; grade 2, mild perivascular cuffing; grade 3, extensive perivascular cuffing; grade 4, extensive perivascular cuffing and severe parenchymal cell infiltration). Demyelinating lesions were assessed using sections stained with the KB method and ED1 for MΦ into five categories (grade 1, trace of perivascular or subpial demyelination; grade 2, focal demyelination; grade 3, demyelination involving a quarter of tissues examined, i.e., the spinal tract, brain stem, cerebellar white matter or optic tract; grade 4, massive confluent demyelination involving half of the tissue; grade 5, extensive demyelination involving the entire tissues) according to Storch et al. (1998) with a few modifications.

Single immunoperoxidase staining was performed as described previously (Matsumoto and Fujiwara, 1987; Ohmori et al., 1992). Briefly, paraffin-embedded sections were deparaffinized and rehydrated. After blocking the endogenous peroxidase activity with methanol containing 0.3% hydrogen peroxide, sections were incubated with mAb ED1 (×200, purified from the hybridoma supernatant) for MΦ for 1 hr at room temperature. In the CNS, ED1 stains fine processes of microglia, whereas the whole cell bodies of MO/MΦ are positive for ED1 (our unpublished observation). Therefore, it can be said that ED1 stains mainly MΦ in diseased spinal cords. After washing, sections were incubated with biotinylated anti-mouse or rabbit IgG (×200, Vector) followed by the horseradish peroxidase-labeled Vectastain Elite ABC Kit (Vector). Horseradish peroxidase binding sites were detected in 0.005% diaminobenzidine and 0.01% hydrogen peroxide. All the procedures were performed at room temperature. To confirm the specificity of staining, primary antibodies were omitted or replaced with normal mouse IgG. The controls did not show any specific staining.

Double immunofluorescence staining was performed as follows. Frozen sections were air-dried and fixed in ether. After washing, the sections were reacted with OX8 (×200, anti-CD8 mAb), followed by incubation with Cy3-labeled anti-mouse IgG (×50, Amersham). The free binding sites of

the secondary antibody were blocked with normal mouse serum and then biotinylated ED1 was applied. Finally, the sections were incubated with Alexa 488-conjugated streptavidin ( $\times 100$ , Molecular Probe). Morphological observations and quantitative analysis were made by microphotographs taken under a confocal microscope (Leica).

### Flow Cytometric Analysis

Leukocytes in the spleen, lymph node (LN), blood (peripheral blood leukocytes, PBL) and spinal cord were collected and stained with unlabeled OX35 ( $\times 200$ , anti-CD4) followed by phycoerythrin (PE)-conjugated anti-mouse IgG ( $\times 50$ , Vector). To saturate free binding sites of the secondary antibody, cells were incubated with normal mouse serum. Then FITC-OX42 ( $\times 20$ , anti-MO/M $\Phi$  mAb) and biotinylated OX8 was applied in the second step. Finally, the cells were incubated with PerCP-streptavidin ( $\times 50$ ). During incubation (15 min each), samples were kept on ice. Ten thousand cells were analyzed in each sample by FACScan (BD Bioscience). We previously confirmed with double immunofluorescence staining that more than 95% of MO/M $\Phi$  in the lymphoid organs are positive for ED1 and OX42 (data not shown).

For the isolation of infiltrating leukocytes in the CNS, rats with or without clinical signs of EAE were killed under ether anesthesia. The spinal cords were removed and minced with scissors and treated with 0.015% collagenase/dispase (Boeringer Mannheim) and 0.015% trypsin (Sigma) for 15 min at 37°C. After passing through a stainless mesh screen, two volumes of the cell suspension were mixed with one volume of 90% Percoll solution and centrifuged at 15,000 rpm for 20 min. By means of this procedure, a myelin cake floated on the top and the infiltrating inflammatory cells were contained in the pellet. The pellet was harvested and used for analysis.

### Isolation of CD8<sup>+</sup> MO/M $\Phi$

CD8<sup>+</sup> MO/M $\Phi$  were isolated with an AutoMACS (Miltenyi Biotec) according to the manufacturer's instructions. In brief, PBL, spleen and LN cells were taken from naive or immunized rats by the density gradient methods and T, B and dendritic cells were removed by negative selection with an antibody cocktail including R73 (anti-T cells), OX33 (anti-B cells) and OX62 (anti-dendritic cells) followed by anti-mouse IgG-Microbeads (Miltenyi Biotec). Then CD4 and CD8 cell populations were negatively selected with OX8 (anti-CD8) and OX35 (anti-CD4), respectively. Finally, bead-free cells were positively selected with OX42 (anti-MO/M $\Phi$ ). The purity of each population was  $>90\%$ .

### Real-time PCR

Total RNA was extracted from the indicated tissues with an RNAqueous Kit (Ambion) and cDNA was then synthesized by reverse transcription with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). SYBR Green real-time PCR reactions were performed on an ABI PRISM 7500 sequence detection system (Applied Biosystems) in a total volume of 25  $\mu$ l with the SYBR Premix Ex Taq (Takara Bio, Otsu, Japan). Each PCR was performed in duplicate

with the following thermocycler conditions: stage 1, 95°C for 10 min for one cycle and stage 2, 95°C for 15 sec and 58°C for 1 min for 50 cycles. All primers were designed on an intron-exon junction to prevent coamplification of genomic DNA and their sequences were shown in previous reports (Matsumoto et al., 2004, 2005). Relative quantification of mRNA was performed by the standard curve method. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as internal control. The absence of nonspecific amplification was confirmed by dissociation curve analysis.

### Statistical Analysis

Data were analyzed by Student's *t*-test or Mann-Whitney's *U*-test. *P* values less than 0.05 were considered statistically significant.

## RESULTS

### Clinical and Pathological Features of Acute and Chronic EAE

To characterize CD8<sup>+</sup> M $\Phi$  that appear in EAE lesions in the CNS, we induced acute EAE in LEW and LEW.1AV1 rats and chronic EAE in LEW.1AV1 rats. Figure 1 summarizes the clinical and pathological features of acute and chronic EAE. The clinical course of acute EAE was monophasic and the onset (day 10), peak (day 14), and recovery (by day 18) were relatively constant (Fig. 1A). This time course was almost identical in acute EAE induced in LEW and LEW.1AV1 rats (data not shown). In contrast, chronic EAE showed a variety of clinical courses including relapsing-remitting and secondary progressive forms as shown in our previous study (Sakuma et al., 2004). The rat depicted in Figure 1B developed clinical signs on postimmunization (PI) day 30 with remission around PI day 42, and died during severe relapse.

Routine pathology with HE and KB staining revealed that acute EAE lesions consisted mainly of small mononuclear leukocytes (Fig. 1C) with minimal demyelination (Fig. 1E), whereas there was dense and extensive "foamy macrophage" infiltration (Fig. 1D) with marked demyelination (Fig. 1F) in chronic EAE lesions. Consistent with these findings, M $\Phi$  staining with ED1 mAb revealed localized perivascular M $\Phi$  infiltration in acute EAE (Fig. 1G) and diffuse M $\Phi$  infiltration in chronic EAE (Fig. 1H).

### A Large Number of CD8<sup>+</sup> M $\Phi$ Infiltrate CNS Lesions in Chronic, but Not in Acute, EAE

We then examined the presence or absence of M $\Phi$  expressing CD8 in CNS lesions at various time points of acute and chronic EAE. Acute EAE was examined on days 9 (preclinical, Fig. 2A-C), 14 (peak, Fig. 2D-F), and 21 (recovery, Fig. 2G-I). As clearly shown, the vast majority of ED1<sup>+</sup> M $\Phi$  did not express the CD8 molecule in acute EAE. Only two cells, indicated by arrows in Figure 1C and Figure 1I, were doubly labeled.

As reported previously (Schroeter et al., 2003) and confirmed here, CD8<sup>+</sup> M $\Phi$  were present in chronic

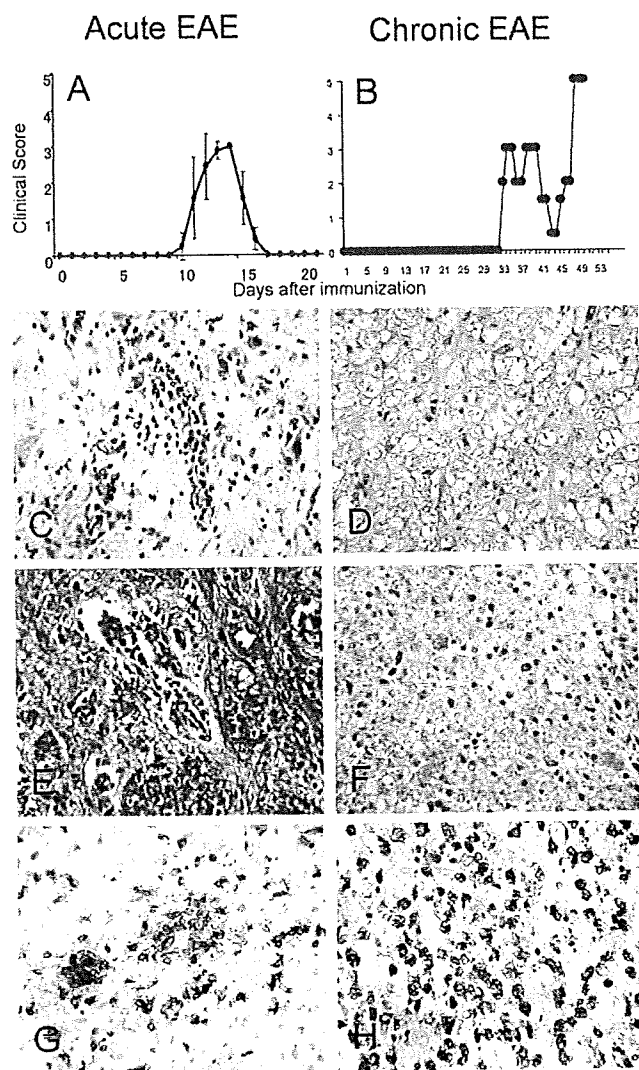


Fig. 1. Clinical and pathological features of acute and chronic EAE. LEW rats that had been immunized with MBP developed acute and monophasic EAE with a relatively constant onset and recovery (A). The mean clinical scores  $\pm$  SD of 9 rats are shown. In contrast, chronic EAE induced by the immunization of LEW.1AV1 rats with MOG showed a variety of clinical courses including relapsing–remitting and secondary progressive EAE. The rat depicted in (B) developed clinical signs on PI day 30 with remission around PI day 42, and died during severe relapse. Note that the timescale in (B) is different from that in (A). Routine pathology with HE and KB staining revealed that acute EAE lesions (PI day 14) in the spinal cord consist mainly of small mononuclear leukocytes (C) with minimal demyelination (E), whereas there is dense and extensive “foamy” M $\Phi$  infiltration (D) with marked demyelination (F) in chronic EAE lesions (PI day 40) in the spinal cord. Consistent with these findings, M $\Phi$  staining with ED1 mAb revealed localized perivascular M $\Phi$  infiltration in acute EAE (G) and diffuse M $\Phi$  infiltration in chronic EAE (H). C and D, HE staining; E and F, KB staining; G and H, ED1 staining. C–H, original magnification, 120 $\times$ .

EAE lesions in the spinal cord (Fig. 3). In addition to the previous study, we found an interesting finding by longitudinal examinations on days 14 (early stage, Fig. 3A–C), 20 (intermediate stage, Fig. 3D–F), and 40 (peak stage, Fig. 3G–I). On day 14, only a small number of double-positive cells were detected. Cells in yellow in Figure 3C were difficult to judge whether they were double-positive cells or simple overlap of two types of cells. However, on day 20, a definite number of M $\Phi$  expressed CD8 (Fig. 3F) and, on PI day 40, the majority of ED1<sup>+</sup> M $\Phi$  were also positive for CD8 (Fig. 3I).

### Quantitative Analysis of CD8<sup>+</sup>ED1<sup>+</sup> M $\Phi$ Infiltrating CNS Lesions in Acute and Chronic EAE

The above findings prompted us to quantitate CD8<sup>+</sup> M $\Phi$  in CNS lesions at various time points during acute and chronic EAE. As shown in Figure 4A, CD8<sup>+</sup> M $\Phi$  in acute EAE lesions (hatched bars) accounted for less than 5% throughout the course of the disease. In sharp contrast, approximately 20% of M $\Phi$  in chronic EAE lesions expressed CD8 in the early stage (solid bar on day 14). CD8<sup>+</sup> M $\Phi$  peaked on PI day 40 ( $58.8 \pm 21.3\%$ ) and remained at a high level even on PI day 58. These findings indicate that CD8<sup>+</sup> M $\Phi$  infiltration in CNS lesions is a characteristic pathological feature of chronic EAE and that the number reaches the maximal level at the peak of chronic EAE (around PI day 40).

During analysis, we found that immunization of LEW.1AV1 rats with MBP elicited acute monophasic EAE and its clinical course was essentially the same as that found in acute EAE in LEW rats (data not shown). With spinal cord sections taken from rats with chronic EAE (LEW.1AV1-MOG in Fig. 4B) and with acute EAE (LEW.1AV1-MBP and LEW-MBP in Fig. 4B), we counted CD8<sup>+</sup> and CD8<sup>-</sup> M $\Phi$  at the indicated time points of disease. In chronic EAE, the proportion and absolute number of CD8<sup>+</sup> M $\Phi$  increased at the peak stage (day 40) compared with the early stage (day 14). The majority of M $\Phi$  forming cluster lesions expressed CD8 (“clustered” in Fig. 4B). The proportion of CD8<sup>+</sup> M $\Phi$  that did not form a cluster lesion (“nonclustered” in Fig. 4B) was relatively low compared with clustered M $\Phi$ . By contrast, in acute EAE, the proportion of CD8<sup>+</sup> M $\Phi$  in the CNS was very small (LEW-MBP). Interestingly, in LEW.1AV1 rats with acute EAE (LEW.1AV1-MBP), the proportion of CD8<sup>+</sup> M $\Phi$  was very low, as in LEW rats. These findings strongly suggest that CD8<sup>+</sup> M $\Phi$  infiltration into the CNS is closely associated with the clinical course of EAE and the used antigen but not with the rat strain.

### Flow Cytometric Analysis of CD8<sup>+</sup> M $\Phi$ in the Lymphoid Organ and Peripheral Blood of Rats With Acute and Chronic EAE

Although it is generally believed that CD8 expression on M $\Phi$  occurs in CNS lesions (Popovich et al., 2003), there is no definite information regarding this

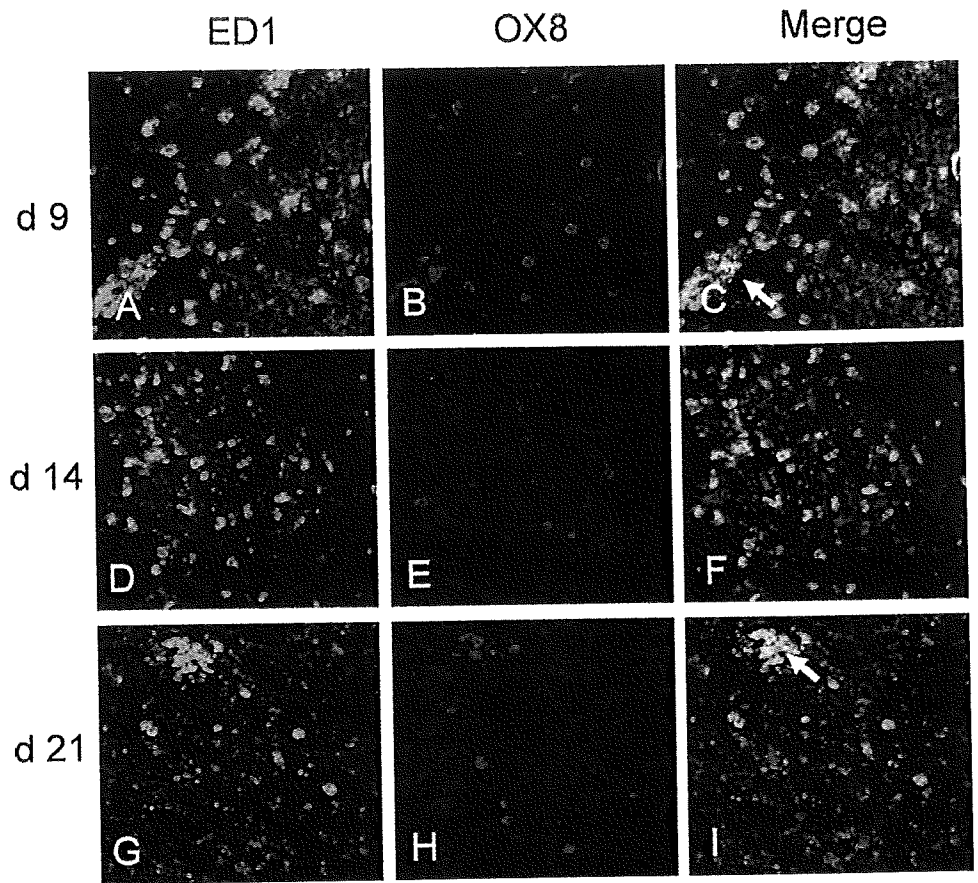


Fig. 2. Identification of CD8<sup>+</sup> MΦ in acute EAE lesions in the spinal cord. Double immunofluorescence staining for CD8 with OX8 mAb and for MΦ with ED1 mAb was performed on days 9 (A-C), 14 (D-F), and 21 (G-I). The majority of MΦ at all the stages examined (A,D,G) are negative for CD8 (B,E,H). Only a few cells indicated by arrows in (C) and (I) are stained positively for CD8 and ED1. Original magnification, A-I, ×200.

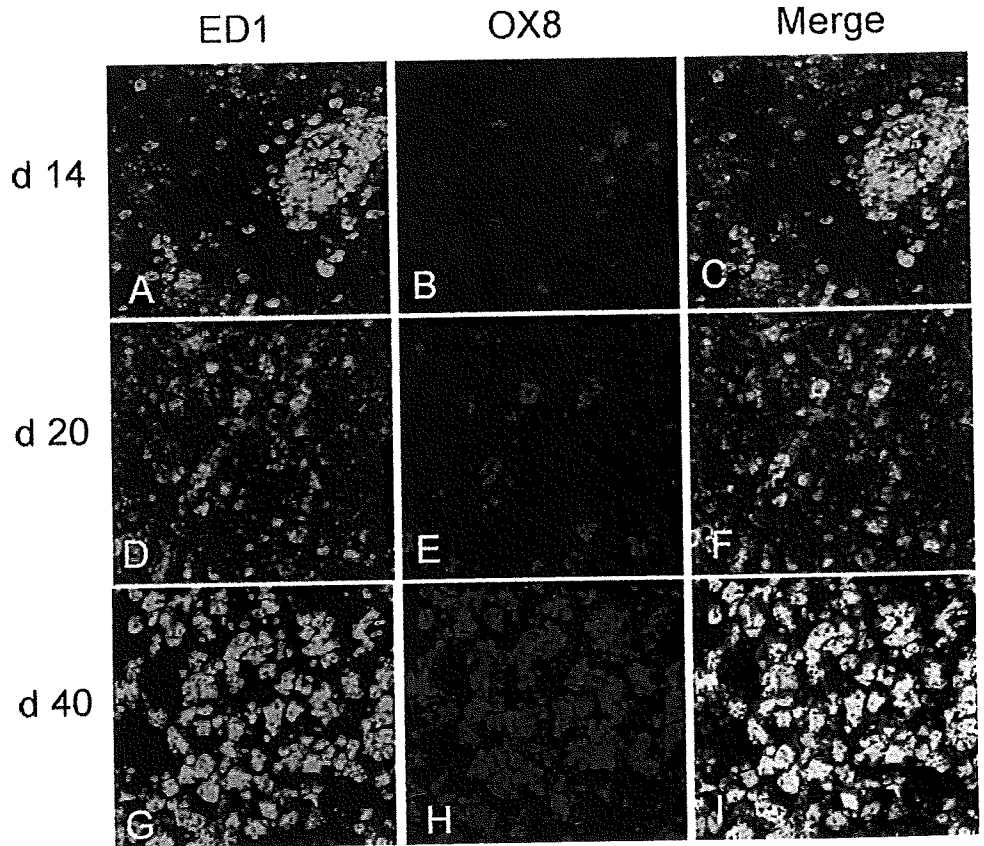


Fig. 3. Longitudinal examination of CD8<sup>+</sup> MΦ in chronic EAE lesions in the spinal cord. Double immunofluorescence staining for CD8 with OX8 mAb and for MΦ with ED1 mAb was performed on days 14 (A-C), 20 (D-F), and 40 (G-I). On day 14, only a small number of double-positive cells were detected. However, on day 20, a definite number of MΦ expressed CD8 (F) and, on day 40, the majority of ED1<sup>+</sup> MΦ were also positive for CD8 (I). Original magnification, A-I, ×200.



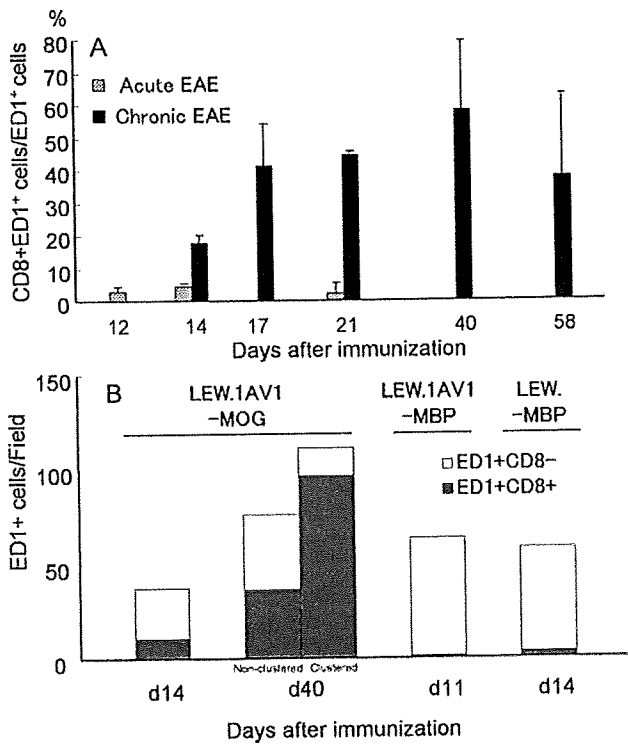


Fig. 4. (A) Quantitation of CD8<sup>+</sup> MΦ in acute (hatched bars) and chronic (solid bars) EAE lesions. At least three fields containing MΦ infiltration were taken as confocal images and all ED1<sup>+</sup> and ED1<sup>+</sup>OX8<sup>+</sup> MΦ were counted and expressed as percentages (OX8<sup>+</sup> MΦ/total MΦ) ± SD. CD8<sup>+</sup> MΦ in acute EAE lesions (hatched bars) accounted for less than 5% throughout the course of the disease. In sharp contrast, approximately 20% of MΦ in chronic EAE lesions expressed CD8 in the early stage (solid bar on day 14). CD8<sup>+</sup> MΦ peaked on day 40 (58.8 ± 21.3%) and remained at a high level even on day 58. There was significant difference in the percentage of CD8<sup>+</sup> MΦ between acute and chronic EAE on days 14 and 21 (*P* < 0.01). (B) Comparison of the numbers of CD8<sup>+</sup> (solid bars) and CD8<sup>-</sup> (open bars) MΦ in the spinal cord at the peak of chronic (LEW.1AV1-MOG) and acute (LEW.1AV1-MBP and LEW-MBP) EAE. In chronic EAE, the proportion and absolute number of CD8<sup>+</sup> MΦ increased at the peak stage (day 40) compared with the early stage (day 14). The majority of infiltrating MΦ formed cluster lesions expressed CD8 (clustered). In contrast, the proportion of CD8<sup>+</sup> MΦ in the acute EAE lesions is very low (LEW.1AV1-MBP and LEW-MBP). The mean values ± SD for ED1<sup>+</sup> CD8<sup>-</sup> cells are 26.8 ± 7.3 (on day 14 of LEW.1AV1-MOG), 47.8 ± 17.3 (nonclustered on day 40 of LEW.1AV1-MOG), 15.3 ± 11.5 (clustered on day 40 of LEW.1AV1-MOG), 62.0 ± 29.4 (on day 11 of LEW.1AV1-MBP) and 54.5 ± 15.8 (on day 11 of LEW-MBP). At each time point, a total of four to six fields from three rats were photographed, and the mean values of the positive cells are shown.

issue. In order to analyze the nature of CD8<sup>+</sup> MΦ in more detail, we examined the peripheral blood, spleen, and LNs of naive, MOG-immunized and MBP-immunized LEW.1AV1 rats at the preclinical stage. In order to surface-label MΦ for flow cytometric analysis, we used OX42 for MO/MΦ instead of ED1 and OX8 for CD8. We previously confirmed by immunohistochemis-

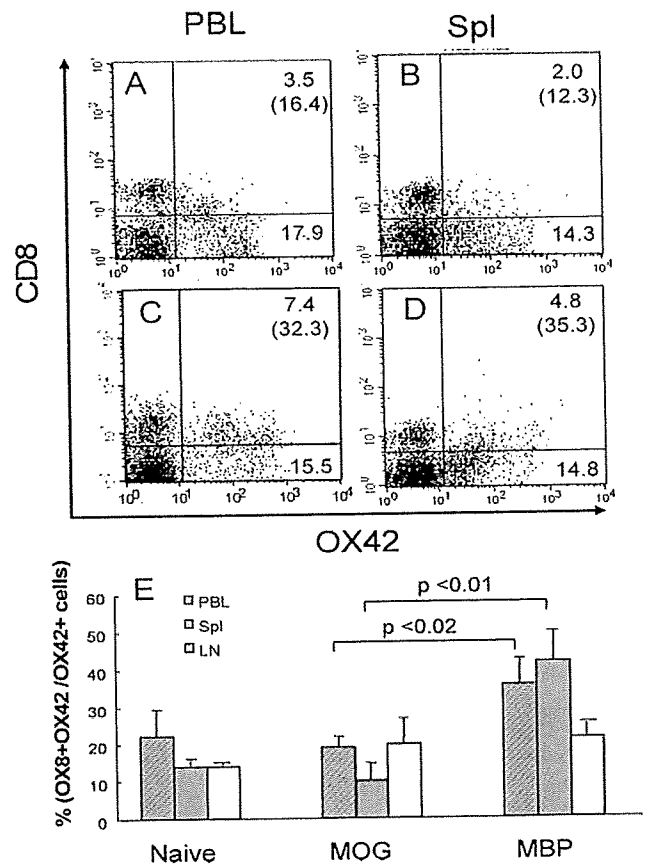


Fig. 5. A–D: Representative profiles of CD8<sup>+</sup> MO/MΦ in PBL (A,C) and the spleen (B,D) of MOG-immunized (A,B) (day 13) and MBP-immunized (C,D) (day 12) LEW.1AV1 rats. The numbers in parentheses are percentages of OX8<sup>+</sup> cells in total OX42<sup>+</sup> cells. E: In naive and MOG-immunized rats, the proportions of CD8<sup>+</sup> MO/MΦ were approximately 15–20% in PBL, spleens, and inguinal LNs. In sharp contrast, CD8<sup>+</sup> MO/MΦ in PBL and spleens of MBP-immunized rats accounted for 35% and 40%, respectively. Three rats were examined in each group, and the percentages of OX8<sup>+</sup>OX42<sup>+</sup> cells in total OX42<sup>+</sup> cells (mean value ± SD) are shown.

try that the majority of MΦ in the lymphoid organs are double-labeled with ED1 and OX42 (data not shown). As mentioned above, immunization of LEW.1AV1 rats with MOG induced chronic EAE, whereas immunization of the same strain with MBP resulted in acute EAE. In the CNS lesions, the proportion of CD8<sup>+</sup> MΦ was high in chronic EAE and very low in acute EAE (Fig. 4B).

In lymphoid organs, a different finding was obtained. As shown in Figure 5, the proportions of CD8<sup>+</sup> MO/MΦ in naive control (Fig. 5E) and MOG-immunized (Fig. 5A,B,E) rats were approximately 15–20% in all organs examined (“Naive” and “MOG” in Fig. 5E). In sharp contrast, CD8<sup>+</sup> MO/MΦ in PBL and spleens of MBP-immunized rats (Fig. 5C–E) accounted for about 35% and 40%, respectively (“MBP” in Fig. 5E). These findings suggest that MBP immunization induces a sufficient number of

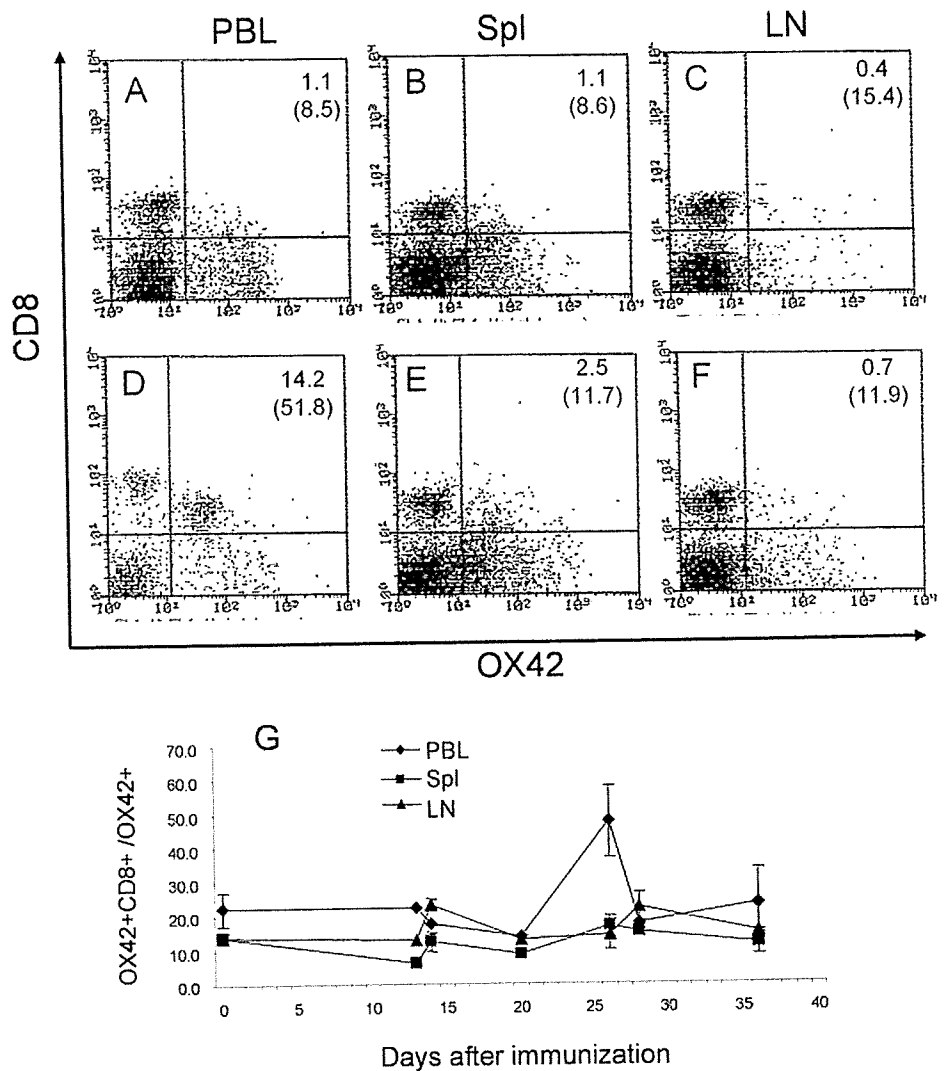


Fig. 6. A–F: Flow cytometric analysis of CD8<sup>+</sup> MO/MΦ in the blood, spleen, and LN of normal (A–C) and chronic EAE (D–F) LEW.1AV1 rats. PBL (A,D), spleen (Spl) (B,E), and LN (C,F) cells were isolated and stained for CD8 with OX8 and for MΦ with OX42. In PBL and spleen of a naive rat (A and B, respectively), CD8<sup>+</sup> MΦ accounted for 10–15%, whereas those in LN of naive rats (C) are relatively few. In a rat with chronic EAE, CD8<sup>+</sup> MO in PBL increased significantly (D), whereas those in the spleen and LNs were not significantly different from a naive rat (E,F). The numbers in parentheses are percentages of OX8<sup>+</sup> cells in total OX42<sup>+</sup> cells. G: During chronic EAE, the numbers of CD8<sup>+</sup> MΦ in all organs remained unchanged in the preclinical stage but CD8<sup>+</sup> MΦ abruptly increased in number around the onset of chronic EAE in PBL (D and diamonds in G). Such change was observed only in PBL and the proportion of CD8<sup>+</sup> MΦ in other organs remained stable throughout the observation period. At each time point, three to four rats were examined.

CD8<sup>+</sup> MO/MΦ in the periphery. However, MOG, but not MBP, immunization may up-regulate factors that recruit CD8<sup>+</sup> MO/MΦ into the CNS more efficiently.

We also performed similar analysis with mononuclear cells isolated from the spinal cord during the peak of chronic EAE. Unfortunately however, it was revealed that harvested CD8<sup>+</sup> cells were always small in both number and size. On the basis of this finding, we judged that the majority of foamy MΦ, the major inflammatory cell population in spinal cord lesions, were destroyed during the processes of isolation and staining for analysis and decided not to use the obtained data.

### Kinetics of CD8<sup>+</sup> MΦ in the Lymphoid Organ and Peripheral Blood in Chronic EAE

Flow cytometric analysis that used PBL obtained from rats on day 12–13 revealed that the proportion of

CD8<sup>+</sup> MO was significantly higher in MBP-immunized rats than in MOG-immunized rats (Fig. 5E). To examine in more detail, we made a longitudinal examination of the numbers of CD8<sup>+</sup> MO/MΦ in the periphery during the course of chronic EAE. The representative profiles of naive control rats and rats with EAE are shown in Figure 6A–F and the summary is in Figure 6G. In PBL, spleens, and LNs (Fig. 6A,B,C, respectively) of naive control rats, CD8<sup>+</sup> MO/MΦ accounted for about 10–20%. During chronic EAE, the numbers of CD8<sup>+</sup> MO/MΦ in all organs remained unchanged at the preclinical stage (Fig. 6G) but CD8<sup>+</sup> MO/MΦ in PBL abruptly increased in number around the onset of chronic EAE (Fig. 6D and diamonds in Fig. 6G). Such change was observed only in PBL and the proportions of CD8<sup>+</sup> MO/MΦ in the spleen and LN remained stable throughout the observation period. Thus, it was demonstrated that CD8<sup>+</sup> MO in PBL was equally up-regulated in both MBP- and MOG-immunized rats but

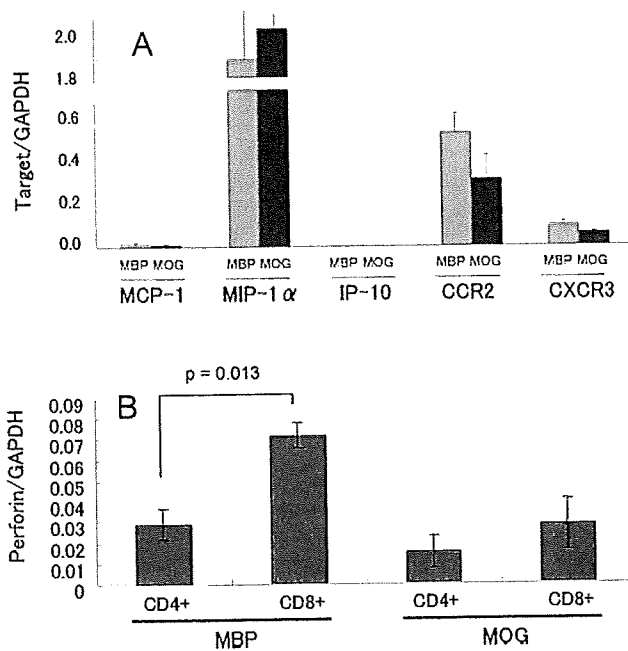


Fig. 7. Real-time PCR analysis of chemokine, chemokine receptor (A) and perforin (B) mRNA of peripheral blood MO. PBL were taken from MBP-immunized (day 12–13, grade 2.5–3,  $n = 4$ ) and MOG-immunized (day 13–20, grade 2.5–3,  $n = 4$ ) rats with clinical EAE. Then CD4<sup>+</sup> and CD8<sup>+</sup> MO were purified with AutoMACS. mRNA levels of the indicated molecules were determined by real-time PCR. The mRNA levels of chemokines and chemokine receptors of CD8<sup>+</sup> MO (A) and of perforin of CD4<sup>+</sup> and CD8<sup>+</sup> MO isolated from MBP- and MOG-immunized rats are shown. GAPDH was used as internal control.

that CD8<sup>+</sup> MO/M $\Phi$  infiltrated the CNS mainly in chronic EAE.

### Quantitative Analysis of Chemokines and Other Bioactive Substances by Real-time PCR

Quantitation of mRNA encoding chemokines and other bioactive substances such as perforin, granzyme B, and Fas-L was performed by using sorted cell populations of PBL. Figure 7A shows levels of chemokines and chemokine receptors in CD8<sup>+</sup> M $\Phi$  in the peripheral blood taken from MBP-immunized and MOG-immunized rats at the peak of acute and chronic EAE. Analysis revealed no significant difference between the two groups. We also examined several bioactive substances related to the cytotoxicity of CD8<sup>+</sup> M $\Phi$  and compared them with those of CD4<sup>+</sup> M $\Phi$ . As shown in Figure 7B, the perforin mRNA level was significantly higher in the CD8 population than in the CD4 population in MBP-immunized rats ( $P = 0.013$ ), and a similar tendency was noted in MOG-immunized rats. Essentially the same results were obtained with regard to granzyme B (data not shown). However, there was no significant difference between MBP-immunized and MOG-immunized rats.

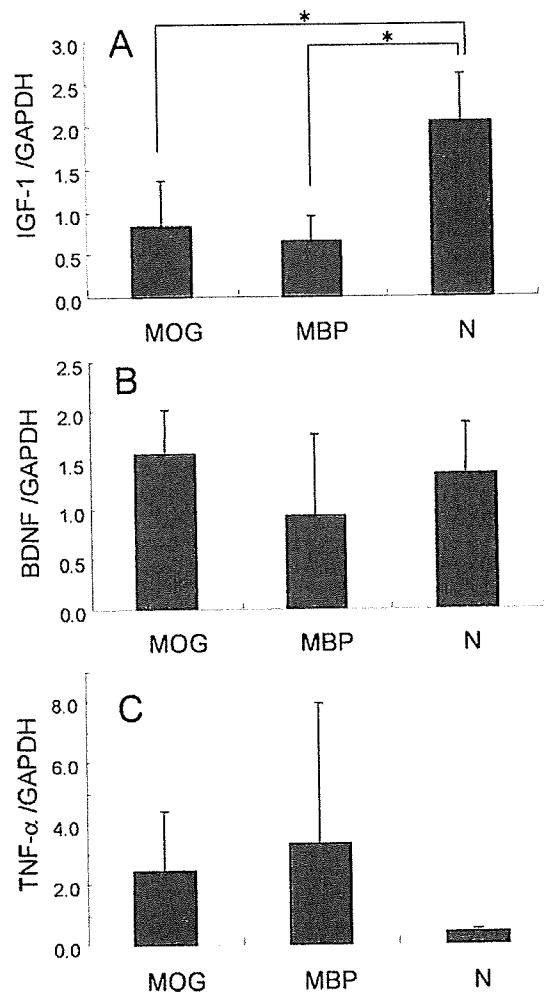


Fig. 8. Real-time PCR analysis of IGF-1 (A), BDNF (B), and TNF- $\alpha$  (C) mRNA of naive control (N) and inflamed (MBP or MOG) spinal cords. LEW.1AV1 rats were immunized with MBP or MOG, and spinal cords were taken from MBP-immunized (PI day 11, grade 4–5,  $n = 3$ ) and MOG-immunized (day 14–20, grade 4–5,  $n = 4$ ) rats with clinical EAE. Then mRNA levels of the indicated molecules were determined by real-time PCR. GAPDH was used as internal control. \* $P < 0.05$ . There was no significant difference in TNF- $\alpha$  levels among three groups.

To learn the role of infiltrating M $\Phi$  in acute and chronic EAE lesions in the spinal cord, we also determined mRNA levels of tissue repair-promoting (insulin-like growth factor [IGF]-1 and brain-derived neurotrophic factor [BDNF]) and neurotoxic (TNF- $\alpha$ ) substances (Fig. 8). IGF-1 was significantly down-regulated in both acute (MBP in Fig. 8A) and chronic (MOG in Fig. 8A) EAE lesions than in control spinal cord tissue. There was no significant difference in BDNF mRNA level among the three groups (Fig. 8B). In contrast, one of major neurotoxic substances, TNF- $\alpha$ , were up-regulated in both acute and chronic EAE lesions. Importantly, there was no significant difference in the levels of IGF-1, BDNF, and TNF- $\alpha$  between acute and chronic EAE

lesions, suggesting that it is less likely that CD8<sup>+</sup> MΦ found in chronic EAE lesions play a role in lesion repair.

## DISCUSSION

It has recently become known that hematogenous MΦ bearing a unique phenotype, i.e., CD8<sup>+</sup> MΦ, infiltrate the CNS under various pathological conditions such as EAE (Schroeter et al., 2003), spinal cord injury (Popovich et al., 2003), focal ischemia (Jander et al., 1998; Schroeter et al., 2001), and Wallerian degeneration (Jander et al., 2001). Although the role of CD8<sup>+</sup> MΦ in CNS lesions is largely unknown, there are common pathological conditions. Furthermore, Schroeter et al. (2003) demonstrated that in chronic EAE, there are a large number of CD8<sup>+</sup> MΦ in the highly demyelinating lesions, whereas only small lymphocyte-like CD8<sup>+</sup> cells were found in acute EAE lesions. These findings strongly suggest that the presence of severely damaged CNS tissues is closely linked with the appearance of CD8<sup>+</sup> MΦ.

In the present study, we have extended previous studies and obtained several intriguing findings. By means of acute and chronic EAE systems, we first made longitudinal examinations of the distribution and number of CD8<sup>+</sup> MΦ in the spinal cord. Consequently, we found that CD8<sup>+</sup> MΦ in chronic EAE lesions increased in number and peaked on PI day 40. At this time point and thereafter, CD8<sup>+</sup> MΦ accounted for about 60% of total MΦ. Contrary to the previous study (Schroeter et al., 2003), a small but definite number of MΦ bearing CD8 were present in acute EAE lesions and accounted for about 5% throughout the disease course.

The kinetic study of CD8<sup>+</sup> MO/MΦ in the peripheral blood and lymphoid organ during chronic EAE revealed that there was a transient but robust increase of CD8<sup>+</sup> MO in the peripheral blood on day 26 when chronic EAE was in the early stage. This suggests that at least a part of MO acquire CD8 molecules outside the CNS and infiltrate the target organ. Although it was previously presumed that the CD8 expression by MΦ takes place in the CNS on the basis of the findings obtained by the treatment experiment with OX8 (Schroeter et al., 2001; Popovich et al., 2003), the present study clearly showed that MO expressing CD8 molecules increase in number in the blood in the early stage of chronic EAE and move into the CNS with EAE lesions. However, this finding does not exclude the possibility of CD8 molecule acquisition by MΦ in CNS lesions.

We also analyzed the numbers and percentages of CD8<sup>+</sup> MO/MΦ in the lymphoid organ and CNS with EAE lesions induced by immunization of LEW and LEW.1AV1 with MBP (acute EAE) and of LEW.1AV1 with MOG (chronic EAE). As a result, we obtained two unexpected findings. First, in the CNS lesion of LEW.1AV1 rats with acute EAE elicited by immunization with MBP, the number of infiltrating CD8<sup>+</sup> MΦ was very small compared with chronic EAE induced in the same strain. This finding clearly indicates that the

appearance of CD8<sup>+</sup> MΦ in the CNS is closely associated with the type of EAE and is totally dependent on the encephalitogenic antigen used for EAE induction. Second and most unexpectedly, there were a large number of CD8<sup>+</sup> MO/MΦ in the blood of LEW.1AV1 rats with acute EAE. This suggests that, in LEW.1AV1 rats, the factors that promote the migration of CD8<sup>+</sup> MO/MΦ from the blood to the inflamed CNS are induced by MOG, but not MBP, immunization. The quantitation of chemokine and chemokine receptor mRNA by real-time PCR in the peripheral blood using sorted cell populations revealed no significant difference in the MO/MΦ phenotype between MBP-immunized and MOG-immunized rats. This finding suggests two possibilities. First, cytokines and chemokines that were not examined in the present study play a role in determining the kinetics of CD8<sup>+</sup> MO/MΦ. Second, factors produced by non-MO/MΦ, such as T and dendritic cells, are critical for the migration of CD8<sup>+</sup> MO/MΦ. In this regard, we are currently investigating more than 30 cytokines and chemokines at both protein and mRNA levels by multiplexed flow cytometric analysis.

The role of CD8<sup>+</sup> MO/MΦ is poorly understood. By means of alveolar MΦ expressing CD8, it was demonstrated that these MΦ produce TNF, IL-1β and nitric oxide after stimulation through the CD8 molecule (Hirje et al., 1998; Lin et al., 2000). Moreover, CD8<sup>+</sup> MΦ were reported to secrete perforin and granzyme B and to kill tumor cells (Baba et al., 2006, 2008). We also evaluated the function of CD8<sup>+</sup> MΦ by examining tissue repair-promoting substances such as IGF-1 and BDNF. However, we were unable to obtain the results suggesting such role. Collectively, it is possible that CD8<sup>+</sup> MΦ show strong cytotoxic effects in the CNS of rats with chronic EAE. Another important issue is related to the presence or absence of the CD8<sup>+</sup> MO/MΦ population in humans. Two groups clearly demonstrated that CD8<sup>+</sup> MO/MΦ are present in PBL of healthy subjects (Baba et al., 2006) and that a substantial number of cells with this phenotype were found in the thyroid gland of patients with autoimmune thyroid disease (Nakamura et al., 2004). Therefore, analysis of MO/MΦ with this subtype provides useful information with regard to elucidation of the pathomechanisms of MS.

In summary, we characterized the nature and kinetics of CD8<sup>+</sup> MO/MΦ during the course of acute and chronic EAE. Although CD8<sup>+</sup> MO/MΦ increased in number in the peripheral organs of rats with both acute and chronic EAE, cells with this subtype infiltrated the CNS mainly in chronic EAE. Targeting of these cells and/or the processes of their migration from the peripheral blood to the CNS could be effective immunotherapies against autoimmune diseases in the CNS.

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# Mutant SOD1 impairs axonal transport of choline acetyltransferase and acetylcholine release by sequestering KAP3

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Mutations in the *superoxide dismutase 1 (sod1)* gene cause familial amyotrophic lateral sclerosis (FALS), likely due to the toxic properties of misfolded mutant SOD1 protein. Here we demonstrated that, starting from the pre-onset stage of FALS, misfolded SOD1 species associates specifically with kinesin-associated protein 3 (KAP3) in the ventral white matter of *SOD1*<sup>G93A</sup>-transgenic mouse spinal cord. KAP3 is a kinesin-2 subunit responsible for binding to cargos including choline acetyltransferase (ChAT). Motor axons in *SOD1*<sup>G93A</sup>-Tg mice also showed a reduction in ChAT transport from the pre-onset stage. By employing a novel FALS modeling system using NG108-15 cells, we showed that microtubule-dependent release of acetylcholine was significantly impaired by misfolded SOD1 species. Furthermore, such impairment was able to be normalized by KAP3 overexpression. KAP3 was incorporated into SOD1 aggregates in human FALS cases as well. These results suggest that KAP3 sequestration by misfolded SOD1 species and the resultant inhibition of ChAT transport play a role in the dysfunction of ALS.

## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal, progressive neurodegenerative disease characterized by motor neuron cell death in the brain and spinal cord accompanied by rapid loss of muscle control and eventually complete paralysis (1,2). The vast majority of ALS cases are of a sporadic nature, while ~10% are familial (FALS). Although the cause of sporadic ALS remains unclear, 15–20% of FALS patients have point mutations in cytosolic Cu<sup>2+</sup>/Zn<sup>2+</sup> superoxide dismutase (SOD1). SOD1 is an antioxidant enzyme ubiquitously expressed in the cytosol, which converts the superoxide anion radical to hydrogen peroxide. More than 115 disease-causing mutations, affecting all regions of the SOD1 gene product, have been identified.

Previous studies using transgenic animal models expressing mutated human SOD1 have shown that the disease is not caused by a loss of its dismutase activity, but by the gain of toxic properties (1). Many mutant SOD1 proteins tend to become easily misfolded and form aggregates, especially under oxidative stress (3,4). Intracellular aggregates containing SOD1 were specifically detected in affected regions of both patients and animal models possessing *sod1* mutations (5–7). In spinal cords of mutant SOD1-Tg mice, misfolded intermediates of mutant SOD1 proteins, which showed a decrease in solubility and increase in size, were found prior to disease onset (8,9). These results suggest that misfolded molecules containing mutant SOD1 may contribute to motor neuron-specific damage in ALS.

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Axonal transport is required for a variety of neural functions including neurotransmitter synthesis, release and recycling (10). Motor neurons often have long axons that can be more than a meter in length in humans. For this reason, orderly function of the axonal transport mechanism should be particularly important in maintaining the structure and signal transmission at the terminals of motor axons. The transport process is carried out by two major families of motor proteins, i.e. kinesin super family for anterograde transport and the cytoplasmic dynein for retrograde transport (11). Defects in axonal transport have been reported as an early sign of motor neuron diseases including ALS (12). In mutant SOD1-Tg mice, anterograde transport of neurofilaments and tubulin is partially impaired long before disease onset, presumably due to an inhibition of certain transport systems (13,14). With respect to retrograde transport, mutations in the dynein heavy chain result in motor-selective dysfunction in mice (15). Moreover, mutation in p150<sup>Glued</sup>, a subunit of dynactin (dynein activator), was identified in a family that developed a lower motor neuron-specific disease (16). These results strongly suggest a link between the integrity of the axonal transport mechanism with the function and survival of motor neurons.

We previously studied the intracellular localization of misfolded SOD1 species by subcellular fractionation of spinal cords from SOD1<sup>G93A</sup>-Tg mice and found a significant accumulation of misfolded SOD1 species within the axon-enriched fraction at disease onset (17). From this result, together with previous reports, we hypothesized that misfolded SOD1 species may bind to proteins required for axonal transport and thereby inhibit normal function of the motor proteins. To prove this hypothesis, we screened axonal transport-related molecules in search for proteins that associate specifically to the misfolded SOD1 species and identified KAP3 as a misfolded SOD1-interacting protein. KAP3 is a component of the kinesin-2 motor complex mediating an axonal transport of choline acetyltransferase (ChAT) in *Drosophila* (18,19). By using an *in vitro* FALS modeling system that we developed, we showed that misfolded SOD1 inhibits axonal transport-dependent release of acetylcholine (ACh). Moreover, such impairment of ACh release could be rescued by the KAP3 overexpression. This evidence strongly suggests that the sequestering of KAP3 and the resultant inhibition of ChAT transport is a novel toxic property of mutant SOD1 proteins that inevitably leads to motor neuron-specific dysfunction.

## RESULTS

### Misfolded SOD1 species is accumulated in ventral white matter of spinal cords in SOD1<sup>G93A</sup>-Tg mice prior to disease onset

We previously showed by using subcellular fractionation analysis that a considerable amount of misfolded SOD1 species is located in a fraction enriched in nucleus and certain kinds of cytoskeletal proteins (i.e. glial fibrillary acidic protein and neurofilament) in SOD1<sup>G93A</sup>-Tg mice spinal cord prior to appearance of characteristic motor symptoms of ALS (17). To analyze more detailed subcellular

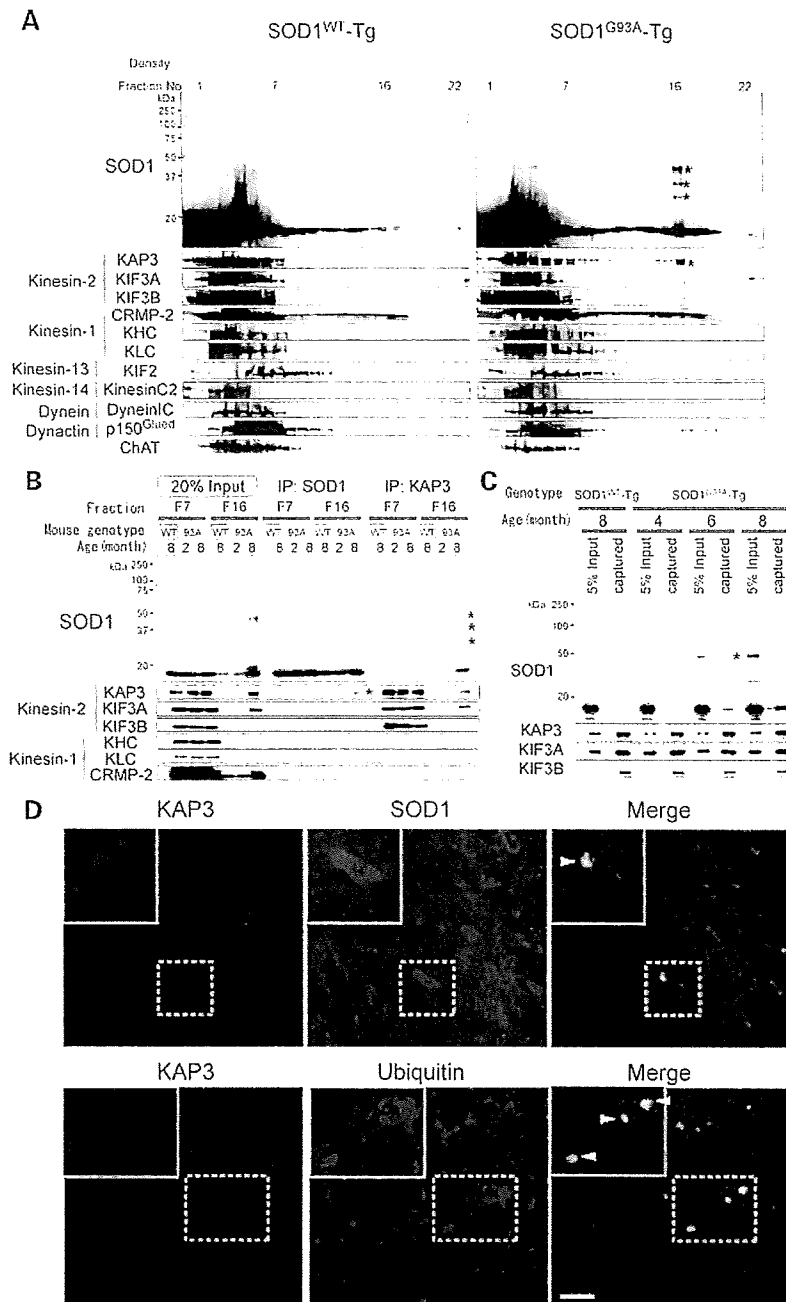
localization of misfolded SOD1 in spinal motor neurons of SOD1<sup>G93A</sup>-Tg mice, we divided the spinal cord into four sub-segments by laser-assisted microdissection (LMD) (summarized in Fig. 1A) and used the lysate from each segment to detect SOD1 by immunoblot analysis. Since we observed that the SOD1<sup>G93A</sup>-Tg mice we used in this study experienced disease onset around the age of 8 months ( $246.5 \pm 5.8$  days) and died around 9.5 months ( $288.5 \pm 7.1$  days, mean  $\pm$  SEM,  $n = 14$ ), we chose to collect samples from mice of 4, 7, 8 and 9 months of age. As expected, the majority of misfolded SOD1 species (high molecular-weight bands/smears immunoreactive for SOD1) was located in the ventral gray matter at 7 months of age, which is 1 month prior to disease onset (Fig. 1B and C). At this age, we also found that a significant amount of misfolded SOD1 species was located in ventral white matter, suggesting that misfolded SOD1 species are localized in motor neuron cell bodies and their axons. We further divided the ventral white matter segment into three subsegments and confirmed a localization of misfolded SOD1 species to a motor axon-enriched segment indicated by ChAT expression (Supplementary Material, Fig. S1). Together, these results suggest that a significant amount of misfolded SOD1 species is located not only in motor neuron cell bodies, but also in motor axons of SOD1<sup>G93A</sup>-Tg mice prior to disease onset.

### Misfolded SOD1 species associates with KAP3, a kinesin-2 component

Previous reports strongly suggest that impairment of the axonal transport mechanism is linked to degeneration of motor neurons (12). Localization of misfolded SOD1 species in motor axons of SOD1<sup>G93A</sup>-Tg mice suggests that misfolded SOD1 species may affect the function of proteins required for axonal transport. Anterograde and retrograde axonal transports are mediated by different protein complexes, which are composed of a large variety of proteins. The kinesin super family for anterograde transport, for instance, is divided into six families (kinesin-1, 2, 3, 4, 13 and 14) consisting of 45 members in human and mouse, and they associate with appropriate cargos via adaptor molecules in most cases (11). To gain more insight into the relationship between misfolded SOD1 and individual components of the molecular motor protein complex, we compared sedimentation of misfolded SOD1 in linear density gradient centrifugation with that of representative molecules that compose motor complexes in motor axon lysate. We asked whether any of these motor complex components co-migrate with misfolded SOD1. Homogenates were prepared from the ventral white matter of SOD1<sup>G93A</sup>-Tg mice at 8 months of age (disease onset) and SOD1<sup>WT</sup>-Tg mice expressing human wild-type *sod1* gene (non-symptomatic control) and subjected to linear Nycodenz density gradient centrifugation. SOD1 immunoreactive bands showing expected molecular weight (representing normally folded SOD1) were observed in fractions nos 1–7 in mice of both genotypes. Misfolded SOD1 species, on the other hand, were found in fractions around no. 16 only in SOD1<sup>G93A</sup>-Tg mice. We then examined migration profiles of 17 major components of the molecular motors from five kinesin families excluding kinesin-4 which is mostly expressed in juvenile brain,







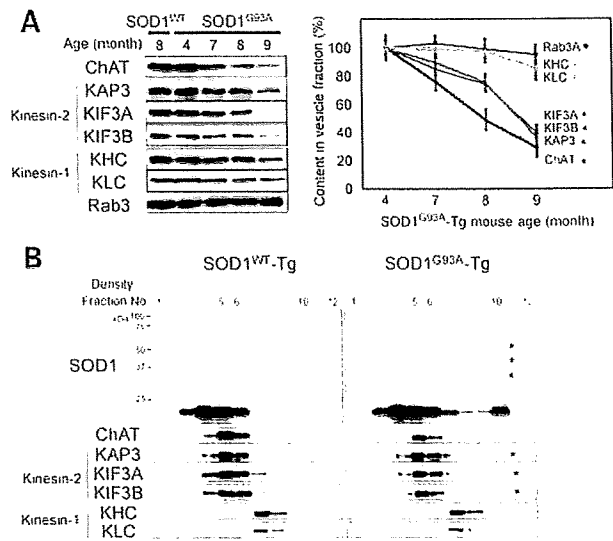
**Figure 2.** Misfolded SOD1 species in ventral white matter of spinal cords specifically associates with KAP3. (A) Fractions obtained from Nycodenz density gradient centrifugation of ventral white matter of 8-month-old *SOD1*<sup>WT</sup>-Tg and *SOD1*<sup>G93A</sup>-Tg spinal cords were analyzed for the presence of indicated proteins (SOD1 and individual components of kinesin and dynein motor complexes) by immunoblot analysis. Each lane (left to right) represents an aliquot collected from the top. Note that misfolded SOD1 species (indicated by asterisks) migrated around fraction no. 16 in *SOD1*<sup>G93A</sup>-Tg spinal cords, where KAP3 co-migrated. (B) Immunoprecipitates of anti-SOD1 and KAP3 antibodies (indicated as 'IP:SOD1' or 'IP:KAP3') from Nycodenz density gradient centrifugation fractions 7 and 16 (F7, F16) of *SOD1*<sup>G93A</sup>-Tg mouse spinal cords prepared above were analyzed for the presence of indicated proteins by immunoblot analysis. Also shown is an immunoblot of samples F7 and F16 (20% of the amount used for immunoprecipitation) examined for expression of the same set of proteins. Note that misfolded SOD1–KAP3 association was detected from F16 but not from F7. (C) Immunoprecipitates of anti-KAP3 antibody from whole spinal cord extracts of 4-, 6- and 8-month-old *SOD1*<sup>G93A</sup>-Tg mice and 8-month-old *SOD1*<sup>WT</sup>-Tg mice were analyzed for the presence of misfolded SOD1 by immunoblot. KIF3A and KIF3B, which are kinesin-2 components, served as controls for immunoprecipitation. Note that misfolded SOD1 species (asterisks) were captured by anti-KAP3 antibody in 6- and 8-month-old *SOD1*<sup>G93A</sup>-Tg extracts. (D) Representative photographs of immunohistochemical co-localization of KAP3 and SOD1 (upper panel), and KAP3 and ubiquitin (lower panel) in a 9-month-old *SOD1*<sup>G93A</sup>-Tg mouse spinal cord. Insets show higher magnification images of boxed area. Arrowheads indicate aggregate-like structures within motor neuron cell bodies, which were positive for both KAP3 and SOD1 (upper panel) and KAP3 and ubiquitin (lower panel). Scale bars=50  $\mu$ m.

ChAT supplied is more critical for the overall production of ACh (21). Therefore, to examine the impact of misfolded SOD1–KAP3 association on the function of motor neurons in *SOD1<sup>G93A</sup>*-Tg mice during the disease progression, we decided to analyze the transport of ChAT in motor neurons. To achieve this, we obtained motor–cargo protein complexes using a previously described method from ventral roots of the caudal spinal cord, which represent motor axons just extended from the spinal cords and examined the presence of ChAT by immunoblot analysis. We found that a significantly lower level of ChAT was present in cauda equina of *SOD1<sup>G93A</sup>*-Tg mice compared with those of *SOD1<sup>WT</sup>*-Tg at 7 months of age or later (Fig. 3A). Furthermore, kinesin-2 motor components, KAP3, KIF3A and KIF3B, all showed a similar decrease in expression during the disease progression in cauda equina of *SOD1<sup>G93A</sup>*-Tg mice. On the other hand, KHC (kinesin heavy chain), KLC (kinesin light chain) (kinesin-1 components) and Rab3 (a synaptic vesicle-associated protein transported by KIF1A) were consistently expressed during the disease progression. Decreased ChAT transport in pre-symptomatic *SOD1<sup>G93A</sup>*-Tg mouse sciatic nerve was also demonstrated by a ligation assay (Supplementary Material, Fig. S2A). These results suggest that the amount of ChAT transported by the kinesin-2 complex significantly decreases in motor axons of *SOD1<sup>G93A</sup>*-Tg mice prior to disease onset.

The results presented in Figure 2A show that ChAT does not co-migrate with the misfolded SOD1–KAP3 complex, suggesting that KAP3 does not bind to ChAT and misfolded SOD1 at the same time. Decreased amounts of ChAT and kinesin-2 motor complex within the axons of motor neurons in *SOD1<sup>G93A</sup>*-Tg mice, together with Figure 2A data, suggest that the misfolded SOD1 species may inhibit kinesin-2-mediated ChAT transportation by trapping the kinesin-2 motor complex in the motor neuron cell body and the surrounding areas. For a better understanding of the misfolded SOD1 species behavior in motor axons of *SOD1<sup>G93A</sup>*-Tg mice, we compared the migration profile in a sucrose density gradient centrifugation of kinesin motor components and ChAT with that of SOD1, by using the axonal motor–cargo protein complexes prepared above as a sample. We found that a small amount of misfolded SOD1 species are present in axonal motor–cargo protein complexes and co-migrate with a small fraction of KAP3, KIF3A (Fig. 3B) and KIF3B, although the detection of KIF3B requires very long exposure (data not shown). We also found that ChAT co-immunoprecipitates with KAP3 in fractions where KAP3 and kinesin-2 components co-migrated. (Supplementary Material, Fig. S2B). These results suggest that misfolded SOD1 species not only trap the kinesin-2 motor complex via association with KAP3 in and around the cell bodies but some of the misfolded SOD1 is also transported to axons in stead of normal cargos such as ChAT.

#### NG108-15 cells serve as an *in vitro* model for FALS

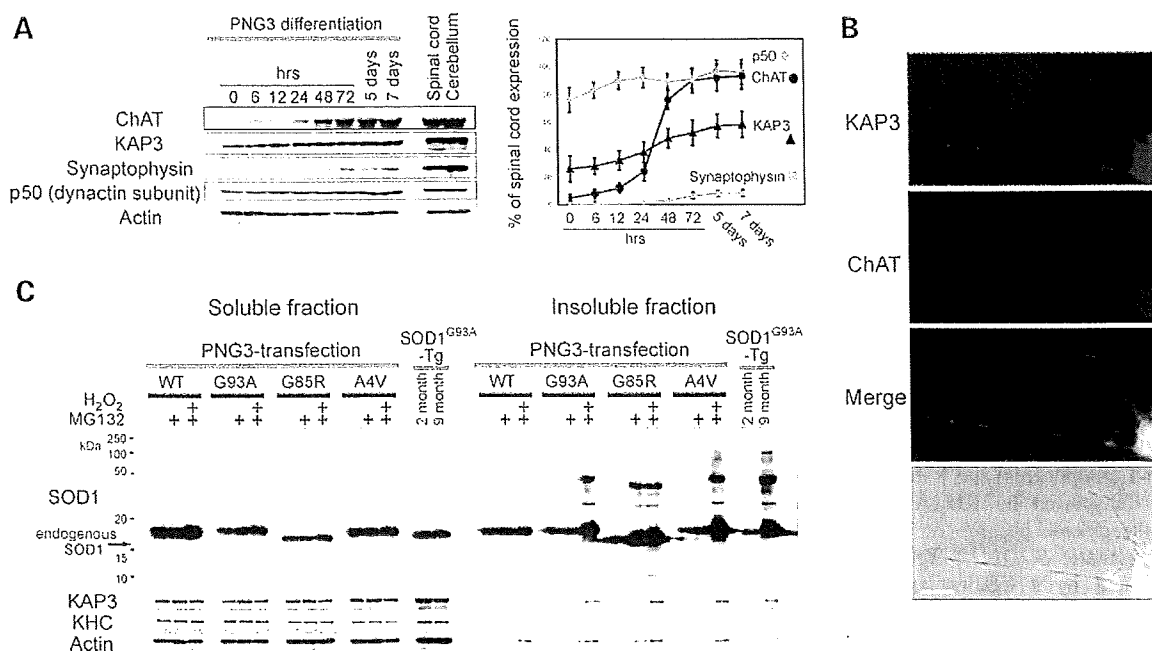
We showed a clear correlation between the association of misfolded SOD1 with KAP3 and the inhibition of ChAT transport within *SOD1<sup>G93A</sup>*-Tg mouse motor axons. To examine the causal relationship between these two phenomena in detail, we developed a novel cell culture model for FALS, in which



**Figure 3.** Axonal transport of ChAT was selectively impaired in spinal motor neurons preceding disease onset in *SOD1<sup>G93A</sup>*-Tg mice. (A) Axonal transport components obtained from ventral roots from caudal spinal cords of *SOD1<sup>WT</sup>*-Tg and *SOD1<sup>G93A</sup>*-Tg mice at indicated ages were analyzed for the presence of indicated proteins by immunoblot analysis (left panel). The expression level of each molecule examined for *SOD1<sup>G93A</sup>*-Tg mice is shown as a percentage of the level at 4 months of age (right panel). Note that the decrease in detection levels of ChAT and kinesin-2 components (KAP3, KIF3A and KIF3B) in *SOD1<sup>G93A</sup>*-Tg mice became apparent at 7 months of age, 1 month before disease onset. (B) Fractions obtained from a sucrose density gradient centrifugation of axonal transported components from 8-month-old mice were analyzed for the presence of indicated proteins (SOD1 and individual components of kinesin motor complexes) by immunoblot analysis. Each lane (left to right) represents an aliquot collected from the top. Note that only kinesin-2 components co-migrated with misfolded SOD1 species (indicated by asterisks on the blots).

cells show cholinergic neuron-like properties—ChAT is transported within axon-like processes, ACh is released by depolarization and mutant SOD1 is misfolded/aggregated. For this purpose, we employed NG108-15 cells, which are of neuron/glia origin and known to differentiate into a cholinergic neuron-like phenotype (22,23). We first cloned a single cell-derived subline of NG108-15 cells, which we termed PNG3 (Purified NG108-15 cell-3), that showed highest expression of ChAT upon differentiation with dibutyl cAMP and dexamethasone (data not shown). We observed that expression of ChAT and synaptophysin (an integral membrane protein found on synaptic vesicles) reaches the maximal level at ~72 h after the stimulation (Fig. 4A). By immunocytochemistry, we found that expression of ChAT and KAP3 were predominantly detected within the extended PNG3 processes in 72 h after the stimulation (Fig. 4B) as other kinesin-2 subunits (data not shown). From these observations, we decided to use PNG3 cells as a model for cholinergic neurons at day 3 after initiating differentiation for all the following experiments.

In order to use PNG3 cells as a cell line model for FALS, misfolding/aggregate formation by mutant SOD1 overexpression needs to be observed. Oxidative stress tends to cause mutant SOD1 proteins misfold and form aggregates (4). Experimentally, mutant SOD1 aggregate formation can be observed and facilitated by inhibiting proteasomal activity (24,25).



**Figure 4.** PNG3 cells served as an *in vitro* model system for studying mutant SOD1 toxicity on cholinergic neurons. (A) Expression of indicated proteins in PNG3 cells during cholinergic neuron-like differentiation was examined by immunoblot analysis. PNG3 cells were lysed at indicated times after induction of differentiation by dibutyryl cAMP and dexamethasone. Representative images of immunoblots (left panel) and quantitative analysis of the blots (right panel) are shown. Expression level is normalized to actin and relative to the level of normal spinal cord at each time point for each molecule. (B) Representative photographs of ChAT and KAP3 expression in differentiated PNG3 cell bodies and processes detected by immunocytochemistry are shown. Scale bar = 25  $\mu$ m. (C) Generation of misfolded SOD1 species in PNG3 cells overexpressing FLAG-tagged SOD1 bearing WT, G93A, G85R or A4V mutation was evaluated by immunoblot analysis. The transfected PNG3 cells were either untreated, treated by MG132 or treated by MG132 followed by H<sub>2</sub>O<sub>2</sub> (as indicated above the images). One percent Triton X-100-soluble and -insoluble fractions were separately analyzed for SOD1-FLAG, KAP3, KHC and actin (for loading control). Spinal cords of 2- and 9-month-old *SOD1*<sup>G93A</sup>-Tg mice were also subjected for the same set of analysis for comparison.

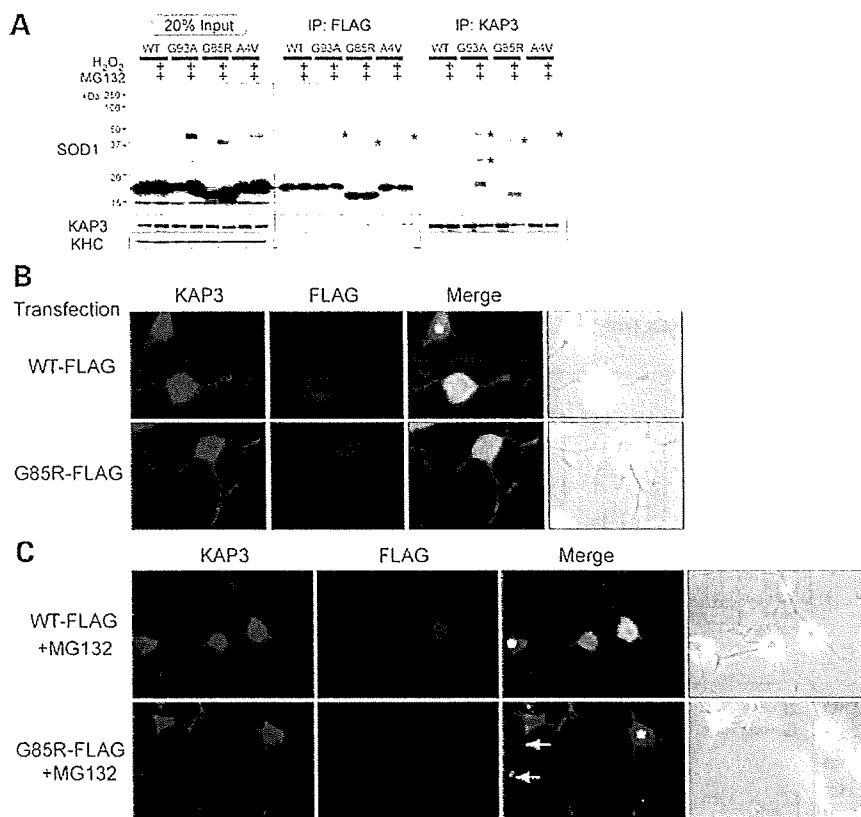
Therefore, we used a combined condition of increased oxidative stress plus decreased proteasomal activity to induce SOD1 misfolding and to enhance an accumulation of those misfolded SOD1 species in differentiated PNG3 cells overexpressing mutant SOD1. We transfected PNG3 cells with previously well-studied G93A, G85R and A4V mutations and wild-type control of human SOD1 for overexpression (3). Misfolded SOD1 species was detected in 1% Triton X-100 insoluble fractions of lysates derived from all three types of FALS-linked SOD1 mutant transfectants but not in wild-type SOD1-overexpressing cells (Fig. 4C). We also found that SOD1 protein bearing the G85R mutation became misfolded without increasing oxidative stress, showing the previously reported unstable nature of this mutant protein (24). These results suggest that mutant SOD1 can form misfolded and/or aggregated molecules in differentiated PNG3 cells, very similar to what is seen in *SOD1*<sup>G93A</sup>-Tg mice by pathological stresses.

For the analysis of pathological significance of misfolded SOD1-KAP3 association in this PNG3 cell model system, we next asked whether association of KAP3 with misfolded SOD1 is observed in this model just as we saw in the *SOD1*<sup>G93A</sup>-Tg mice. To prove the association of KAP3 with misfolded SOD1 directly, we performed immunoprecipitation experiments using lysates of PNG3 cells transfected with either wild-type or mutant SOD1, and then treated with hydrogen peroxide to induce the mutant SOD1 misfolding.

We found that anti-KAP3 immunoprecipitates contained misfolded SOD1 species, and the antibody against mutant SOD1-Flag immunoprecipitated misfolded SOD1 together with KAP3 (Fig. 5A). To visualize the misfolded SOD1-KAP3 association in PNG3 cells, we performed immunocytochemical analysis for localization of KAP3 and mutant SOD1. We found mutant SOD1-immunoreactive aggregates were prominent in cell bodies as well as the processes of MG132-treated differentiated PNG3 cells expressing G85R SOD1, and those aggregates were also positive for KAP3 (Fig. 5B and C). These observations together suggest that the cellular FALS model that we developed here using PNG3 cells recapitulates the association of KAP3 and misfolded SOD1 observed in *SOD1*<sup>G93A</sup>-Tg mice and therefore is suitable to analyze the pathological impact of the misfolded SOD1-KAP3 association on motor neurons.

#### Microtubule-dependent fraction of depolarization-induced ACh release from PNG3 cells requires KAP3

In *Drosophila* neurons, the kinesin-2 motor complex is responsible for anterograde axonal transport of ChAT (18,19). To examine whether the same is true in mammals, we first developed an assay model to detect a depolarization-induced microtubule-dependent release of ACh as a result of ChAT transport. Differentiated PNG3 cells were metabolically



**Figure 5.** KAP3 associates with misfolded SOD1 species in differentiated PNG3 cells. (A) Immunoprecipitates of anti-FLAG and KAP3 antibodies (labeled as 'IP:FLAG' or 'IP:KAP3') from lysates of PNG3 cells transfected with expression plasmids for wild-type SOD1 or SOD1 with G93A, G85R or A4V mutation with or without induction of misfolding by MG132/H<sub>2</sub>O<sub>2</sub> were analyzed for the presence of indicated proteins by immunoblot analysis. Immunoblot of lysates used for immunoprecipitation (20% of the amount) of the expression of the same set of proteins is also shown. Note that misfolded SOD1–KAP3 association was detected in all three mutant SOD1 transfectants (indicated by asterisks). (B and C) Photographs of KAP3 and FLAG-tagged SOD1 in differentiated PNG3 cells transfected with either FLAG-tagged wild-type SOD1 or SOD1 bearing a G85R mutation before and after MG132 treatment are shown in (B) and (C), respectively. Phase contrast images are also shown to the right of each set. Asterisks indicate untransfected cells. Note that prominent aggregate formation is only in the mutant SOD1-expressing cells and processes after MG132 treatment. Scale bars=25  $\mu$ m.

labeled with [<sup>3</sup>H]choline, and depolarization-induced [<sup>3</sup>H]ACh release was measured. We observed that PNG3 cells released [<sup>3</sup>H]ACh in a KCl dose-dependent manner (Fig. 6A). To distinguish 'nerve terminal' ACh release from exocytosis at other locations, we examined ACh release with and without colchicine, a microtubule destabilizing reagent that inhibits axonal transport (18). From ACh release experiments with different trial amounts of colchicine, we decided to use colchicine at 2.5  $\mu$ M for the best differentiation of microtubule-dependent release representing the release from the terminal of differentiated PNG3 cellular processes. Treating the cells with this concentration converted nearly 20% of the tubulin from filamentous to free form in PNG3 cells (Supplementary Material, Fig. S3A). Then to analyze the involvement of KAP3/kinesin-2 in ChAT transport in the mammalian neuron using this assay system, we examined the effect of KAP3 mRNA down-regulation in differentiated PNG3 cells. Although NG108-15 cells are mouse–rat hybrid, transfection of siRNA for mouse KAP3 in PNG3 cells decreased the KAP3 protein level to  $\sim$ 15% after 2 days (Supplementary Material, Fig. S3B). We found that siRNA-mediated down-

regulation of KAP3 significantly reduced the microtubule-dependent fraction of ACh release from PNG3 cells (Fig. 6B). This reduction in ACh release was rescued by the co-transfection of full-length human KAP3 which is not affected by the mouse siRNA we used. However, the ACh release was not rescued by the human KAP3 lacking C-terminal sequence (513–792 amino acids), which is required for the interaction with KIF3A/B (26). These results suggest that KAP3 is required for ChAT transport along microtubules and the subsequent release of ACh from nerve terminals in mammalian neurons.

As described above, we observed decreased ChAT axonal transport in *SOD1*<sup>G93A</sup>-Tg mice. To examine whether misfolded SOD1 inhibits ChAT transport also in this FALS model system in culture, we measured microtubule-dependent ACh release from differentiated PNG3 cells after induced production of misfolded SOD1 species (procedure is summarized in Fig. 6C). We found that induction of SOD1 misfolding by MG132 treatment resulted in a significant decrease of microtubule-dependent ACh release from mutant SOD1-expressing PNG3, but not from wild-type SOD1-expressing cells