Two days before admission, brain computed tomography (CT) did not show any abnormalities. On neurological examination, he was alert and oriented. No cerebellar signs were observed. His routine laboratory examinations were normal. The serum β-D-glucan was not increased. Three times of rapid antigen test for influenza virus A and B were negative. A brain CT revealed low density areas in the slightly swollen left cerebellar hemisphere. A cerebrospinal fluid (CSF) study showed 404 cells/mm^3 with 94% lymphocytes; protein was 144 mg/dl; and glucose 50 mg/dl. Bacterial culture, viral culture, and cryptococcal antigen were negative. CSF polymerase chain reaction (PCR) analysis did not detect Herpes simplex virus DNA. He progressively developed nausea, vomiting, photophobia, nuchal rigidity, and severe headache. Brain magnetic resonance imaging (MRI) revealed cerebellitis of the left hemisphere and obstructive hydrocephalus (Fig. 1). The serum antibody and PCR analysis for *Coxiella burnetii* did not indicate Q fever. A CSF study did not show an increase in the concentration of myelin basic protein or an oligoclonal band; however, the anti-GluRδ2 antibodies were detected in the serum (Table 1). He was treated with 1 g/day of intravenous methylprednisolone for three days. His meningeal signs soon disappeared; however, he transiently showed mild dysmetria and dysdiadochokinesis on the left side. He left hospital on the 21st day of his illness without any sequelae. After six months, his neurological signs were normal and the serum anti-GluRδ2 antibodies were negative, but his brain MRI showed mild atrophy of the left cerebellar hemisphere (Table 1, Fig. 2).

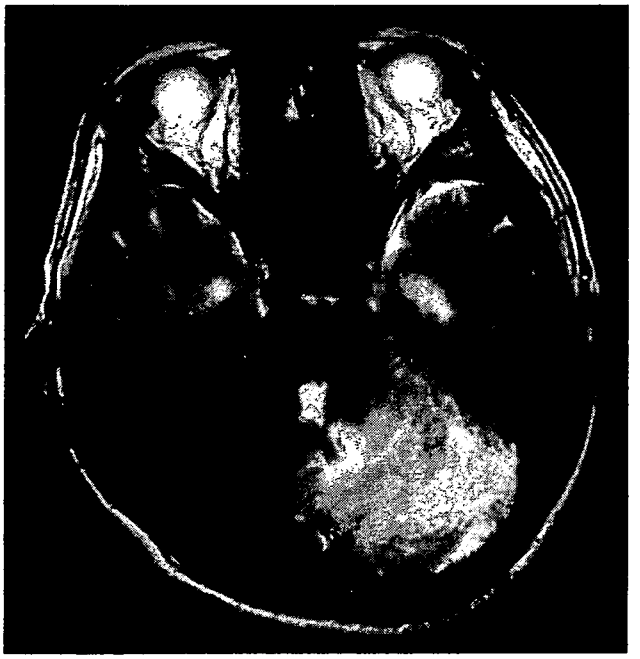

Fig. 1. Axial T2-weighted image on the 11th day of illness shows increased signal intensity in the left cerebellar hemisphere and compression of the fourth ventricle.

Table 1
Autoantibodies against glutamate receptor ε and δ2

Sample	The day after onset	IgG-ε2	IgM-ε2	IgG-δ2	IgM-δ2
CSF	Day 11	–	–	–	–
Serum	Day 10	–	–	+	–
Serum	6 month	–	–	–	–

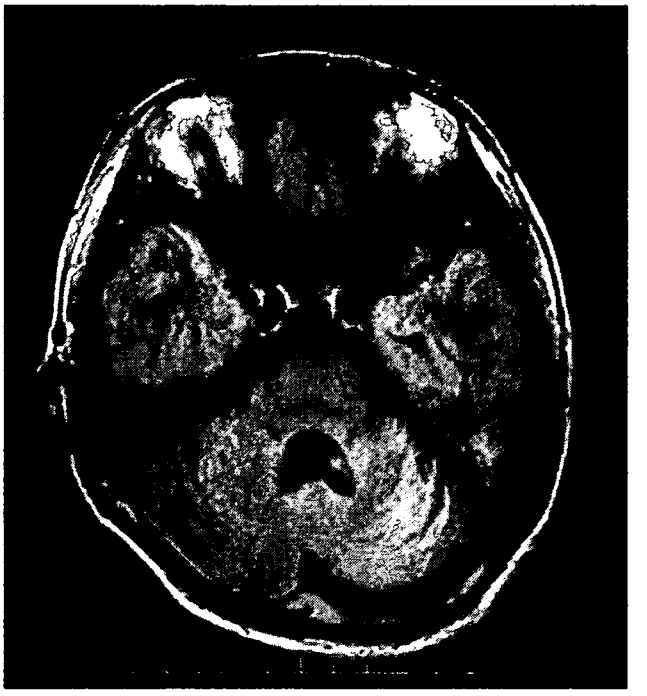

Fig. 2. Axial fluid attenuated inversion recovery image after six months shows increased signal intensity and mild atrophy in the left cerebellar hemisphere.

3. Discussion

The patient had fever and headache as initial symptoms and developed cerebellar signs after steroid therapy. Patients with acute cerebellitis initially show headache, vomiting, and disturbances of consciousness, similar to our patient [1]. Fever and stiff neck might be also seen in acute cerebellitis, but cerebellar signs are sometimes hidden by those symptoms in the first stage of illness [1]. Pleocytosis in the CSF and stiff neck indicated meningitis in our patient, and dilatation of the ventricles and high signal intensity areas around the lateral ventricles on the T2 weighted MRI image suggested increased intracranial pressure caused by cerebellar swelling and obstruction of the aqueduct. Both pathological conditions induced headache and disturbance of consciousness, which were critical points to segregate acute cerebellitis from ACA.

Neither viral nor bacterial agents have been isolated in our patient, as reported in 70% patients with acute cerebellitis. EB virus and Varicella-zoster virus are responsible for acute cerebellitis [1]. Interestingly, anti-ganglioside antibodies (GM1, GM2, and GT1b) have been detected in the serum from a patient with meningo-encephalitis and cerebellitis due to *Mycoplasma pneumoniae* infection [2]. Gangliosides are the sphingoglycolipids with sialic acid, which are located predominantly in nuclear areas of gray matter, and a small amount in the myelin of white matter [3]. Although the mechanism by which antibodies to glycolipids are induced by Mycoplasma pneumoniae infection is not clear, common epitopes might exist in neurons and Mycoplasma pneumoniae [2]. In our case, serologic test for Mycoplasma pneumoniae was not performed because of lacking overt pneumonia signs. Recently, molecular mimicry has been shown between the bacterial lipo-oligosaccharide, the cell-surface structure of *Campylobacter jejuni*, which is the most frequent antecedent pathogen for axonal type of Guillain-Barré syndrome, and the human gangliosides, GM1 or GD1a, expressed in the peripheral nerves [4]. Efforts to detect a specific pathogen and comparative analysis of the molecular structure between the pathogen and cerebellar neurons seem to be important to understand the pathogenesis of acute cerebellitis.

Besides anti-ganglioside antibodies, several types of autoantibodies have been detected in neurological conditions involved in the cerebellum, e.g., anti-Yo or anti-Hu antibodies in paraneoplastic cerebellar ataxia and anti-ZIC1 antibody in cerebellar degeneration [5,6]. Autoantibodies against glutamate receptors have been reported in the chronic form of epilepsy syndrome, such as Rasmussen encephalitis or epilepsia partialis continua [7]. The IgG type of anti-GluRδ2 antibodies were identified in the sera, but not in the CSF in our patient. So far, anti-GluRδ2 antibody has been found in patients with a chronic course of cerebellitis [8] and acute encephalitis [9]. In a patient with chronic cerebellitis, both IgG and IgM of GluRδ2 antibodies were detected in the CSF from the initial stage [8], which was different from our patient. Autoimmunity against GluRδ2 was not likely a cause for acute cerebellitis in our patient because of the absence of anti-GluRδ2 antibodies in the CSF. Although the precise mechanism by which anti-GluRδ2 antibodies were induced is unclear, infection by an unknown agent might have caused meningitis and cerebellitis, which led to a massive leakage of cerebellar antigens, including GluRδ2, and consequently induced autoantibodies against GluRδ2. The patient with chronic cerebellitis received intravenous immunoglobulin treatment at one month after the onset of the disease [8]. Early treatment may prevent the development of the injury by the immunological reaction. It was reported that high dose steroid therapies might improve the symptoms and avoid neurosurgical decompression [10]. Early steroid treatment for acute cerebellitis is likely effective to prevent the progress of cerebellitis and to improve the prognosis. Further investigation would be required to understand the role of autoantibodies in the pathogenesis of acute cerebellitis.

References

[1] Sawaishi Y, Takada G. Acute cerebellitis. Cerebellum 2002;1:223–8.
[2] Komatsu H, Kuroki S, Shimizu Y, Takada H, Takeuchi Y. *Mycoplasma pneumoniae* meningoencephalitis and cerebellitis with antiganglioside antibodies. Pediatr Neurol 1998;18:160–4.
[3] Svennerholm L. The Gangliosides. J Lipid Res 1964;5: 145–55.
[4] Yuki N. Carbohydrate mimicry: a new paradigm of autoimmune diseases. Curr Opin Immunol 2005;17:577–82.
[5] Voltz R. Paraneoplastic neurological syndromes: an update on diagnosis, pathogenesis, and therapy. Lancet Neurol 2002;1: 294–305.
[6] Bataller L, Wade DF, Fuller GN, Rosenfeld MR, Dalmau J. Cerebellar degeneration and autoimmunity to zinc-finger proteins of the cerebellum. Neurology 2002;59:1985–7.
[7] Takahashi Y, Mori H, Mishina M, Watanabe M, Kondo N, Shimomura J, et al. Autoantibodies and cell-mediated autoimmunity to NMDA-type GluRepsilon2 in patients with Rasmussen's encephalitis and chronic progressive epilepsia partialis continua. Epilepsia 2005;46(Suppl. 5):152–8.
[8] Sugiyama N, Hamano S, Mochizuki M, Tanaka M, Takahashi Y. A case of chronic cerebellitis with anti-glutamate receptor delta 2 antibody (in Japanese). No To Hattatsu 2004;36:60–3.
[9] Mochizuki Y, Mizutani T, Isozaki E, Ohtake T, Takahashi Y. Acute limbic encephalitis: a new entity? Neurosci Lett 2006;394: 5–8.
[10] Gohlich-Ratmann G, Wallot M, Baethmann M, Schaper J, Roggendorf M, Roll C, et al. Acute cerebellitis with near-fatal cerebellar swelling and benign outcome under conservative treatment with high dose steroids. Eur J Paediatr Neurol 1998;2:157–62.

Anti-aquaporin 4 antibody in selected Japanese multiple sclerosis patients with long spinal cord lesions

K Tanaka[1], T Tani[1], M Tanaka[2], T Saida[2], J Idezuka[3], M Yamazaki[4], M Tsujita[4], T Nakada[5], K Sakimura[4] and M Nishizawa[1]

Multiple sclerosis (MS) in Asian populations is often characterized by the selective involvement of the optic nerve (ON) and spinal cord (SP) (OSMS) in contrast to classic MS (CMS), where frequent lesions are observed in the cerebrum, cerebellum or brainstem. In Western countries, inflammatory demyelinating disease preferentially involving the ON and SP is called neuromyelitis optica (NMO). Recently, Lennon *et al.* discovered that NMO-IgG, shown to bind to aquaporin 4 (AQP4), could be a specific marker of NMO and also of Japanese OSMS whose clinical features were identical to NMO having long spinal cord lesions extending over three vertebral segments (LCL). To examine this antibody in larger populations of Japanese OSMS patients in order to know its epidemiological and clinical spectra, we established an immunohistochemical detection system for the anti-AQP4 antibody (AQP4-Ab) using the AQP4-transfected human embryonic kidney cell line (HEK-293) and confirmed AQP4-Ab positivity together with the immunohistochemical staining pattern of NMO-IgG in approximately 60% of Japanese OSMS patients with LCL. Patients with OSMS without LCL and those with CMS were negative for this antibody. Our results accorded with those of Lennon *et al.* suggest that Japanese OSMS with LCL may have an underlying pathogenesis in common with NMO. *Multiple Sclerosis* 2007; **13**: 850–855. http://msj.sagepub.com

Key words: aquaporin 4 water channel; long spinal cord lesion; neuromyelitis optica; NMO-IgG; opticospinal multiple sclerosis

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) probably caused by autoimmune mechanisms. MS in Asian populations is often characterized by the selective involvement of the optic nerve and spinal cord with few or no lesions observed in the cerebrum or cerebellum. This optic-spinal MS (OSMS) is in marked contrast to classic MS (CMS), where lesions are frequently observed in the cerebrum, cerebellum or brainstem, as well as in the optic nerve and spinal cord, though the definition of OSMS tends to be wider, to include small cerebral/brainstem lesions [1].

In Western countries, the inflammatory disease of the CNS that preferentially affects the optic nerve and spinal cord while virtually sparing the brain is called neuromyelitis optica (NMO), also called Devic's syndrome [2–4].

Clinicopathological features that constitute the core of OSMS [5] and NMO [4] are very similar and the question has been raised whether these two conditions are the same.

Recently, Lennon *et al.* have reported that the serum immunoglobulin G antibody (NMO-IgG) that recognizes the aquaporin-4 water channel is a specific marker of NMO [6,7]. To define the frequency and clinical characteristics of Japanese MS with this

[1] Department of Neurology, Brain Research Institute, Niigata University, Niigata Japan
[2] Department of Neurology, Utano National Hospital, Kyoto, Japan
[3] Department of Neurology, Ojiya Sakura Hospital, Ojiya, Japan
[4] Department of Cellular and Neurobiology, Brain Research Institute, Niigata University, Niigata, Japan
[5] Center for Integrated Human Brain Science, Brain Research Institute, Niigata University, Niigata, Japan
Author for correspondence: Keiko Tanaka, Department of Neurology, Brain Research Institute, Niigata University, 1-757 Asahimachi-Dori, Niigata City, Niigata 951-8585, Japan. E-mail: keiko@bri.niigata-u.ac.jp
Received 29 August 2006; accepted 16 January 2007

 10.1177/1352458507076976

antibody, we established a detection system for the anti-aquaporin-4 antibody (AQP4-Ab) immunohistochemically using AQP4-transfected culture cells together with the immunohistochemical staining of rodent and human brains and spinal cord sections (NMO-IgG detection).

Since NMO-IgG-positive patients shown in the study by Lennon *et al.* most frequently had MRI spinal-cord lesion extending over three vertebral segments (LCL), we selected MS patients with LCL for the AQP4-Ab test to validate our detection system comparing them with those without LCL or other inflammatory neurological diseases. We found that approximately 60% of MS patients with LCL have both the AQP4-Ab and immunohistochemically proven NMO-IgG, whereas no MS patients without LCL or other controls have this antibody. Our results were similar to the frequency of NMO-IgG found in NMO patients and also in Japanese OSMS patients reported by Nakashima *et al.* [8], and confirmed the results of Lennon *et al.* [6,7]. Our AQP4-Ab detection system could be a useful tool for the clinical study of MS.

Subjects and methods

Patients

To validate our AQP4-Ab detection system and compare the results with NMO-IgG study, we examined sera from 26 OSMS patients with LCL (one man and 25 women; mean age, 43.5 ± 12.3 years) mainly presenting with optic nerve and spinal cord lesions, six OSMS patients without LCL (three men and three women; mean age, 41.2 ± 10.5 years). For controls, the sera from 21 CMS patients mainly presenting with cerebral lesions (four men and 17 women; mean age, 39.9 ± 14.2 years), 28 patients with other inflammatory neuromuscular diseases such as systemic lupus erythematosus (SLE), Sjögren's syndrome (Sjs), mixed connective tissue disease (MCTD), Lambert Eaton myasthenic syndrome (LEMS), dermatomyositis, sarcoidosis and polyarteritis nodosa (seven men and 21 women; mean age, 36.1 ± 14.0 years) and 10 healthy volunteers (two men and eight women; mean age, 37.7 ± 10.3 years) were examined. Sera and CSF were obtained after receiving spoken consent for testing, and the antibody testing was performed blinded.

AQP4-Ab production

We raised a polyclonal antibody against human AQP4 in rabbits immunized with a synthetic peptide (C-terminal of human AQP4 at amino acids 301–318) and affinity-purified it. The procedures used for immunization and antibody purification were reported previously [9]. This AQP4-Ab cross-reacted with mouse AQP4; therefore, its specificity was confirmed using an AQP4 knockout mouse. To examine staining patterns, paraffin-embedded sections or cryostat sections of the human cerebrum, cerebellum and spinal cord were stained with the raised antibody.

Cloning of human AQP4 and expression in cultured cells

Total RNA was extracted from an adult human cerebellum using ISOGEN (Nippongene). cDNA encoding human aquaporin 4 (AQP4 M23 isoform; GenBank accession number U63623 [10]) was cloned by the reverse-transcription polymerase chain reaction (RT-PCR) technique. First-strand cDNA was synthesized from human brain total RNA using an Advantage RT-for-PCR kit (Clontech). Full-length cDNA was inserted into the *Xba*I site of a pEF-BOS expression vector. HEK 293 cells were seeded in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) one day before transfection at 3×10^4 cells per well in a poly-L-lysine-coated eight-well chamber slide (BD BioCoat). The cells were transfected with an AQP4 expression vector or a control empty vector using lipofectamine reagent (Invitrogen) according to the manufacturer's instructions.

Immunohistochemistry

Thirty hours after transfection, the HEK-293 cells were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 20 minutes. Then, non-specific binding was blocked with 10% goat serum/PBS, and the cells were incubated with a patient's serum (1:20–1:1000), cerebrospinal fluid (CSF, 1:1–1:10) or the rabbit antibody for the human AQP4-peptide (0.3 μg/mL) for 60 minutes at room temperature, then incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human IgG (BD Biosciences) for a patient's serum or CSF and FITC-conjugated goat anti-rabbit IgG for a rabbit serum, for 30 minutes. Then, a *SlowFade* Gold antifade reagent (Molecular Probes, USA) was applied to the slide. Between steps, the wells were washed with PBS three times.

To confirm the coincidence of staining pattern between MS sera and AQP4-Ab raised in rabbits, rhodamine-conjugated goat anti-rabbit IgG (BD Biosciences) was used as the secondary antibody for the latter.

Cryostat sections (8 μm thick) of the rat cerebrum, cerebellum or human spinal cord were immunostained as follows: tissue sections were air-dried, fixed in cold acetone for 10 minutes, and blocked with 10% goat serum/PBS, MS or control serum (1:20–1:1000) for 60 minutes, then with FITC-anti-human IgG (BD Biosciences), followed by a *SlowFade* Gold antifade reagent was applied. Between steps, the sections were washed with PBS three times.

Results

The AQP4-Ab raised against the AQP4 peptide in the rabbit reacted with the microvessel wall, pia, subpia and margin of the Virchow-Robin space in the human cerebrum and cerebellum, producing a staining pattern the same as that of NMO-IgG [6]; thus, we used this antibody as the positive control.

The AQP4-Ab in the sera of MS patients reacted with the AQP4-transfected HEK-293 cells, showing a dotlike staining pattern on the cell surface and along cell processes, but not with the non-AQP4-transfected HEK cells (*Figure 1*). The efficiency of AQP4 transfection in the HEK-293 cells was very stable between transfections, at 35.8 ± 1.2%, determined with the same serum used as the positive control for each staining examination.

Rat or human cerebrum, cerebellum and spinal cord cryostat sections were incubated with the AQP4-Ab-positive MS serum examined by AQP4-transfected HEK-293 cell immunostaining and gave similar staining patterns to NMO-IgG (Figure 2).

AQP4-Ab positivity was observed in 16 of 26 patients diagnosed as OSMS with LCL but in none of the six OSMS patients without LCL. The patients with CMS and other inflammatory neuromuscular diseases and the healthy controls were all negative for AQP4-Ab. No sera reacted with the non-AQP4-transfected HEK-293 cells.

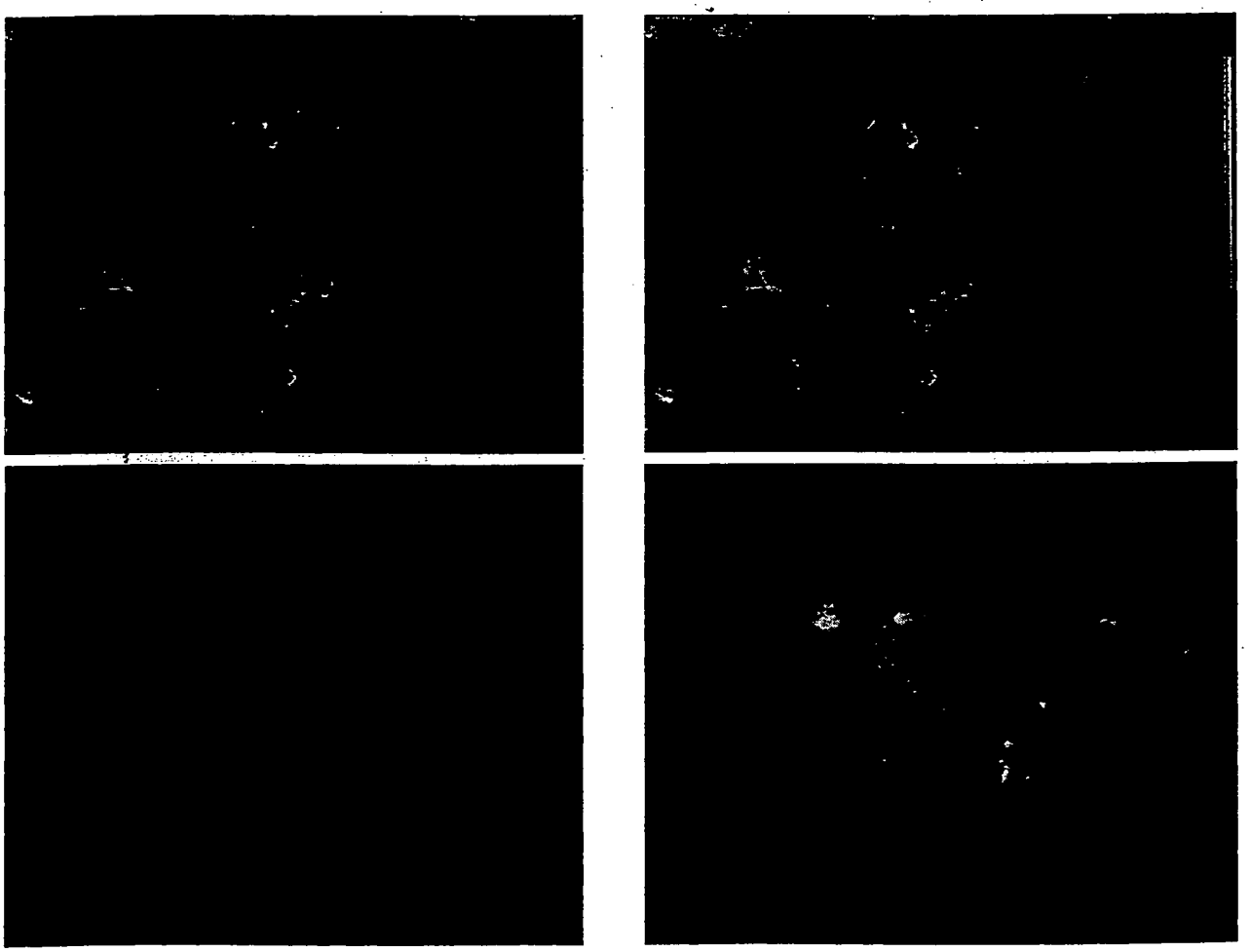

Figure 1 Immunostaining of AQP4-transfected HEK-293 cells with AQP4-Ab. LCL(+)MS serum (1 : 400)/FITC-anti-human IgG (top left), affinity-purified rabbit IgG specific for human AQP4 C-terminal peptide (0.3 μg/mL)/Rhodamine-anti-rabbit IgG (top right), merged figure (bottom left) and high power figure of LCL(+)MS serum/FITC-anti-human IgG.

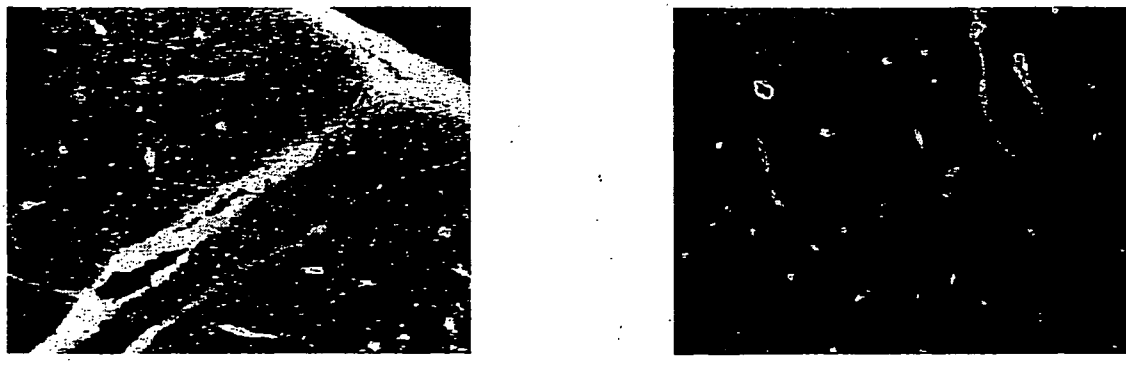

Figure 2 Immunostaining of rat cerebrum with LCL(+)MS serum (1 : 1000). The pia, subpia and perivascular area (high power) were identical to NMO-IgG staining.

All 16 AQP4-Ab-positive patients had LCL extending over three or more vertebral longitudinal segments (LCL(+)MS), whereas 10 LCL(+)MS patients were AQP4-Ab-negative, that is 61.5% of LCL(+)MS patients were AQP4-Ab-positive (Table 1).

Comparison of AQP4-Ab-positive and AQP4-Ab-negative LCL(+)MS patients showed that the former group comprised only women with severe visual disturbance (seven were blind), whereas the latter group comprised one man and nine women, of which only one was blind. The duration of the disease was also longer in the former group at the point of this study (AQP4-Ab-positive: 12.1 ± 9.2 years; AQP4-Ab-negative: 5.9 ± 2.5 years). However the examined sera from six of the patients of this group were obtained at the early stage of the disease (less than two years from onset) so the antibody production is not secondary to a long disease course. Other factors such as mean age (AQP4-Ab-positive: 49.1 ± 13.5 years; AQP4-Ab-negative: 42.4 ± 14.8 years), severity of limb and truncal disabilities (as shown by mean value of expanded disability status scale (EDSS) score; AQP4-Ab-positive: 8.1 ± 2.1; AQP4-Ab-negative: 7.3 ± 2.5), existence of cerebral lesions (AQP4-Ab-positive: eight of 16 patients; AQP4-Ab-negative: five of 10) did not differ between the two groups (Table 2).

Table 1 Clinical phenotype of AQP4-Ab (+) patients. Sixteen of 26 OSMS patients with LCL were positive for the AQP4-Ab, while other phenotypes were all negative for the antibody

	AQP4-Ab (+)
OSMS with LCL	16/26 (61.5%)
OSMS without LCL	0/6
CMS without LCL	0/21
OND	0/28
Healthy controls	0/10

OND: other inflammatory neurological diseases.

Table 2 Clinical features of AQP4-Ab-positive or AQP4-Ab-negative LCL(+)MS patients

Study parameter	AQP4-Ab (+)	AQP4-Ab (−)
Sex (male : female)	0 : 16	1 : 9
Age at examination	49.1 ± 13.5	42.4 ± 14.8
Years from onset of disease	12.1 ± 9.2	5.9 ± 2.5
EDSS score	8.1 ± 2.1	7.3 ± 2.5
No. of patients with cerebral lesions	8/16	5/10
No. of blind patients	7/16	1/10

Of the AQP4-Ab-positive patients, half were in the active stage of the disease and some had been treated with several cycles of 1000 mg of methylprednisolone per day for three days just before sera were taken, and the other half were in a chronic stable stage without corticosteroid or immunosuppressive treatment when sera were taken. The sera were taken several times in the active and remission stages in three of the patients and the AQP4-Ab was positive in all the sera taken in the active and chronic stages.

Discussion

It has long been considered that relapsing-remitting MS in Japan could be classified into two groups: CMS and OSMS. Compared with CMS, OSMS has some distinctive features similar to those of NMO: 1) older age at onset; 2) high female-to-male ratio; 3) high relapse frequency; 4) severe disability; 5) few cerebral lesions but tend to have long spinal cord lesions; 6) pleocytosis and absence of oligoclonal bands in the cerebrospinal fluid; 7) continuous shift towards a Th1 phenotype even in the remission phase, but only in the exacerbation phase in CMS, as suggested by results obtained using several biomarkers; 8) difference in spinal

cord neuropathology, that is, swelling and cavitation observed macroscopically and large numbers of polymorphonuclear cells containing neutrophils and eosinophils with a hyalinized appearance of medium-sized arteries observed microscopically; and 9) association with systemic autoimmune disorders such as hypothyroidism, Sjögren's syndrome or other types of connective tissue disease with multiple autoantibodies [11]. With these features, Wingerchuck *et al.* concluded that NMO is distinct from MS with different underlying pathogenesis, and incorporated the new findings on NMO-IgG in their concept [12].

Following the recent reports by Lennon *et al.* [6,7], we established an AQP4-Ab detection system together with the NMO-IgG test and confirmed their results in Japanese MS: 61.5% of Japanese LCL(+)MS patients were positive for AQP4-Ab, which was similar in the frequency of NMO-IgG positivity in seven of 12 Japanese MS patients reported under the designation of OSMS (58%) [6] and also in 12 of 19 patients (63%) [8]. In the report of Nakashima *et al.*, they described two NMO-IgG-positive CMS patients [8]. They diagnosed the two patients as having CMS because they had extensive brain lesions, although they otherwise had typical features of OSMS. In our series, almost half of the LCL(+)MS patients with or without AQP4-Ab showed cerebral MRI lesions, which were unlike typical CMS, and optic or spinal cord lesions preceded several years before cerebral MRI lesions were detected. Among them, one patient showed a large cystic lesion in the cerebral white matter. The proposed diagnostic criteria for NMO exclude patients with lesions outside the optic nerve and spinal cord. However, Pittock *et al.* included patients with cerebral lesions in NMO in their recent study [13], and Wingerchuk *et al.* [11] proposed revised diagnostic criteria for NMO that will include patients with MRI brain lesions. These revised criteria brought NMO closer to LCL(+)MS.

The AQP4-Ab-positive patients tended to show severe paraplegia in the early stage of the disease; 50% of these patients became blind shortly thereafter. Only one of seven AQP4-Ab-negative patients with LCL(+)MS was blind in our series.

Whether AQP4-Ab is directly related to the pathogenesis of LCL(+)MS and NMO remains to be elucidated. It is not necessarily related directly to the presence of active demyelinating lesions because patients at the chronic stage of the disease, who showed only an atrophic spinal cord without active inflammatory lesions (no cord swelling nor gadolinium-enhanced lesions), were also AQP4-Ab-positive. This indicates that the antibody does not simply block or disturb the function of water channels, or cause edema in nervous tissues only at the acute stage. However, the presence of the antibody might disrupt the formation of severe inflammatory lesions in nervous tissues once initial pathomechanisms develop.

We used both the immunohistochemical staining pattern of CNS tissues (NMO-IgG) and a confirmatory test on specific binding to AQP4-Ab, since the NMO-IgG staining pattern itself is not equal to AQP4 antigen detection, because other proteins colocalized with AQP4 could show similar immunohistochemical staining patterns. Thus our antibody test using full-length human AQP4 cDNA-transfected HEK 293 cells is more specific for defining AQP4-Ab positivity than the immunohistochemical detection system of NMO-IgG.

AQP4 is a water-selective transporter expressed strongly in astrocytes and ependymal cells throughout the brain and spinal cord, particularly at sites of fluid transport at the pial and ependymal surfaces in contact with the cerebrospinal fluid [14,15]. AQP4 has also been shown to be expressed at the optic chiasm [16]. AQP4 is thought to be involved in the development of brain edema. There is accumulating evidences that it is related to glial migration and neural signal transduction, as obtained through many experimental studies [17,18]. In the immunohistochemical study of the MS brain using the AQP4-Ab raised against a synthetic peptide homologous to the C-terminal end of the cytoplasmic domain of the rat AQP4, the staining intensity was prominent at the periphery of the plaques of recent foci; however, this perilesional accentuation of AQP4 expression is evident even at later stages with glial scarring without edema [19]. We could not find a similar study of LCL(+)MS brains and the role of the AQP4-Ab on lesion formation in this group is not yet known. The preferential involvement of the optic nerve and spinal cord in LCL(+)MS is not yet understood. AQP4 has been shown to be expressed at a high level in the gray matter in the spinal cord where numerous AQP4-dense processes are found in direct contact with neuronal cell bodies and synapses [20,21]. There is as yet no direct comparison of AQP4 densities and their distribution in the cerebrum and the spinal cord. The interpretation of the significance of AQP4-Ab in LCL(+)MS needs to wait until more basic knowledge of AQP4 is obtained.

Acknowledgements

This study was supported in part by the Ministry of Education, Culture, Sports, Science, and Technology, Japan and a Neuroimmunological Disease Research Committee grant from the Ministry of Health, Labor, and Welfare, Japan.

References

1. **Saida T, Tashiro K, Itoyama Y, Sato T, Ohashi Y, Shao Z** *et al.* Interferon beta-1b is effective in Japanese RRMS patients: A randomized, multicenter study. *Neurology* 2005; **64**: 621–30.
2. **Cree BA, Goodin DS, Hauser SL.** Neuromyelitis optica. *Semin Neurol* 2002; **22**: 105–22.
3. **O'Riordan JI, Gallagher HL, Thompson AJ, Howard RS, Kingsley DP, Thompson EJ** *et al.* Clinical, CSF, and MRI findings in Devic's neuromyelitis optica. *J Neurol Neurosurg Psychiatry* 1996; **60**: 382–87.
4. **Wingerchuk DM, Hogancamp WF, O'Brien PC, Weinshenker BG.** The clinical course of neuromyelitis optica (Devic's syndrome). *Neurology* 1999; **53**: 1107–14.
5. **Kira J.** Multiple sclerosis in the Japanese population. *Lancet Neurol* 2003; **2**: 117–27.
6. **Lennon VA, Wingerchuk DM, Kryzer TJ, Pittock SJ, Lucchinetti CF, Fujihara K** *et al.* A serum autoantibody marker of neuromyelitis optica: distinction from multiple sclerosis. *Lancet* 2004; **364**: 2106–12.
7. **Lennon VA, Kryzer TJ, Pittock SJ, Verkman AS, Hinson SR.** IgG marker of optic-spinal multiple sclerosis binds to the aquaporin-4 water channel. *J Exp Med* 2005; **202**: 473–77.
8. **Nakashima I, Fujihara K, Miyazawa I, Misu T, Narikawa K, Nakamura M** *et al.* Clinical and MRI features of Japanese MS patients with NMO-IgG. *J Neurol Neurosurg Psychiatry* 2006; **77**: 1073–75.
9. **Watanabe M, Fukaya M, Sakimura K, Manabe T, Mishina M, Inoue Y.** Selective scarcity of NMDA receptor channel subunits in the stratum lucidum (mossy fibre-recipient layer) of the mouse hippocampal CA3 subfield. *Eur J Neurosci* 1998; **10**: 478–87.
10. **Lu M, Lee MD, Smith BL, Jung JS, Agre P, Verdijk MAJ** *et al.* The human *AQP4* gene: definition of the locus encoding two water channel polypeptides in brain. *Proc Natl Acad Sci USA* 1996; **93**: 10908–12.
11. **Wingerchuk DM, Lennon VA, Pittock SJ, Lucchinetti CF, Weinshenker BG.** Revised diagnostic criteria for neuromyelitis optica. *Neurology* 2006; **66**:1485–89.
12. **Wingerchuk DM.** Neuromyelitis optica. In Freedman MS ed. *Advances in neurology* (Vol 98). Lippincott Williams & Wilkins, 2006: 319–33.
13. **Pittock SJ, Lennon VA, Krecke K, Wingerchuk DM, Lucchinetti CF, Weinshenker BG.** Brain abnormalities in neuromyelitis optica *Arch Neurol* 2006; **63**: 390–96.
14. **Nielsen S, Nagelhus ER, Amiry-Moghaddam M, Bourque C, Agre P, Ottersen OP.** Specialized membrane domains for water transport in glial cells: High-resolution immunogold cytochemistry of aquaporin-4 in rat brain *J Neurosci* 1997; **17**: 171–80.
15. **Rash JE, Yasumura T, Hudson CS, Agre P, Nielsen S.** Direct immunogold labeling of aquaporin-4 in square arrays of astrocyte and ependymocyte plasma membranes in rat brain and spinal cord *Proc Natl Acad Sci USA* 1998; **95**: 11981–86.
16. **Venero JL, Vizuete ML, Ilundáin AA, Machado A, Echevarria M, Cano J.** Detailed localization of aquaporin-4 messenger RNA in the CNS: preferential expression in periventricular organs *Neurosci* 1999; **94**: 239–50.
17. **Verkman AS.** More than just water channels: unexpected cellular roles of aquaporins. *J Cell Sci* 2005; **118**: 3225–32.
18. **Verkman AS, Binder DK, Bloch O, Auguste K, Papadopoulos MC.** Three distinct roles of aquaporin-4 in brain function revealed by knockout mice. *Biochim Biophys Acta* 2006; (online).
19. **Aoki-Yoshino K, Uchihara T, Duyckaerts C, Nakamura A, Hauw J-J, Wakayama Y.** Enhanced expression of aquaporin 4 in human brain with inflammatory diseases. *Acta Neuropathologica* 2005; **110**: 281–88.
20. **Oshio K, Binder DK, Yang B, Schecter S, Verkman AS, Manley GT.** Expression of aquaporin water channels in mouse spinal cord. *Neurosci* 2004; **127**: 685–93.
21. **Vitellaro-Zuccarello L, Mazzetti S, Bosisio P, Monti C, De Biasi S.** Distribution of Aquaporin 4 in rodent spinal cord: Relationship with astrocyte markers and chondroitin sulfate proteoglycans. *Glia* 2005; **51**: 148–59.

SHORT REPORT

Anti-aquaporin 4 antibody in Japanese multiple sclerosis: the presence of optic–spinal multiple sclerosis without long spinal cord lesions and anti-aquaporin 4 antibody

Masami Tanaka, Keiko Tanaka, Mika Komori, Takahiko Saida

J Neurol Neurosurg Psychiatry 2007;**78**:990–992. doi: 10.1136/jnnp.2006.114165

Background: Anti-aquaporin 4 (AQP4) antibodies were found in patients with neuromyelitis optica (NMO) and Japanese optic–spinal multiple sclerosis (OSMS).
Objective: To review the clinical features and investigate anti-AQP4 antibodies of Japanese patients with multiple sclerosis (MS), with or without long spinal cord lesions (LCL).
Methods: Anti-AQP4 antibodies were examined in the sera of 128 consecutive Japanese patients by the immunofluorescence method using AQP4 transfected cells.
Results: The 45 LCL-MS patients included 28 with a long spinal cord lesion extending contiguously over three vertebral segments on sagittal T2 weighted images (long T2 lesion) and 17 with segmental cord atrophy extending more than three vertebral segments. We identified 25 patients with anti-AQP4 antibody with LCL and anti-AQP4 antibody. Anti-AQP4 antibody was found in 12/17 (70.6%) LCL-MS patients with segmental cord atrophy, and in 13/28 (46.4%) LCL-MS patients without segmental long cord atrophy ($p=0.135$, Fisher's exact test). Seropositive MS patients with LCL had more relapses than seronegative patients ($p=0.0004$, Mann–Whitney U test). 9 patients with OSMS were negative for anti-AQP4 antibody who did not show LCL.
Conclusion: These results suggest that an anti-AQP4 antibody is found not only in MS patients with long T2 lesions but also in patients with segmental cord atrophy extending more than three vertebral segments. It is a marker of LCL-MS showing frequent exacerbations. Japanese OSMS cases comprised those that were identical to NMO cases and those that were more closely related to classic MS.

Multiple sclerosis (MS) is a chronic autoimmune disorder of the central nervous system. Japanese MS patients have been classified into two phenotypes: classic MS (CMS) and optic–spinal MS (OSMS).[1] OSMS has been recognised since the 1950s.[2] Patients with OSMS have symptoms and MRI findings in which the main lesions are confined to the optic nerve and spinal cord. In patients with OSMS, there is a higher female/male ratio; neuropathologically necrotic lesions; pleocytosis with a predominance of polymorphonuclear cells and a low frequency of oligoclonal IgG bands in CSF; a high incidence of autoantibodies in sera; long spinal cord lesions (LCL) extending more than three vertebral segments in MRI scans; and an association with a human leucocyte antigen class II allele (DPB1*0502).[3]

Neuromyelitis optica (NMO) has been described as Devic disease, but its clinical definition has frequently been revised.[4] LCL extending contiguously over three vertebral segments on sagittal T2 weighted images (long T2 lesion) is a disease marker. Current NMO criteria include unilateral optic neuritis, no restriction on onset of optic neuritis and myelitis, relapsing course and brain involvement.[4 5] Recent NMO criteria stress the presence of both brain MRI abnormalities that do not meet diagnostic criteria for MS and NMO-IgG,[4] a highly specific biomarker of NMO,[6] and its target antigen is the aquaporin 4 (AQP4) water channel.[7]

The incidence of NMO-IgG seropositivity in Japanese patients with OSMS (6/11 cases, 54%) was similar to that in NMO (33/45, 73%), and OSMS was thought to be the same disease.[6] However, in that study,[6] the Japanese OSMS patients had been selected using 1999 NMO criteria (Fujihara K, personal communication) that are not the same as the clinical definition of OSMS widely used in Japan.[8] Their recent report showed that serum NMO-IgG was found in 12 of 19 patients with OSMS (63%).[9] We established an AQP4 antibody assay system and identified nine seropositive MS patients with LCL-MS.[10]

PATIENTS AND METHODS

A total of 128 consecutive Japanese patients (36 men, 92 women), aged 21–75 years, who had definite relapsing–remitting MS clinically, according to McDonald's criteria, were considered for enrolment in our study. Patients with primary progressive MS, spinal MS and relapsing optic neuritis were excluded. Time since onset was 1–35 years. Kurtzke's Expanded Disability Status Scale (EDSS) score[11] was used for the clinical ratings.

Patients who showed selective involvement of the spinal cord and optic nerves but no clinical signs indicative of cerebrum, cerebellum or brainstem involvement were classified as having OSMS, while those with estimated lesions in the cerebrum, cerebellum or brainstem were defined as CMS.[1] Patients with a long cord lesion on spinal cord MRI (long T2 lesion or segmental cord atrophy) extending over three vertebral segments were designated as having LCL-MS.

Anti-AQP4 antibody measurement

Anti-AQP4 IgG antibody in MS patient sera was measured using an indirect immunofluorescence method reported previously[10] with HEK-293 cells transfected with an AQP4 expression vector containing full length cDNA of human AQP4. Sera were obtained during the clinically stable stage. Anti-AQP4 antibody was measured by one of the authors (KT) who was blind to all clinical information, including clinical phenotype.

The statistical significance of differences between the two groups were determined using the Mann–Whitney U test or Fisher's exact test for difference in percentage anti-AQP4 antibodies between patients with and without segmental long spinal cord atrophy.

Abbreviations: AQP4, aquaporin 4; CMS, classic multiple sclerosis; EDSS, Expanded Disability Status Scale; LCL, long spinal cord lesions; MS, multiple sclerosis; NMO, neuromyelitis optica; OSMS, optic–spinal multiple sclerosis

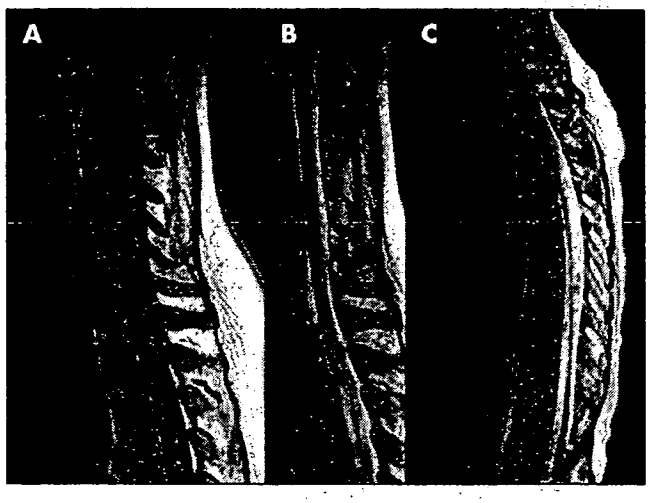

Figure 1 MRI findings of patients with long spinal cord lesions–multiple sclerosis (LCL-MS). The patient shows discontinuous enhanced lesions from C1 to Th5 (A) and discontinuous T2 high intensity lesions in the cervical segment and T2 high intensity lesions from Th1 to Th5 (long T2 lesion) (B). Another patient shows segmental spinal cord atrophy from C7 to Th11 (C).

This study was approved by the Medical Ethics Committee of Utano National Hospital. All participants gave written informed consent.

RESULTS

The 45 LCL-MS patients (five men and 40 women) included 28 with long T2 lesions (four men and 24 women) and 17 with segmental cord atrophy extending more than three vertebral segments (two men and 15 women) (fig 1). Anti-AQP4 antibodies were found in 12/17 (70.6%) LCL-MS patients with segmental cord atrophy and in 13/28 (46.4%) LCL-MS patients without segmental long cord atrophy ($p = 0.135$, Fisher's exact test) (table 1). Excluding those with LCL-MS, patients did not have anti-AQP4 antibodies. There was a relationship between blindness and seropositivity against AQP4 ($p<0.0005$) (table 2). Eleven of 128 patients were blind, nine of whom had LCL-MS. Seven of the nine blind patients had LCL and anti-AQP4 antibodies.

The number of relapses in 22 patients with LCL-MS and anti-AQP4 antibodies (0–7; mean 3.64) was greater than in 18 seronegative patients with LCL-MS (0–6; 1.39) in the year before determination of antibodies ($p = 0.0004$). Time from onset for seropositive patients (mean 14.3) was not different from that of seronegative patients (9.1) ($p = 0.1518$). There was no other difference in clinical phenotype between patients with and without anti-AQP4 antibodies.

We found nine OSMS patients who had a 5 year minimum follow-up period after onset and did not have LCL or anti-AQP4 antibodies (OSMS(−)). Age of OSMS(−) onset ranged from 7 to 51 years (mean 27.0), which did not differ from that of LCL-MS cases (2–62 years; 35.2) ($p = 0.1281$). The period after onset of OSMS (−) was 5–24 years (mean 11.4), which did not differ from that of LCL-MS cases (1–30 years; 12.0) ($p = 0.6685$). Kurtzke's EDSS for OSMS(−) (0–4.0; mean 1.50), however, was lower than that for LCL-MS (0–9.5; 6.63) ($p<0.0001$). At disease onset, one of three patients had asymptomatic subcortical white matter lesions on brain MRI, while the other two had normal brain MRI findings. However, most patients who had optic neuritis as the first event did not undergo MRI. Patients with OSMS(−) had no clinical manifestations except for symptoms or signs caused by lesion of the optic nerves or spinal cord. All showed asymptomatic brain lesions on T2 weighted brain MRI scans.

Table 1 Number of seropositive patients with multiple sclerosis

	n	No of anti-AQP4 antibody positive (%)
Total LCL-MS	45	25 (55.6%)
With segmental cord atrophy	17	12 (70.6%)
Without segmental cord atrophy	28	13 (46.4%)
OSMS	22	2 (9.1%)
Including LCL-MS	3	2 (66.7%)
CMS	64	0 (0%)
Less than 5 y after onset	29	4 (13.8%)
Including LCL-MS	9	4 (44.4%)
OSMS	9	0 (0%)

AQP4, aquaporin 4; CMS, classic multiple sclerosis—patients with estimated lesions in the cerebrum, cerebellum or brainstem; LCL-MS, long spinal cord lesions-multiple sclerosis—patients with a long spinal cord lesion on MRI (long T2 lesion or segmental cord atrophy) extending over three vertebral segments; OSMS, optic-spinal multiple sclerosis—patients with selective involvement of the spinal cord and optic nerves but no clinical signs indicative of cerebrum, cerebellum or brainstem involvement;. The 128 patients include 45 patients with LCL-MS, 12 with OSMS and 71 with CMS.

DISCUSSION

Anti-AQP4 antibody is a good diagnostic biomarker for LCL-MS as 25 of 45 LCL-MS patients (56%) had anti-AQP4 antibodies but patients who did not have LCL-MS did not have antibodies.

The Mayo Clinic Group has reported that long T2 lesions was a disease marker of NMO[4] but they did not mention spinal cord atrophy, which would also be a marker of NMO. Segmental cord atrophy seems to be caused by severe necrotic lesions in a similar way as long T2 lesions on spinal cord MRI in patients with NMO. The percentage of anti-AQP4 antibodies in patients with segmental long spinal atrophy was not different from that in patients with long T2 lesions, which also supports the hypothesis that the pathomechanism of segmental cord atrophy is similar to that of long T2 lesions.

Similar findings of LCL on spinal cord MRI and seropositivity against AQP4 in NMO and OSMS seem to indicate that these diseases fall into the same clinical spectrum. Some Japanese OSMS patients, however, did not fulfil NMO criteria[4] because they did not have LCL or anti-AQP4 antibodies. Patients with CMS may present with only optic neuritis and myelitis for the first few years after onset, whereas the diagnosis of OSMS requires a 5 year follow-up period after onset.[3] Several patients with OSMS differed from those with NMO, with the disease being clinically restricted to the optic nerves and spinal cord for a period of 5–24 years after the first clinical event. This may be the same clinical phenotype as the benign form of OSMS.[3] All of our patients with OSMS(−) had brain lesions but no brain symptoms or signs. Moreover, they had lower EDSS scores than patients with LCL-MS, indicating that the higher LCL-MS scores may have been caused by LCL.

The role of anti-AQP4 antibody as the primary cause of tissue destruction has not been demonstrated and there were more

Table 2 Relationship between blindness and seropositivity against aquaporin 4 ($p<0.0005$).

	Blindness	
	+	−
Anti-AQP4 antibody		
Positive	7	16
Negative	4	101

relapses in patients with anti-AQP4 antibodies than in seronegative patients. This does not exclude the possibility of a secondary immune response to damage. However, NMO IgG is a known marker of relapse after myelitis with LCL.[12] This suggest that anti-AQP4 antibody/NMO IgG is not the secondary product of tissue destruction, rather it causes necrotic tissue damage and is a marker of a condition related to frequent exacerbation.

Authors' affiliations
Masami Tanaka, Mika Komori, Takahiko Saida, MS Centre, Utano National Hospital, Kyoto, Japan
Keiko Tanaka, Department of Neurology, Brain Research Institute, Niigata University, Niigata, Japan

This work was supported in part by a Neuroimmunological Disease Research Committee grant from the Ministry of Health, Labour and Welfare, Japan.

Competing interests: Researchers from Mayo MS group. Professor Kira, Kyushu University, Japan.

Correspondence to: Dr Masami Tanaka, 8 Ondoyama, Narutaki, Ukyo-ku, Kyoto, 616-8255, Japan; tanaka@unh.hosp.go.jp

Received 5 January 2007
Revised 15 April 2007
Accepted 25 April 2007

REFERENCES

1 **Saida T,** Tashiro K, Itoyama Y, *et al.* Interferon Beta-1b Multiple Sclerosis Study Group of Japan. Interferon beta-1b is effective in Japanese RRMS patients: a randomized, multicenter study, *Neurology* 2005;64:621–30.
2 **Okinaka S,** Tsubaki T, Kuroiwa Y, *et al.* Multiple sclerosis and allied diseases in Japan: clinical characteristics. *Neurology* 1958;8:756–63.
3 **Misu T,** Fujihara K, Nakashima I, *et al.* Pure optic-spinal form of multiple sclerosis in Japan. *Brain* 2002;125:2460–8.
4 **Wingerchuk DM,** Lennon VA, Pittock SJ, *et al.* Revised diagnostic criteria for neuromyelitis optica. *Neurology* 2006;66:1485–9.
5 **Pittock SJ,** Lennon VA, Krecke K, *et al.* Brain abnormalities in neuromyelitis optica. *Arch Neurol* 2006;63:390–6.
6 **Lennon VA,** Wingerchuk DM, Kryzer TJ, *et al.* A serum autoantibody marker of neuromyelitis optica: distinction from multiple sclerosis. *Lancet* 2004;364:2106–12.
7 **Lennon VA,** Kryzer TJ, Pittock SJ, *et al.* IgG marker of optic-spinal multiple sclerosis binds to the aquaporin-4 water channel. *J Exp Med* 2005;202:473–7.
8 **Kira J,** Kanai T, Nishimura Y, *et al.* Western versus Asian types of multiple sclerosis: immunogenetically and clinically distinct disorders. *Ann Neurol* 1996;40:569–74.
9 **Nakashima I,** Fujihara K, Miyazawa I, *et al.* Clinical and MRI features of Japanese patients with multiple sclerosis positive for NMO-IgG. *J Neurol Neurosurg Psychiatry* 2006;77:1073–5.
10 **Tanaka K,** Tani T, Tanaka M, *et al.* Anti-aquaporin 4 antibody in Japanese opticospinal multiple sclerosis. *Mult Scler* in press.
11 **Kurtzke JF.** Rating neurologic impairment in multiple sclerosis: An expected disability status scale (EDSS). *Neurology* 1983;33:1444–52.
12 **Weinshenker BG,** Wingerchuk DM, Vukusic S, *et al.* Neuromyelitis optica IgG predicts relapse after longitudinally extensive transverse myelitis. *Ann Neurol* 2006;59:566–9.

Resistance to Experimental Autoimmune Encephalomyelitis and Impaired T Cell Priming by Dendritic Cells in Src Homology 2 Domain-Containing Protein Tyrosine Phosphatase Substrate-1 Mutant Mice[1]

Takeshi Tomizawa,[2]* Yuka Kaneko,[2†‡] Yoriaki Kaneko,[3]* Yasuyuki Saito,* Hiroshi Ohnishi,† Jun Okajo,* Chie Okuzawa,* Tomomi Ishikawa-Sekigami,* Yoji Murata,† Hideki Okazawa,† Koichi Okamoto,‡ Yoshihisa Nojima,* and Takashi Matozaki[3†]

Src homology 2 domain-containing protein tyrosine phosphatase (SHP) substrate-1 (SHPS-1) is a transmembrane protein that binds the protein tyrosine phosphatases SHP-1 and SHP-2 through its cytoplasmic region and is expressed on the surface of CD11c dendritic cells (DCs) and macrophages. In this study, we show that mice that express a mutant form of SHPS-1 lacking most of the cytoplasmic region are resistant to experimental autoimmune encephalomyelitis (EAE) in response to immunization with a peptide derived from myelin oligodendrocyte glycoprotein (MOG (35o55)). The MOG (35o55)-induced proliferation of, and production of IFN- , IL-2, and IL-17, by T cells from immunized SHPS-1 mutant mice were reduced compared with those apparent for wild-type cells. The abilities of splenic DCs from mutant mice to stimulate an allogenic MLR and to prime Ag-specific T cells were reduced. Both IL-12-stimulated and TLR-dependent cytokine production by DCs of mutant mice were also impaired. Finally, SHPS-1 mutant mice were resistant to induction of EAE by adoptive transfer of MOG (35o55)-specific T cells. These results show that SHPS-1 on DCs is essential for priming of naive T cells and the development of EAE. SHPS-1 is thus a potential therapeutic target in inflammatory disorders of the CNS and other autoimmune diseases. *The Journal of Immunology,* **2007, 179: 869o877.**

Dendritic cells (DCs)[4] are professional APCs and play a central role in the induction of immune responses to pathogens (1, 2). When immature DCs, such as Langerhans cells, which reside in nonlymphoid tissues, encounter exogenous Ags, they engulf and process them for presentation of Ag-derived peptides in complexes with MHC molecules on the cell surface. The DCs subsequently migrate to draining lymph nodes (LNs) and make contact with naive T cells. During such migration, DCs mature and express costimulatory molecules such as CD80, CD86, and CD40 in addition to MHC class II molecules on their surface. The mature DCs thus present MHC-peptide complexes to naive T cells together with costimulatory molecules that are essential for priming of the T cells by DCs (3). However, the detailed molecular mechanism of priming of naive T cells by DCs remains largely unknown.

Src homology 2 domain-containing protein tyrosine phosphatase (SHP) substrate-1 (SHPS-1) (4), also known as signal-regulatory protein (5, 6) or brain Ig-like molecule with tyrosine-based activation motifs (BIT) (7), is a transmembrane protein whose extracellular region comprises three Ig-like domains and whose cytoplasmic region contains four tyrosine phosphorylation sites that mediate the binding of the protein tyrosine phosphatases SHP-1 and SHP-2. Tyrosine phosphorylation of SHPS-1 is regulated by various growth factors and cytokines as well as by integrin-mediated cell adhesion to extracellular matrix proteins (8, 9). SHPS-1 thus functions as a docking protein to recruit and activate SHP-1 or SHP-2 at the cell membrane in response to extracellular stimuli. CD47 is a ligand for the extracellular region of SHPS-1 (10, 11). This protein, which was originally identified in association with $_{v}$ $_{3}$ integrin, is also a member of the Ig superfamily, possessing an Ig-V-like extracellular domain, five putative membrane-spanning segments, and a short cytoplasmic tail (12). Among hemopoietic cells, SHPS-1 is especially abundant in DCs, macrophages, and neutrophils, being barely detectable in T or B lymphocytes (11, 13–16). In contrast, CD47 is expressed in a variety of hemopoietic cells, including RBCs and T cells (12). Indeed, the interaction of CD47 on RBCs with SHPS-1 on macrophages is thought to prevent phagocytosis of the former cells by the latter through activation of SHP-1, which forms a complex with SHPS-1 (17–19). Similarly, SHPS-1, through its interaction with CD47, is also thought to play a negative role in the immune

*Department of Medicine and Clinical Science, Gunma University Graduate School of Medicine, Gunma, Japan; †Laboratory of Biosignal Sciences, Institute for Molecular and Cellular Regulation, Gunma University, Gunma, Japan; and ‡Department of Neurology, Gunma University Graduate School of Medicine, Gunma, Japan

Received for publication December 11, 2006. Accepted for publication May 8, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *adveStTement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas Cancer, a Grant-in-Aid for Scientific Research (B) and (C), a grant of Initiatives for Attractive Education in Graduate Schools, and a grant of the 21st Century Center of Excellence Program from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

[2] T.T., and Yu.K. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Takashi Matozaki, Laboratory of Biosignal Sciences, Institute for Molecular and Cellular Regulation, Gunma University, 3-39-15 Showa-Machi, Maebashi, Gunma 371-8512, Japan; E-mail address: matozaki@showa.gunma-u.ac.jp or Dr. Yoriaki Kaneko, Department of Clinical Science, Gunma University, Graduate School of Medicine, 3-39-22 Showa-Machi, Maebashi, Gunma 371-8512, Japan; E-mail address: ykaneko@showa.gunma-u.ac.jp

[4] Abbreviations used in this paper: DC, dendritic cell; BMDC, DC derived from bone marrow; EAE, experimental autoimmune encephalomyelitis; LN, lymph node; MOG, myelin oligodendrocyte glycoprotein; ODN, oligodeoxynucleotide; SHP, Src homology 2 domain-containing protein tyrosine phosphatase; SHPS-1, SHP substrate-1; WT, wild type.

system (15). Ligation of SHPS-1 by CD47-Fc fusion proteins suppressed the phenotypic and functional maturation of immature DCs and inhibited cytokine production by mature DCs (15), suggesting that SHPS-1 (on DCs), through its interaction with CD47 (on T cells), prevents activation of DCs. In contrast, interaction of CD47 with SHPS-1 promotes the proliferation of T cells as well as contributes to the activation of Ag-specific CTL by DCs in vitro (16). Moreover, SHPS-1 also stimulates NO production by macrophages (20), suggesting that SHPS-1 would play a positive role in the immune system. These effects of SHPS-1 were demonstrated in vitro, however, with the physiological roles of SHPS-1 in the immune system remaining largely unknown. Furthermore, the possible role of SHPS-1 in autoimmune disease has not previously been investigated.

We previously generated mice that express a mutant version of SHPS-1 that lacks most of the cytoplasmic region (19, 21). This mutant protein does not undergo tyrosine phosphorylation or form a complex with SHP-1 or SHP-2. Furthermore, the cellular abundance of the mutant protein is markedly reduced compared with that of the full-length protein in wild-type (WT) cells (19, 21). With the use of these SHPS-1 mutant mice, we have now investigated the roles of SHPS-1 in the immune system and in the development of autoimmune disease.

Materials aOd Methods

Abs and reagents

Hybridoma cells producing the rat P84 mAb to SHPS-1 were provided by C. Lagenaur (University of Pittsburgh, Pittsburgh, PA); the mAb was purified from culture supernatants and conjugated to sulfo-NHS-LC biotin (sulfosuccinimidyl-6-(biotinamido) hexanoate; Pierce). Rabbit polyclonal Abs to SHPS-1 were obtained from ProSci. The mAbs to mouse CD16/32 (2.4G2) were prepared from the culture supernatants of hybridoma cells (provided by K. Okumura, Juntendo University, Tokyo, Japan). FITC-conjugated mAbs to mouse CD11c (HL3), CD4 (L3T4), or CD8 (Ly-2) as well as a biotin-conjugated mAb to mouse CD11c, biotin-conjugated rat IgG to trinitrophenol (isotype control), and PE- or FITC-conjugated streptavidin were from BD Pharmingen. FITC-conjugated mAbs to mouse NK1.1 (PK136) or B220 (RA3-6B2); biotin-conjugated mAbs to mouse MHC class I (28-14-8), MHC class II (M5/114.15.2), CD80 (16-10A1), CD86 (GL1), CD25 (PC61), or CD40 (3/23); and FITC-conjugated rat IgG to trinitrophenol (isotype control) were from eBioscience. Murine rIL-12 and rGM-CSF were from PeproTech, and murine rIFN- was from Pierce. RPMI 1640 medium (Sigma-Aldrich) was supplemented with 10% heat-inactivated FBS, 50 M 2-ME, 2 mM L-glutamine, 10 mM HEPES-NaOH (pH 7.4), penicillin (100 U/ml), streptomycin (100 g/ml), and 1 mM sodium pyruvate to yield complete medium.

Mice

Mice that express a mutant version of SHPS-1 that lacks most of the cytoplasmic region were described previously (19, 21) and were backcrossed onto the C57BL/6 background for five or six generations as well as for nine generations. Mice were bred and maintained at the Institute of Experimental Animal Research of Gunma University under specific pathogen-free conditions and were handled in accordance with the animal care guidelines of Gunma University.

Induction of experimental autoimmune encephalomyelitis (EAE) and histological analysis

EAE was induced in 6- to 12-wk-old male C57BL/6 WT or SHPS-1 mutant mice by s.c. injection (into the base of the tail and rump) with a total of 100 g of a peptide derived from mouse (rat) myelin oligodendrocyte glycoprotein (MOG (35–55)) (MEVGWYRSPFSRVVHLYRNGK) in CFA containing 400 g of heat-inactivated *Mycobacterium tuberculosis* (Difco) (22, 23). Pertussis toxin (200 ng) (List Biological Laboratories) was also injected i.p. on days 0 and 2. Animals were observed daily for clinical signs of EAE for up to 35 days after immunization, and neurological effects were quantified on an arbitrary clinical scale: 0, no disease; 1, limp tail; 2, hindlimb weakness; 3, total hindlimb paralysis; 4, hind- and forelimb paralysis; and 5, death. The mean clinical score was calculated by averaging the scores of all mice in each group, including animals that did not develop EAE. For histological analysis, mice were perfused transcardially with 4% paraformaldehyde in PBS, after which the spinal cord was removed, embedded in paraffin, sectioned, and stained with Mayer's H&E.

Cell proliferation and cytokine production in spleen or LN cells

The spleen or LNs were removed from mice 10 days after immunization with MOG. The tissue was gently ground with autoclaved frosted glass slides in PBS, and RBCs were hypotonically lysed by incubation of the resulting cell suspension with Gey's solution. The remaining lymphocytes from LNs were washed twice with PBS, and the cells (5 10^5 per well) were then cultured with various concentrations of MOG (35–55) for 72 h under an atmosphere of 5% CO_2 at 37°C in 96-well, round-bottom microplates (Falcon). The cells were exposed to 1 Ci of [^{3}H]TdR for the final 14 h of the incubation and were then harvested on glass fiber filters with the use of an automated sample harvester (PerkinElmer). The incorporated radioactivity was measured with a scintillation spectrometer (PerkinElmer). For measurement of cytokine production from spleen cells, the culture supernatants obtained after incubation of the cells for 72 h with the MOG peptide were collected and assayed for IFN- (BD Pharmingen), IL-2 (BD Pharmingen), IL-10 (BD Pharmingen), and IL-17 (R&D Systems) with the use of ELISA kits.

Assay for serum Abs to MOG

The relative titers of Abs to MOG in serum samples were measured, as described previously (24). Serum samples were obtained from blood of MOG-primed WT and SHPS-1 mutant mice 40 days after immunization. A 96-well flat-bottom plate was coated with 5 g/ml MOG (35–55) peptide at 4°C overnight. After washing the wells with PBS containing 0.05% Tween 20, the wells were blocked with 0.25% skim milk in PBS for 1 h at 37°C. After washing, diluted serum samples (diluted 1/30 or 1/300 in PBS) were added and incubated for 2 h at 37°C. Thereafter, HRP-conjugated goat polyclonal Abs to mouse IgG (Zymed Laboratories), IgG1, and IgG2a (Bethyl Laboratories) were added (concentration 1/4,000 or 1/40,000 in PBS). Plates were incubated for 2 h at 37°C. Tetramethylbenzidine substrate reagent set (BD Pharmingen) was used to develop the plates, and the reaction was stopped with stop solution (BD Pharmingen), and read at 450 nm.

Preparation of splenic DCs and T cells

Immature or mature splenic CD11c DCs were prepared from collagenase-digested spleen tissue, as described (25, 26), with minor modifications. In brief, splenocytes were released by homogenization of the spleen and subsequent exposure (with repeated passage through the tip of a pipette) of the tissue for 30 min at room temperature to collagenase (WAKO) at 400 U/ml in the presence of 5 mM EDTA. The undigested fibrous material was removed by filtration through a nylon mesh, and RBCs in the filtrate were lysed with Gey's solution. The remaining cells were washed twice with PBS, suspended in 2 ml of Ca^2 - and Mg^2 -free HBSS (Invitrogen Life Technologies) containing 17% Optiprep (Nycomed), and overlaid consecutively with 2 ml of 12% Optiprep in 10 mM HEPES-NaOH (pH 7.4), 0.88% NaCl, 1 mM EDTA, and 0.5% BSA, and with 2 ml of Ca^2 - and Mg^2 -free HBSS. The resulting gradient was centrifuged at 700 *g* for 15 min at 20°C, after which cells at the interface of the top two layers were collected and washed twice with PBS. Immature CD11c DCs were then isolated from the washed cells with the use of magnetic beads coated with a mAb to CD11c and a MACS column (Miltenyi Biotec). For isolation of mature DCs, the washed cells were suspended in RPMI 1640 complete medium and cultured for 2 h in a plastic dish; the nonadherent cells were then removed, and the remaining adherent cells were incubated for an additional 18 h. The newly nonadherent cells were then harvested, and mature CD11c DCs were isolated from this fraction with the use of magnetic beads coated with a mAb to mouse CD11c. Purified DCs were routinely 95% CD11c as determined by flow cytometry. For preparation of splenic CD4 or CD8 T cells (27, 28), the spleen was gently ground with autoclaved frosted glass slides in PBS, and the released cells were exposed to Gey's solution, washed twice with PBS, and filtered through nylon wool. Cells in the filtrate were then subjected to purification with the use of magnetic beads coated with mAbs to mouse CD4 or CD8 and a MACS column (Miltenyi Biotec). The purity of the isolated CD4 or CD8 T cells was 95% as determined by flow cytometry.

Flow cytometric and immunoblot analysis of DCs

For examination of the surface expression of SHPS-1 on DCs, immature splenic CD11c DCs (1 10^6) were first incubated with a mAb to mouse CD16/32 (1 g/ml) to avoid nonspecific binding of labeled mAbs to FcR . The cells were then washed and incubated consecutively with a biotin-conjugated mAb to SHPS-1 (1 g/ml) and streptavidin FITC (0.2 g/ml)

before flow cytometric analysis with a FACSCalibur instrument and CellQuest software (BD Biosciences). For immunoblot analysis, immature splenic CD11c DCs were homogenized on ice in a solution containing 20 mM Tris-HCl (pH 7.6), 140 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM PMSF, aprotinin (10 g/ml), and 1 mM sodium vanadate. The lysates were centrifuged at 10,000 *g* for 15 min at 4°C, and the resulting supernatants were subjected to immunoblot analysis (18).

MLRs

Immature CD11c DCs prepared from WT or SHPS-1 mutant mice were exposed to -radiation (30 Gy) and then plated in 96-well, round-bottom microplates. CD4 or CD8 T cells were purified from BALB/c splenocytes by magnetic cell separation. Purified responder T cells (1 10^5 per well) were then cultured with the stimulator cells for 72 h, with [^{3}H]TdR (1 Ci per well) being added for the final 12 h. Cells were harvested on glass fiber filters, and the incorporated radioactivity was measured. The culture supernatants were also assayed for IFN- , IL-2, and IL-10. In another set of experiments, irradiated immature CD11c DCs from BALB/c mice were incubated with CD4 or CD8 T cells from WT or SHPS-1 mutant mice, and proliferation of the responder cells was determined.

Proliferation of T cells from OT-II mice

For assay of the proliferation of OT-II CD4 T cells in vitro (29), 8- to 12-wk-old male WT or SHPS-1 mutant mice were injected i.v. with 3 mg of OVA (Calbiochem) or vehicle. Twelve hours after the injection, immature CD11c DCs were purified from the spleen and cultured for 72 h at various densities with OT-II CD4 T cells (1 10^5 per well) in 96-well, round-bottom microplates; the final 12 h of culture were performed in the additional presence of [^{3}H]TdR (1 Ci per well), and the cell-associated radioactivity was subsequently measured with a scintillation spectrometer.

Assay of TLR- or IL-12-induced cytokine production

For the preparation of DCs derived from bone marrow (BMDCs), bone marrow cells were isolated from the femur and tibia of WT or SHPS-1 mutant mice with the use of a syringe fitted with a 23-gauge needle (30, 31). The cells (1 10^6/ml) were seeded onto a 24-well culture plate in RPMI 1640 complete medium supplemented with GM-CSF (10 ng/ml), and the culture medium was changed every 2 days to remove granulocytes. After culture for 6–7 days, loosely adherent and clustered cells were collected as immature BMDCs. For assay of cytokine production by BMDCs (30), the cells were stimulated for 20 h with LPS (Sigma-Aldrich) at 1 g/ml or 1 M phosphorothioate oligodeoxynucleotide (ODN) with a CpG motif (CpG ODN) 1826 (5 -TCCATGACGTTCCTGACGTT-3) (Japan Bio Services) in the absence or presence of IFN- (10 ng/ml). Culture supernatants were then assayed for IL-12p70 (R&D Systems) as well as TNF- and IL-6 (BD Pharmingen) with ELISA kits. In other experiments, mature splenic CD11c DCs (1 10^5 per well of 96-well, round-bottom microplates) were cultured for 72 h in RPMI 1640 complete medium with various concentrations of IL-12. Culture supernatants were than assayed for IFN- with an ELISA kit.

Adoptive transfer

Adoptive transfer of T cells from WT mice was performed, as described previously (22). In brief, WT mice were immunized with 100 g of MOG (35–55), and, after 10 days, draining LNs were harvested for isolation of lymphocytes. The cells were cultured with MOG (35–55) (10 g/ml) and IL-12 (5 ng/ml) for 4 days, washed with PBS, and resuspended in PBS for transfer. WT or SHPS-1 mutant mice were injected i.v. with the lymphocytes (1 10^7) as well as with 200 ng of pertussis toxin (both immediately and 2 days after cell transfer). The animals were observed daily, and neurological effects were quantified, as described above.

4tatistical analysis

Data are presented as means SE and were analyzed by Student's *t* test with the use of Stat View 5.0 software (SAS Institute). A *p* value of 0.05 was considered statistically significant.

Results

Resistance of 4) P4-1 mutant mice to EAE

We first examined the susceptibility of the SHPS-1 mutant mice to EAE, an animal model of multiple sclerosis. EAE was induced by immunizing mice; those were backcrossed onto the C57BL/6 background for five or six generations, with MOG (35–55) (32, 33). Fourteen of 15 (93%) WT mice developed typical EAE 14 days after immunization with MOG (35–55) and pertussis toxin. In marked contrast, none of the 15 similarly immunized SHPS-1 mutant mice developed EAE. The time course and severity of EAE in WT mice compared with data for SHPS-1 mutant mice in a typical experiment are shown in Fig. 1*A*. Histological analysis of the spinal cord revealed pronounced infiltration of mononuclear cells around blood vessels 20 days after immunization in WT mice, whereas no such inflammatory response was observed in SHPS-1 mutant mice (Fig. 1*B*). Furthermore, we also examined the susceptibility of the SHPS-1 mutant mice to EAE by the use of mice; those were backcrossed onto the C57BL/6 background for nine generations. We found that such SHPS-1 mutant mice were also resistant to EAE, compared with WT mice (Fig. 1*C*). Thirteen of 14 (93%) WT mice developed typical EAE with MOG (35–55) and pertussis toxin. In contrast, only 4 of the 13 SHPS-1 mutant

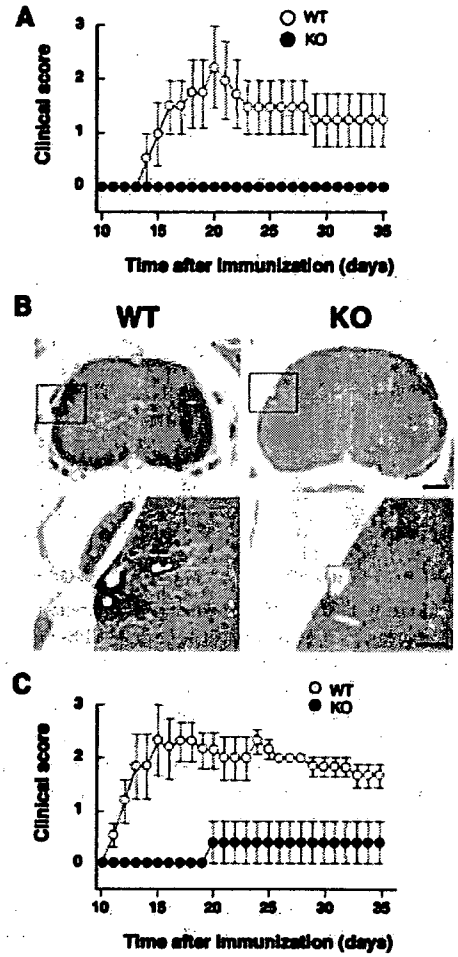

FIGURE 1. Resistance of SHPS-1 mutant mice to EAE. *A*, Time course of the clinical score of WT and SHPS-1 mutant (knockout (KO)) mice; those were backcrossed on the C57BL/6 background for five to six generations and immunized with MOG (35–55), as described in *Materials and Methods*. Data are means SE (*n* 4) and are representative of four independent experiments. *B*, Histopathology of the spinal cord of WT and SHPS-1 mutant (KO) mice 20 days after immunization with MOG (35–55). The boxed regions of the *upper panels* are shown in the *lower panels*. H&E staining shows marked infiltration of mononuclear cells around the blood vessels in WT mice, but not in the mutant mice. Scale bars: 200 m (*upper panels*) and 50 m (*lower panels*). *C*, Time course of the clinical score of WT and SHPS-1 mutant (KO) mice; those were backcrossed on the C57BL/6 background for nine generations and immunized with MOG (35–55). Data are means SE (*n* 6) and are representative of three independent experiments.

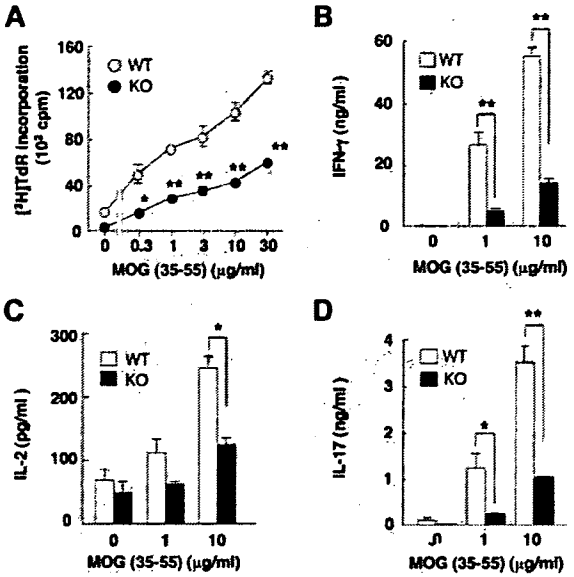

FIGURE 2. Impaired MOG (35–55)-induced proliferation of, and production of cytokines by, T cells from immunized SHPS-1 mutant mice. Lymphocytes (*A*) or splenocytes (*B–D*) prepared from WT or SHPS-1 mutant (knockout (KO)) mice 10 days after immunization with MOG (35–55) were assayed for cell proliferation (*A*) and production of IFN- (*B*), IL-2 (*C*), or IL-17 (*D*) after exposure to various concentrations of MOG (35–55). Data are means SE of triplicate determinations and are representative of three independent experiments. , p 0.05; , p 0.01 vs WT mice or for the indicated comparisons (Student's t test).

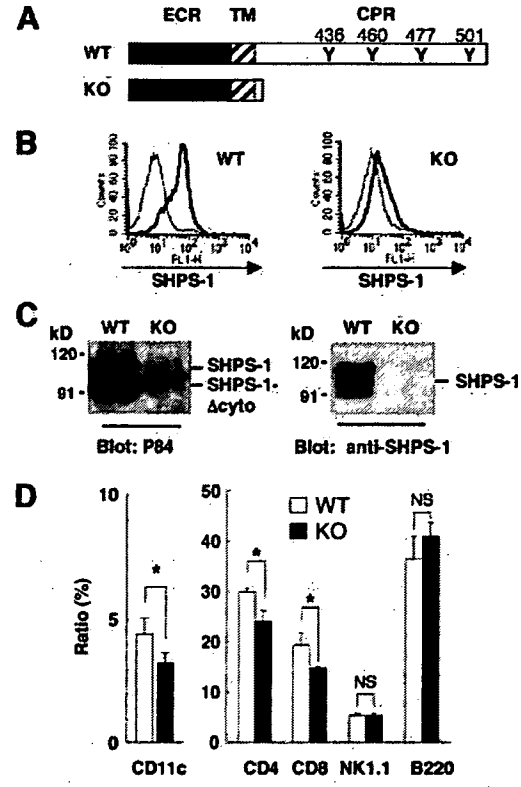

FIGURE 3. Flow cytometric and immunoblot analysis of SHPS-1 expression in CD11c DCs and reduced numbers of CD11c DCs as well as CD4 or CD8 T cells in SHPS-1 mutant mice. *A*, Schematic representations of WT and mutant (knockout (KO)) forms of mouse SHPS-1. ECR, extracellular region; TM, transmembrane region; CPR, cytoplasmic region; Four putative tyrosine (Y) phosphorylation sites in the cytoplasmic region are indicated. *B*, Immature CD11c DCs prepared from the spleen of WT or SHPS-1 mutant (KO) mice at 6 wk of age were stained with biotinylated mAb P84 (thick trace) or an isotype control mAb (thin trace) and then with FITC-conjugated streptavidin. The expression of SHPS-1 was then examined by flow cytometry. *C*, Lysates of splenic immature CD11c DCs derived from WT or SHPS-1 mutant (KO) mice at 6 wk of age were subjected to immunoblot analysis with a mAb to the extracellular region of SHPS-1 (P84) or with polyclonal Abs to the cytoplasmic region of SHPS-1 (anti-SHPS-1), as indicated. The positions of WT (SHPS-1) and mutant (SHPS-1- cyto) forms of SHPS-1 are indicated. *D*, The percentages of CD11c DCs, CD4 T cells, CD8 T cells, NK1.1 cells, and B220 B cells in the spleen of WT or SHPS-1 mutant (KO) mice at 6 wk of age were determined by flow cytometry. Data are means SE of values from six mice of each genotype. , p 0.05 for the indicated comparisons (Student's t test).

mice developed EAE, and the clinical score for such SHPS-1 mutant mice was lower than that for WT mice. We therefore next compared the MOG-specific proliferative response of and cytokine production by T cells derived from MOG-primed animals. The cell proliferation and production of IFN- in response to MOG were markedly reduced in lymphocytes derived from MOG-primed SHPS-1 mutant mice compared with the responses of WT cells (Fig. 2, *A* and *B*). The production of IL-2 in response to MOG was also reduced in splenic lymphocytes derived from MOG-primed SHPS-1 mutant mice compared with the responses of WT cells (Fig. 2*C*). In contrast, the production of IL-10 in response to MOG was minimal in splenic lymphocytes derived from MOG-primed WT or SHPS-1 mutant mice (data not shown). The production of IL-17 by MOG-primed Th17 cells was recently shown to be essential for development of EAE (34–36). The MOG-induced production of IL-17 was also greatly reduced in splenic lymphocytes derived from MOG-primed SHPS-1 mice compared with that apparent with WT cells (Fig. 2*D*). We also examined the relative titers of Abs to MOG in serum samples from MOG-primed WT and SHPS-1 mutant mice 40 days after immunization. However, no marked difference was observed in IgG, IgG1, and IgG2a titers between WT and mutant mice (data not shown).

Reduced numbers of CD11c DCs as well as CD4 or CD8 T cells in 4) P4-1 mutant mice

Induction of EAE requires T cell responses that are initiated by priming of naive CD4 T cells by DCs. SHPS-1 mutant mice express a mutant version of SHPS-1 that lacks most of the cytoplasmic region (Fig. 3*A*) (19, 21). Flow cytometry and immunoblot analysis with the P84 mAb to the extracellular region of SHPS-1 confirmed that SHPS-1 is expressed on CD11c DCs (Fig. 3, *B* and *C*). The abundance of SHPS-1 was substantially reduced in CD11c DCs from the spleen of SHPS-1 mutant mice compared with that apparent for WT cells (Fig. 3, *B* and *C*). In addition, immunoblot analysis with polyclonal Abs specific for the cytoplasmic region of SHPS-1 yielded no signal with splenic DCs from the homozygous mutant mice (Fig. 3*C*). These results suggested that the minimal susceptibility of SHPS-1 mutant mice to EAE may be attributable to a functional defect of DCs in priming of naive CD4 T cells. We therefore characterized the functions of DCs in SHPS-1 mutant mice. We first showed that the number of CD11c DCs in the spleen was reduced in the mutant animals (Fig. 3*D*), although the total number of splenic cells was not altered (data not shown) (19). Small decreases in the numbers of CD4 or CD8 T cells were also apparent in the spleen of SHPS-1 mutant mice, whereas the numbers of B220 B cells or NK1.1 NK cells (or NK T cells) did not differ between the mutant and WT

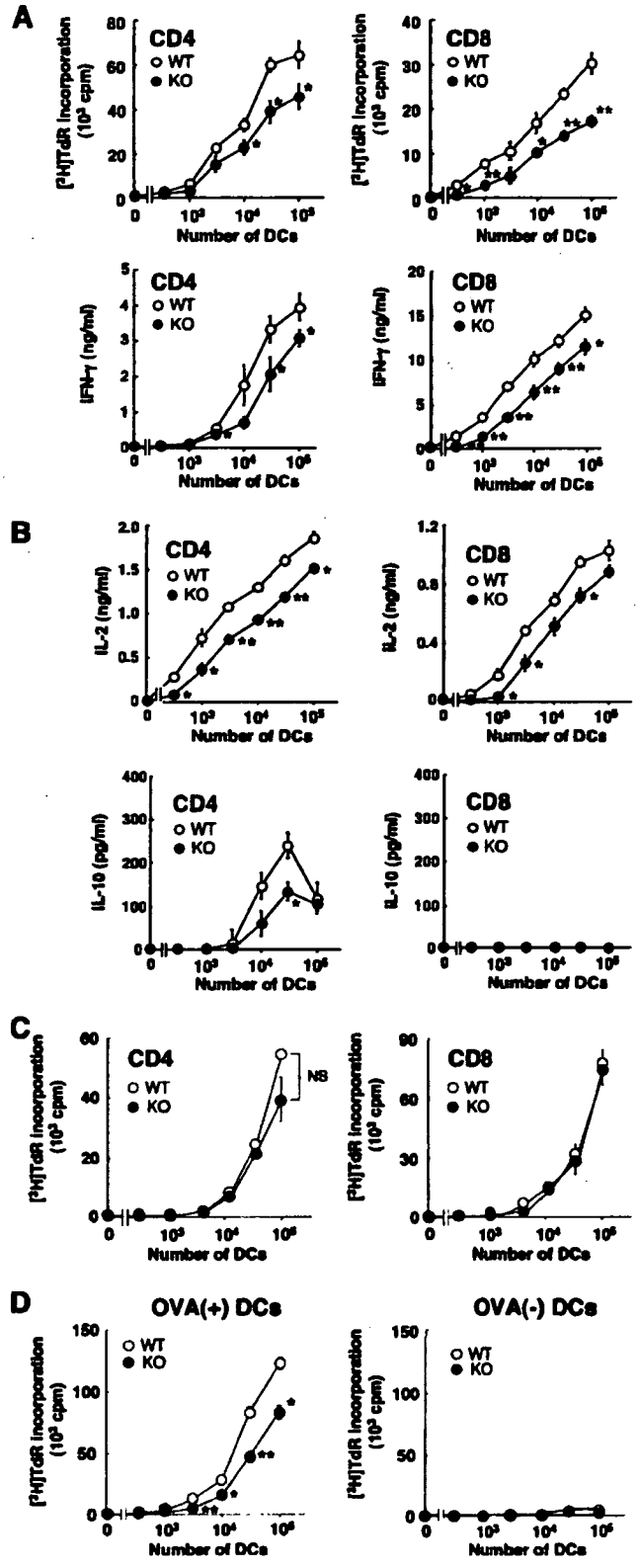

FIGURE 4. Impaired function of CD11c DCs from SHPS-1 mutant mice in an allogenic MLR and in priming of OVA-specific CD4 T cells. *A*, Cell proliferation (*upper panels*) and IFN- production (*lower panels*) were determined in MLRs with various numbers of irradiated immature CD11c DCs of WT or SHPS-1 mutant (knockout (KO)) mice and with splenic CD4 or CD8 T cells (1 10^5) from BALB/c mice. *B*, The production of IL-2 or IL-10 was determined in MLRs with various numbers of irradiated immature CD11c DCs of WT or SHPS-1 mutant (KO) mice and with splenic CD4 or CD8 T cells (1 10^5) from BALB/c mice. *C*, Cell proliferation in MLRs with various numbers of irradiated immature CD11c DCs of BALB/c mice and with splenic CD4 or CD8 T cells (1 10^5) from WT or SHPS-1 mutant mice. *D*, Immature CD11c DCs derived from the spleen of WT or SHPS-1 mutant (KO) mice injected with OVA (*left panel*) or vehicle (*right panel*) were cultured at various densities with CD4 T cells (1 10^5) from OT-II mice, after which cell proliferation was determined. Data in all panels are means SE of values from triplicate determinations and are representative of three separate experiments. , p 0.05; , p 0.01 vs WT (Student's t test).

animals (Fig. 3*D*). These data suggested that SHPS-1 regulates the number of CD11c DCs as well as those of T cells in the spleen.

Reduced abilities of DCs from 4) P4-1 mutant mice to stimulate an allogenic MLR and the proliferation of Ag-specific CD4 T cells

We next examined the ability of DCs from SHPS-1 mutant mice to stimulate alloreactive CD4 or CD8 T cells. In MLRs with CD11c DCs isolated from SHPS-1 mutant mice, the proliferation of CD4 or CD8 T cells from BALB/c mice was markedly reduced, compared with that apparent with DCs from WT mice (Fig. 4*A*). Consistent with this finding, the production of IFN- was also reduced on incubation of the CD4 or CD8 T cells with DCs from SHPS-1 mutant mice (Fig. 4*A*). In addition, the production of IL-2 was reduced on incubation of the CD4 or CD8 T cells with DCs from SHPS-1 mutant mice (Fig. 4*B*). The production of IL-10 was slightly reduced on incubation of the CD4 T cells with DCs from SHPS-1 mutant mice, whereas it was not detected on incubation of the CD8 cells with DCs from either WT or SHPS-1 mutant mice (Fig. 4*B*). In contrast, the proliferation of CD4 or CD8 T cells from SHPS-1 mutant mice was similar to that of the corresponding WT cells on incubation with DCs from BALB/c mice (Fig. 4*C*). These results suggested that DCs, but not CD4 or CD8 T cells, of SHPS-1 mutant mice are functionally defective in an allogenic MLR.

To characterize further the dysfunction of DCs in SHPS-1 mutant mice, we examined the proliferation of OVA-specific CD4 T cells (prepared from OT-II transgenic mice (37)) in response to culture with OVA-pulsed CD11c DCs. The proliferation of OVA-specific CD4 T cells cultured with DCs from SHPS-1 mutant mice was markedly impaired compared with that of those cultured with DCs from WT mice (Fig. 4*D*), suggesting that priming of Ag-specific T cells by DCs is indeed defective in SHPS-1 mutant mice.

Impaired TLR-dependent cytokine production and IL-12-induced IFN- release in DCs from 4) P4-1 mutant mice

We therefore next examined what specific activity of DCs might be impaired in SHPS-1 mutant mice. For priming of naive T cells, DCs first capture exogenous Ags and then migrate from peripheral tissues to draining LNs, where they encounter naive T cells and present MHC-peptide complexes (1, 2). During this process, DCs also mature and express multiple costimulatory molecules, such as CD80, CD86, and CD40, on their surface. They also secrete IL-12, a key cytokine that promotes the production of IFN- not only by Th1 cells, but also by DCs themselves. However, neither the migratory response of, nor the uptake of FITC-labeled BSA by, BMDCs differed substantially between SHPS-1 mutant and WT mice (Fig. 5, *A* and *B*). In addition, the up-regulation of surface molecules, including MHC class I and II as well as CD80, CD86, and CD40, on splenic CD11c DCs during their maturation also appeared similar for WT and SHPS-1 mutant mice (Fig. 5*C*).

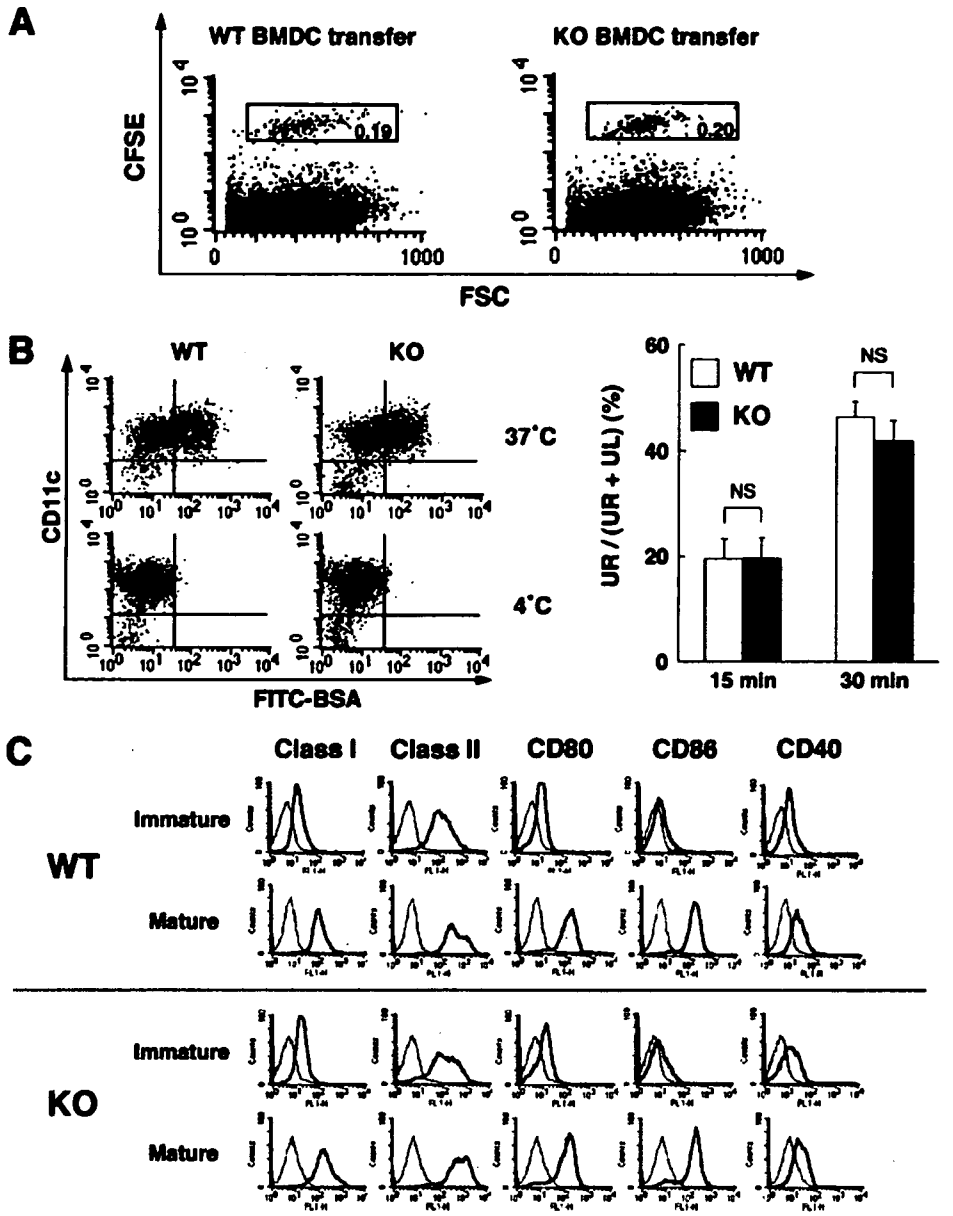

FIGURE 5. Migration, Ag uptake, and differentiation in DCs from WT and SHPS-1 mutant mice. *A*, BMDCs derived from WT or SHPS-1 mutant (knockout (KO)) mice were labeled with 10 M CFSE for 10 min at 37°C in RPMI 1640, as described (31), washed three times, and then resuspended (5 10^6 cells in 40 l) in PBS and injected into a hind footpad of WT mice. Three days after injection, lymphocytes were isolated from the draining LNs and subjected to flow cytometric analysis for determination of the percentage of cells labeled with CFSE (gated area). Data are from a representative experiment. The mean SE values for the percentage of migrating cells were 0.17 0.01 and 0.11 0.05 for WT and SHPS-1 mutant mice, respectively (n 3, p 0.05). FSC, forward scatter. *B*, BMDCs from WT or SHPS-1 mutant (KO) mice were incubated with FITC-BSA (5 g/ml) (Sigma-Aldrich) at 37°C or 4°C (control) for 15 or 30 min, washed, and then stained with biotin-conjugated Abs to mouse CD11c and PE-conjugated streptavidin. FITC-BSA uptake by CD11c DCs was then monitored by flow cytometry. Data shown in the *left panels* were obtained with cells incubated for 30 min and are representative of three separate experiments. The uptake of FITC-BSA by CD11c DCs at 15 or 30 min and 37°C was also determined as the percentage of CD11c DCs that had incorporated FITC-BSA (cells in the *upper right* (UR) *quadrant*) among total CD11c DCs (sum of the cells in the *upper right* and *upper left* (UL) *quadrants*), as shown in the *right panel*; data are means SE of values from three separate experiments. *C*, Immature or mature CD11c DCs prepared from the spleen of WT or SHPS-1 mutant (KO) mice were incubated first with biotin-conjugated mAbs to mouse MHC class I, MHC class II, CD80, CD86, or CD40 (thick traces), or with an isotype control mAb (thin traces) and then with FITC-conjugated streptavidin. They were then analyzed by flow cytometry. Data are representative of three separate experiments.

We then evaluated the TLR-dependent production of cytokines by BMDCs. The production of 70-kDa heterodimeric IL-12 (IL-12p70) in response to either LPS or CpG ODN was slightly impaired in BMDCs from SHPS-1 mutant mice (Fig. 6*A*). Moreover, the production of TNF- and IL-6 in response to either LPS or CpG ODN was markedly impaired in BMDCs from the mutant animals (Fig. 6*A*). The IL-12-induced production of IFN- by DCs is important for DC functions (25, 26, 38). We found that the IL-12-induced production of IFN- in splenic CD11c DCs from SHPS-1 mutant mice was greatly reduced compared with that

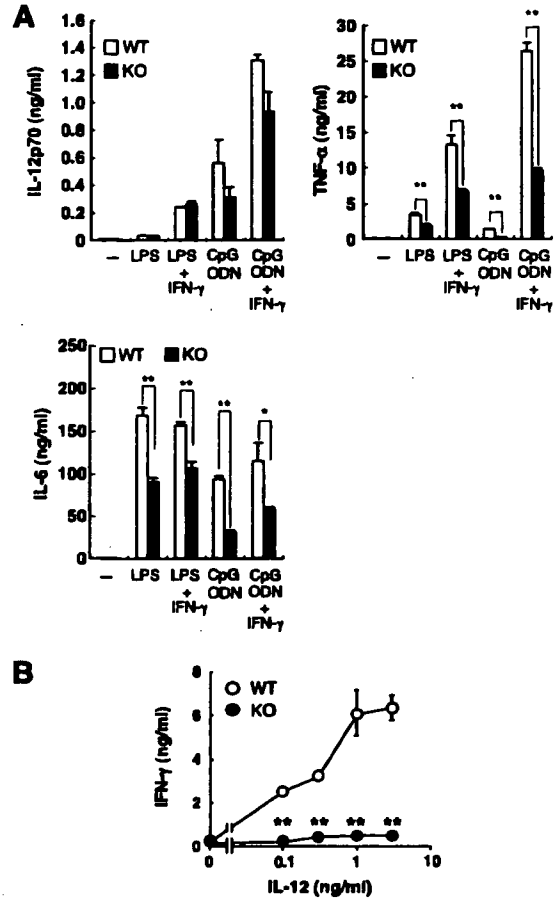

FIGURE 6. Impaired TLR-dependent cytokine production and IL-12-induced IFN- production in DCs from SHPS-1 mutant mice. *A*, BMDCs from WT or SHPS-1 mutant (knockout (KO)) mice were stimulated with LPS (1 g/ml) or CpG ODN (1 M) in the absence or presence of IFN- (10 ng/ml) for 20 h, after which the levels of the indicated cytokines in culture supernatants were determined. *B*, Mature CD11c DCs from the spleen of WT or SHPS-1 mutant (KO) mice were cultured with various concentrations of IL-12 for 72 h, after which the concentration of IFN- in culture supernatants was determined. All data are means SE of values from triplicate determinations and are representative of three separate experiments. , p 0.05; , p 0.01 vs WT or for the indicated comparisons (Student's t test).

apparent with WT cells (Fig. 6*B*). These data thus suggested that SHPS-1 is essential for TLR-dependent cytokine production as well as the IL-12-induced production of IFN- by DCs.

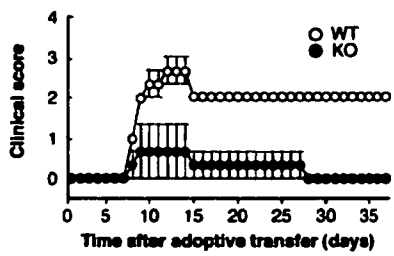

FIGURE 7. Impaired induction of EAE in SHPS-1 mutant mice by adoptive transfer of T cells from WT donor mice. Time course of the clinical score of WT or SHPS-1 mutant (knockout (KO)) mice injected with MOG-specific lymphocytes prepared from WT mice, as described in *Materials and Methods*. Data are means SE (n 3) and are representative of three separate experiments.

Impaired induction of EAE in 4) P4-1 mutant mice by adoptive transfer of T cells from WT donor mice

Given that development of EAE was blocked in SHPS-1 mutant mice, it was possible that these animals have a defect in the effector phase of this condition as well as in the priming of T cells by DCs. We therefore performed adoptive transfer of T cells derived from MOG-primed WT mice into either WT or SHPS-1 mutant mice. Adoptive transfer of MOG-primed T cells from WT donor mice induced EAE in WT mice, but this effect was markedly diminished in SHPS-1 mutant mice (Fig. 7).

DiscussioO

We have shown in this study that SHPS-1 mutant mice are resistant to EAE. Moreover, the MOG-induced proliferation of and production of IFN- as well as of IL-2 by T cells from immunized SHPS-1 mutant mice were markedly reduced compared with those apparent for WT cells. It was recently shown that IL-17 produced by Th17 cells in response to TGF- , IL-6, or IL-23 is essential for development of EAE (34, 35). Indeed, IL-17-deficient mice also manifest minimal susceptibility to EAE (36). We found that the MOG-induced production of IL-17 by T cells from immunized SHPS-1 mutant mice was reduced compared with that apparent for WT cells. Induction of EAE requires T cell responses that are initiated by priming of naive CD4 T cells by DCs. Given that SHPS-1 is expressed selectively in DCs, these results suggest that the resistance of SHPS-1 mutant mice to EAE is attributable to a defect in priming of CD4 T cells by DCs.

This notion was further supported by the observations that, in an allogenic MLR with CD11c DCs from SHPS-1 mutant mice, the proliferation of and production of IFN- by CD4 or CD8 T cells from BALB/c mice were markedly reduced, compared with those apparent with DCs from WT mice. Furthermore, the proliferation of OVA-specific CD4 T cells primed with DCs from SHPS-1 mutant mice was markedly impaired compared with that of those primed with DCs from WT mice. These results thus suggest that priming of either allogenic or Ag-specific T cells by DCs from SHPS-1 mutant mice is indeed defective. The interaction of SHPS-1 on macrophages with CD47 on RBCs prevents phagocytosis of RBCs by macrophages in a manner dependent on SHP-1, which binds the cytoplasmic region of SHPS-1 (17–19, 39). Moreover, SHPS-1 on DCs, through its interaction with CD47 on T cells, prevents activation of DCs (15), suggesting that SHPS-1 negatively regulates functions of macrophages and DCs. However, our present results demonstrate a positive regulatory role for SHPS-1 in efficient priming by DCs of naive T cells both in vivo and in vitro.

We investigated what specific function of DCs is actually impaired in SHPS-1 mutant mice. The abilities to migrate and to take up Ag were not markedly impaired in BMDCs from SHPS-1 mutant mice. In contrast, the TLR-dependent production of cytokines, including TNF- and IL-6, was defective in BMDCs from the mutant animals. Moreover, the IL-12-stimulated production of IFN- by DCs from SHPS-1 mutant mice was greatly impaired compared with that in WT cells. This effect of IL-12 is thought to be important for priming and activation of naive T cells by DCs (25, 26, 38). The impairment in priming of T cells by DCs of SHPS-1 mutant mice may thus be attributable, at least in part, to the defects in IL-12-induced production of IFN- and in TLR-dependent cytokine production in DCs. The molecular mechanism by which SHPS-1 positively regulates the effect of IL-12 on IFN- production by DCs remains unknown. The JAK2-STAT4 signaling pathway and the p38 isoform of MAPK are implicated in this effect of IL-12 (26, 40). Given that SHP-2 positively regulates

activation of the JAK-STAT pathway and MAPK signaling (41) and complex formation of SHPS-1 with SHP-2 is specifically defective in the SHPS-1 mutant mice, it is possible that the SHPS-1–SHP-2 complex positively regulates the action of IL-12 as well as that of other related cytokines that activate the JAK-STAT pathway and p38 MAPK in DCs.

Interaction of SHPS-1 (on DCs) with CD47 (on T cells) may contribute to the activation of T cells in a manner functionally similar to that apparent for costimulatory molecules on DCs such as B7 (which interacts with CD28 on T cells) and OX40 ligand (which interacts with OX40 on T cells). Mice deficient in either B7 or OX40 ligand indeed manifest a reduced susceptibility to EAE (22, 42). In addition, it was recently shown that delayed-type hypersensitivity to 2,4-dinitro-1-fluorobenzene, which is also thought to be mediated by Th1 cells, is markedly diminished in CD47-deficient mice (43). Conversely, we found that such delayed-type hypersensitivity is greatly reduced in extent in SHPS-1 mutant mice (44). Moreover, ligation of CD47 on T cells is thought to generate costimulatory signals for T cell activation (45). Together with the present results, these observations suggest a notion that interaction of SHPS-1 (on DCs) with its ligand CD47 (on T cells) is essential for priming of T cells by DCs.

We showed that induction of EAE by adoptive transfer of MOG-specific T cells from WT donor mice was impaired in SHPS-1 mutant mice, suggesting that these animals have a defect in the effector phase of EAE development as well as in the priming of T cells by DCs. It is possible that the production of chemokines or cytokines from various cell types, including macrophages and neutrophils, in the effector phase of EAE is impaired in SHPS-1 mutant mice. Secondary priming of T cells by APCs surrounding blood vessels of the CNS is also important for the development of EAE (46, 47). The reduced effect of adoptive transfer of MOG-specific T cells in SHPS-1 mutant mice may thus be attributable to a defect in secondary priming of the transferred T cells by host DCs.

Overall, our present study clearly indicates that SHPS-1 positively regulates the priming of T cells by DCs, and hence, that this protein is essential for the development of EAE. We found that SHPS-1 is required for multiple functions of DCs, including the IL-12-induced production of IFN- as well as the TLR-dependent production of cytokines by these cells. Our results also implicate SHPS-1 on DCs in the activation of T cells (presumably through interaction with CD47 on T cells) in a manner functionally similar to that apparent for other costimulatory molecules on DCs. We have recently shown that SHPS-1 mutant mice also manifest minimal susceptibility to collagen-induced arthritis, an animal model of rheumatoid arthritis (C. Okuzawa, Y. Kaneko, and T. Matozaki, unpublished observation), suggesting that SHPS-1 may contribute to development of various inflammatory autoimmune diseases. Given that the function of SHPS-1 is susceptible to regulation by ligands such as Abs, this protein is a potential therapeutic target for inflammatory autoimmune diseases in general.

AckOwledgmeOts

We thank C. F. Lagenaur for the mAb to SHPS-1; K. Okumura for the mAbs to CD16/32; D. Kamimura, M. Murakami, and T. Hirano for OT-II mice; S. Koyasu and T. Takai for suggestions and discussion; as well as A. Morita, Y. Niwayama, Y. Hayashi, and R. Koitabashi for technical assistance.

Disclosures

The authors have no financial conflict of interest.

RefereOces

1. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *NatuSe* 392: 245–252.
2. Moser, M., and K. M. Murphy. 2000. Dendritic cell regulation of T_H1-T_H2 development. *Nat. Immunol.* 1: 199–205.
3. Crow, M. K. 2006. Modification of accessory molecule signaling. *4pSnHŁS 4e-min. Immunopathol.* 27: 409–424.
4. Fujioka, Y., T. Matozaki, T. Noguchi, A. Iwamatsu, T. Yamao, N. Takahashi, M. Tsuda, T. Takada, and M. Kasuga. 1996. A novel membrane glycoprotein, SHPS-1, that binds the SH2-domain-containing protein tyrosine phosphatase SHP-2 in response to mitogens and cell adhesion. *Mol. Cell. Biol.* 16: 6887–6899.
5. Kharitonenkov, A., Z. Chen, I. Sures, H. Wang, J. Schilling, and A. Ullrich. 1997. A family of proteins that inhibit signalling through tyrosine kinase receptors. *NatuSe* 386: 181–186.
6. Van Beek, E. M., F. Cochrane, A. N. Barclay, and T. K. van den Berg. 2005. Signal regulatory proteins in the immune system. *J. Immunol.* 175: 7781–7787.
7. Ohnishi, H., M. Kubota, A. Ohtake, K. Sato, and S. Sano. 1996. Activation of protein-tyrosine phosphatase SH-PTP2 by a tyrosine-based activation motif of a novel brain molecule. *J. Biol. Chem.* 271: 25569–25574.
8. Timms, J. F., K. Carlberg, H. Gu, H. Chen, S. Kamatkar, M. J. Nadler, L. R. Rohrschneider, and B. G. Neel. 1998. Identification of major binding proteins and substrates for the SH2-containing protein tyrosine phosphatase SHP-1 in macrophages. *Mol. Cell. Biol.* 18: 3838–3850.
9. Oshima, K., A. R. Ruhul Amin, A. Suzuki, M. Hamaguchi, and S. Matsuda. 2002. SHPS-1, a multifunctional transmembrane glycoprotein. *FEB4 Lett.* 519: 1–7.
10. Jiang, P., C. F. Lagenaur, and V. Narayanan. 1999. Integrin-associated protein is a ligand for the P84 neural adhesion molecule. *J. Biol. Chem.* 274: 559–562.
11. Seiffert, M., C. Cant, Z. Chen, I. Rappold, W. Brugger, L. Kanz, E. J. Brown, A. Ullrich, and H. J. Buhring. 1999. Human signal-regulatory protein is expressed on normal, but not on subsets of leukemic myeloid cells and mediates cellular adhesion involving its counterreceptor CD47. *Blood* 94: 3633–3643.
12. Brown, E. J., and W. A. Frazier. 2001. Integrin-associated protein (CD47) and its ligands. *TSendT Cell Biol.* 11: 130–135.
13. Adams, S., L. J. van der Laan, E. Vernon-Wilson, C. Renardel de Lavalette, E. A. Dopp, C. D. Dijkstra, D. L. Simmons, and T. K. van den Berg. 1998. Signal-regulatory protein is selectively expressed by myeloid and neuronal cells. *J. Immunol.* 161: 1853–1859.
14. Veillette, A., E. Thibaudeau, and S. Latour. 1998. High expression of inhibitory receptor SHPS-1 and its association with protein-tyrosine phosphatase SHP-1 in macrophages. *J. Biol. Chem.* 273: 22719–22728.
15. Latour, S., H. Tanaka, C. Demeure, V. Mateo, M. Rubio, E. J. Brown, C. Maliszewski, F. P. Lindberg, A. Oldenborg, A. Ullrich, et al. 2001. Bidirectional negative regulation of human T and dendritic cells by CD47 and its cognate receptor signal-regulator protein- : down-regulation of IL-12 responsiveness and inhibition of dendritic cell activation. *J. Immunol.* 167: 2547–2554.
16. Seiffert, M., P. Brossart, C. Cant, M. Cella, M. Colonna, W. Brugger, L. Kanz, A. Ullrich, and H. J. Buhring. 2001. Signal-regulatory protein (SIRP) but not SIRP is involved in T-cell activation, binds to CD47 with high affinity, and is expressed on immature CD34 CD38 hematopoietic cells. *Blood* 97: 2741–2749.
17. Oldenborg, P. A., A. Zheleznyak, Y. F. Fang, C. F. Lagenaur, H. D. Gresham, and F. P. Lindberg. 2000. Role of CD47 as a marker of self on red blood cells. *4cience* 288: 2051–2054.
18. Okazawa, H., S. Motegi, N. Ohyama, H. Ohnishi, T. Tomizawa, Y. Kaneko, P. A. Oldenborg, O. Ishikawa, and T. Matozaki. 2005. Negative regulation of phagocytosis in macrophages by the CD47-SHPS-1 system. *J. Immunol.* 174: 2004–2011.
19. Ishikawa-Sekigami, T., Y. Kaneko, H. Okazawa, T. Tomizawa, J. Okajo, Y. Saito, C. Okuzawa, M. Sugawara-Yokoo, U. Nishiyama, H. Ohnishi, et al. 2006. SHPS-1 promotes the survival of circulating erythrocytes through inhibition of phagocytosis by splenic macrophages. *Blood* 107: 341–348.
20. Alblas, J., H. Honing, C. R. de Lavalette, M. H. Brown, C. D. Dijkstra, and T. K. van den Berg. 2005. Signal regulatory protein ligation induces macrophage nitric oxide production through JAK/STAT- and phosphatidylinositol 3-kinase/Rac1/NAPDH oxidase/H_2O_2-dependent pathways. *Mol. Cell. Biol.* 25: 7181–7192.
21. Inagaki, K., T. Yamao, T. Noguchi, T. Matozaki, K. Fukunaga, T. Takada, T. Hosooka, S. Akira, and M. Kasuga. 2000. SHPS-1 regulates integrin-mediated cytoskeletal reorganization and cell motility. *EMBO J.* 19: 6721–6731.
22. Chang, T. T., C. Jabs, R. A. Sobel, V. K. Kuchroo, and A. H. Sharpe. 1999. Studies in B7-deficient mice reveal a critical role for B7 costimulation in both induction and effector phases of experimental autoimmune encephalomyelitis. *J. Exp. Med.* 190: 733–740.
23. Bakker, A. B., R. M. Hoek, A. Cerwenka, B. Blom, L. Lucian, T. McNeil, R. Murray, L. H. Phillips, J. D. Sedgwick, and L. L. Lanier. 2000. DAP12-deficient mice fail to develop autoimmunity due to impaired antigen priming. *Immunity* 13: 345–353.
24. Chitnis, T., N. Najafian, C. Benou, A. D. Salama, M. J. Grusby, M. H. Sayegh, and S. J. Khoury. 2001. Effect of targeted disruption of STAT4 and STAT6 on the induction of experimental autoimmune encephalomyelitis. *J. Clin. InveTt.* 108: 739–747.
25. Fukao, T., S. Matsuda, and S. Koyasu. 2000. Synergistic effects of IL-4 and IL-18 on IL-12-dependent IFN- production by dendritic cells. *J. Immunol.* 164: 64–71.
26. Fukao, T., D. M. Frucht, G. Yap, M. Gadina, J. J. O'Shea, and S. Koyasu. 2001. Inducible expression of Stat4 in dendritic cells and macrophages and its critical role in innate and adaptive immune responses. *J. Immunol.* 166: 4446–4455.
27. Fang, D., C. Elly, B. Gao, N. Fang, Y. Altman, C. Joazeiro, T. Hunter, N. Copeland, N. Jenkins, and Y. C. Liu. 2002. Dysregulation of T lymphocyte

function in itchy mice: a role for itch in T$_H$2 differentiation. *Nat. Immunol.* 3: 281o287.
28. Kumanogoh, A., T. Shikina, K. Suzuki, S. Uematsu, K. Yukawa, S. Kashiwamura, H. Tsutsui, M. Yamamoto, H. Takamatsu, E. P. Ko-Mitamura, et al. 2005. Nonredundant roles of Sema4A in the immune system: defective T cell priming and Th1/Th2 regulation in Sema4A-deficient mice. *Immunity* 22: 305o316.
29. Li, M., G. M. Davey, R. M. Sutherland, C. Kurts, A. M. Lew, C. Hirst, F. R. Carbone, and W. R. Heath. 2001. Cell-associated ovalbumin is cross-presented much more efficiently than soluble ovalbumin in vivo. *J. Immunol.* 166: 6099o6103.
30. Fukao, T., M. Tanabe, Y. Terauchi, T. Ota, S. Matsuda, T. Asano, T. Kadowaki, T. Takeuchi, and S. Koyasu. 2002. PI3K-mediated negative feedback regulation of IL-12 production in DCs. *Nat. Immunol.* 3: 875o881.
31. Ujike, A., K. Takeda, A. Nakamura, S. Ebihara, K. Akiyama, and T. Takai. 2002. Impaired dendritic cell maturation and increased T$_H$2 responses in PIR-B $^{-/-}$ mice. *Nat. Immunol.* 3: 542o548.
32. Pedotti, R., J. J. De Voss, L. Steinman, and S. J. Galli. 2003. Involvement of both Allergic' and Autoimmune' mechanisms in EAE, MS and other autoimmune diseases. *TSendT Immunol.* 24: 479o484.
33. Sospedra, M., and R. Martin. 2005. Immunology of multiple sclerosis. *Annu. Rev. Immunol.* 23: 683o747.
34. Park, H., Z. Li, 9. O. Yang, S. H. Chang, R. Nurieva, Y. H. Wang, Y. Wang, L. Hood, Z. Zhu, 2. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* 6: 1133o1141.
35. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner, and V. K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector T$_H$ 17 and regulatory T cells. *NatuSe* 441: 235o238.
36. Komiyama, Y., S. Nakae, T. Matsuki, A. Nambu, H. Ishigame, S. Kakuta, K. Sudo, and Y. Iwakura. 2006. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J. Immunol.* 177: 566o573.
37. Barnden, M. J., J. Allison, W. R. Heath, and F. R. Carbone. 1998. Defective TCR expression in transgenic mice constructed using cDNA-based - and -chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol.* 76: 34o40.
38. Frucht, D. M., T. Fukao, C. Bogdan, H. Schindler, J. J. O'Shea, and S. Koyasu. 2001. IFN- production by antigen-presenting cells: mechanisms emerge. *TSendT Immunol.* 22: 556o560.
39. Oldenborg, P. A., H. D. Gresham, and F. P. Lindberg. 2001. CD47-signal regulatory protein (SIRP) regulates Fc and complement receptor-mediated phagocytosis. *J. Exp. Med.* 193: 855o862.
40. Watford, W. T., B. D. Hissong, J. H. Bream, Y. Kanno, L. Muul, and J. J. O'Shea. 2004. Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4. *Immunol. Rev.* 202: 139o156.
41. Gadina, M., L. M. Stancato, C. M. Bacon, A. C. Larner, and J. J. O'Shea. 1998. Involvement of SHP-2 in multiple aspects of IL-2 signaling: evidence for a positive regulatory role. *J. Immunol.* 160: 4657o4661.
42. Ndhlovu, L. C., N. Ishii, K. Murata, T. Sato, and K. Sugamura. 2001. Critical involvement of O9 40 ligand signals in the T cell priming events during experimental autoimmune encephalomyelitis. *J. Immunol.* 167: 2991o2999.
43. Hagnerud, S., P. P. Manna, M. Cella, A. Stenberg, W. A. Frazier, M. Colonna, and P. A. Oldenborg. 2006. Deficit of CD47 results in a defect of marginal zone dendritic cells, blunted immune response to particulate antigen and impairment of skin dendritic cell migration. *J. Immunol.* 176: 5772o5778.
44. Fukunaga, A., H. Nagai, 9. Yu, S. Oniki, H. Okazawa, S. Motegi, R. Suzuki, N. Honma, T. Matozaki, C. Nishigori, and T. Horikawa. 2006. Src homology 2 domain-containing protein tyrosine phosphatase substrate 1 regulates the induction of Langerhans cell maturation. *EuS. J. Immunol.* 36: 3216o3226.
45. Reinhold, M. I., F. P. Lindberg, G. J. Kersh, P. M. Allen, and E. J. Brown. 1997. Costimulation of T cell activation by integrin-associated protein (CD47) is an adhesion-dependent, CD28-independent signaling pathway. *J. Exp. Med.* 185: 1o11.
46. Greter, M., F. L. Heppner, M. P. Lemos, B. M. Odermatt, N. Goebels, T. Laufer, R. J. Noelle, and B. Becher. 2005. Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. *Nat. Med.* 11: 328o334.
47. Heppner, F. L., M. Greter, D. Marino, J. Falsig, G. Raivich, N. Hovelmeyer, A. Waisman, T. Rulicke, M. Prinz, J. Priller, et al. 2005. Experimental autoimmune encephalomyelitis repressed by microglial paralysis. *Nat. Med.* 11: 146o152.