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Functional Blocking Monoclonal Antibodies against IL-12p40 Homodimer Inhibit Adoptive Transfer of Experimental Allergic Encephalomyelitis

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IL-12p70 (p40:p35) and IL-23 (p40:p19) are bioactive cytokines and their role in experimental allergic encephalomyelitis (EAE), an animal model for multiple sclerosis, are becoming clear. On the other hand, the IL-12p40 homodimer (p402) was considered as an inactive or inhibitory molecule and its functions are poorly understood. To facilitate the studies on p402, we have recently generated neutralizing mAb against mouse p402. The present study demonstrates the effectiveness of p402 mAb in treating the disease process of relapsing-remitting EAE in female SJL/J mice. The p402 mAb ameliorated clinical symptoms and disease progression of EAE in recipient mice and suppressed the generation of encephalitogenic T cells in donor mice. Histological and blood-brain barrier (BBB) and blood-spinal cord barrier (BSB) permeability studies reveal that p402 mAb effectively inhibited the infiltration of mononuclear cells into brain and spinal cord and improved the integrity of BBB and BSB in EAE mice. Consequently, p402 mAb also suppressed the expression of proinflammatory molecules, normalized the expression of myelin genes, and blocked demyelination in the CNS of EAE mice. On the other hand, recombinant mouse p402 increased the infiltration of mononuclear cells into the CNS, enhanced the permeability through BBB and BSB, stimulated CNS expression of proinflammatory molecules, and aggravated the disease process of EAE. Taken together, our results suggest that p402 participates in the pathogenesis of EAE and that neutralization of p402 may be beneficial in multiple sclerosis patients. The Journal of Immunology, 2009, 182: 5013–5023.

Multiple sclerosis (MS) is the most common human demyelinating disease of the CNS. Although the etiology is poorly understood, several studies on MS patients suggest that it is a T cell-mediated autoimmune response (1). Experimental allergic encephalomyelitis (EAE) is an animal model of MS. Adoptively transferred EAE mimics the relapsing-remitting MS, the most common form of MS found in patients. In this model, neuroantigen-specific autoimmune T cells first contact a naïve intact blood-brain barrier (BBB) and are able to extravasate through the BBB due to their activated status. These cells are retained in the CNS due to presentation of appropriate Ag and undergo further activation (2). This is followed by the recruitment of non-Ag-specific lymphocytes and activated macrophages from the blood into this site, accompanied by activation of resident glial cells and further disruption of the BBB. Detection of a wide variety of proinflammatory molecules, such as proinflammatory cytokines (IL-1β, IFN-γ, IL-6, and TNF-α), proinflammatory chemokines (MCP-1/CCL2 and IP-10/CXCL10), proinflammatory enzymes (inducible NO synthase (iNOS), cyclooxygenase 2), and proinflammatory transcription factors (NF-κB and C/EBP) in CNS leukocytes during the inflammation process (3–7).

IL-12 plays a critical role in the early inflammatory response to infection and in the generation of Th1 cells (8). IL-12 consists of a H chain (p40) and an L chain (p35) linked covalently by disulfide bonds to give rise to the so-called bioactive heterodimeric (p70) molecule (9, 10). Recently, p40 has been shown to pair with p19 to form a newly discovered cytokine, IL-23. IL-23 has biological functions that are similar to as well as distinct from IL-12. For example, similar to IL-12, IL-23 also enhances the proliferation of Th1 cells and increases their IFN-γ production (11–13). However, in contrast to IL-12, IL-23 aids in the proliferation of memory T cells (11–13). Apart from forming heterodimers (IL-12 and IL-23), the p40 subunit is also secreted as monomer (p40) and homodimer (p402) (9). Because all of these cytokines (IL-12, IL-23, p40, and p402) contain the common p40 subunit, these cytokines can better be grouped into the p40 family of cytokines.

Due to the fact that MS and its animal model EAE are T cell-mediated autoimmune diseases and that IL-12 is capable of inducing T cell activation and Th1 differentiation, IL-12 has long been considered essential in MS and EAE (14, 15). For example, IL-12 treatment increased the severity of EAE induced by adoptive transfer of proteolipid protein (PLP)-primed lymph node cells in mice (16, 17). Furthermore, an Ab to IL-12 prevented the induction or progression of disease in a murine model of relapsing-remitting EAE (18, 19). However, recent data demonstrate that the so-called important role of IL-12 in CNS inflammatory demyelination is...
actually due to IL-23 (12, 14). According to Cua et al. (12), p19 ("p") mice do not develop EAE.

In contrast, the role of p40* and p40 in the disease process of EAE is not known. It was known that p40* was inhibitory to bioactive cytokine IL-12 and/or biologically inactive until we demonstrated the induction of NO synthase (iNOS) and TNF-α by p40* in microglia and macrophages (20, 21). We have recently demonstrated that p40*, but not IL-12p70, induces the expression of IL-16, a leukocyte chemotractant factor, in microglia and macrophages (22). Among various stimuli tested, p40* has been found as the most potent one followed by p40 monomer, IL-16, and IL-23 in inducing the activation of IL-16 promoter in microglial cells (22). Furthermore, we have also reported that p40*, but not IL-12p70, is capable of inducing the expression of lymphotoxin α in various immune cells (23). Among various stimuli (p40*, IL-12p70, IL-23, TNF-α, IFN-γ, IL-1β, LPS, dsRNA, HIV-1 gp120, and IL-16) tested, p40* has emerged as the most potent followed by IL-16, LPS, and dsRNA in inducing the activation of LI-α promoter in microglial cells (23). It is often quite straightforward to consider a knockout mouse model to investigate the role of a candidate molecule in any disease process. However, p40 cannot be used in this case because knocking out the p40 gene will knock out IL-12, IL-23, p40*, and p40. Therefore, to investigate the role of p40* and p40 in EAE, the only feasible approach is to use neutralizing mAb against these molecules.

Recently, we have generated neutralizing mAbs against mouse p40*, and established a sandwich ELISA to quantify p40* (24). By direct ELISA, we have demonstrated that Abs produced from clones a3-1d and d7-12c specifically recognize p40*, but not p40, IL-12, and IL-23 (24). In this study, we demonstrate that the level of p40* goes up in serum, spleen, brain, and spinal cord of EAE mice and that p40* mAb a3-1d attenuates clinical symptoms of EAE by reducing perivascular cuffing, inflammation, and demyelination in adoptively transferred EAE mice. On the other hand, recombinant p40* aggravated the disease process of EAE by increasing perivascular cuffing. These results raise a possibility that neutralization of p40* may be a therapeutic step in MS.

Materials and Methods

Reagents

Bovine myelin basic protein (MBP), t-glutamine, and 2-ME were obtained from Invitrogen. FBS and RPMI 1640 were from Mediatech. Heat-killed Mycobacterium tuberculosis (H37RA) was purchased from Difco. IFA was obtained from Calbiochem. Solvent Blue 38, cresyl violet acetate, and lithium carbonate were purchased from Sigma-Aldrich.

Induction of EAE

Specific pathogen-free female SJL/J mice (3–4 wk old) were purchased from Harlan Sprague Dawley. EAE was elicited by passive transfer of MAb-reactive T cells as described earlier (25–27). Donor mice were immunized s.c. with 400 μg of bovine MBP and 60 μg of M. tuberculosis in IFA. Animals were killed 10–12 days after immunization, and the draining lymph nodes were harvested. Single-cell suspensions were treated with RBC lysis buffer (Sigma-Aldrich), washed, and cultured at a concentration of 4–5 × 10⁶ cells/ml in 6-well plates in RPMI 1640 supplemented with 10% FBS, 50 μg/ml MBP, 50 μM 2-ME, 2 mM t-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. On day 4, cells were harvested and resuspended in HBSS. A total of 2 × 10⁵ viable cells in a volume of 200 μl was injected into the tail vein of naive mice. Pertussis toxin (150 μg/mouse; Sigma-Aldrich) was injected once via i.p. route on day 0 posttransfer (dpt) of cells. Cells isolated from donor mice immunized with CFA or IFA alone were not viable after 4 days in culture with MBP and therefore were not transferred. Animal maintenance and experimental protocols were approved by the Rush University Medical Center. Animals were observed daily for clinical symptoms. Experimental animals were scored by a masked investigator as follows: 0, no clinical disease; 0.5, piloerection; 1, tail weakness; 1.5, tail paralysis; 2, hind limb weakness; 3, hind limb paralysis; 3.5, forelimb weakness; 4, forelimb paralysis; and 5, moribund or death.

Treatment with recombinant p40* and Abs against p40*

mAbs against p40* (clone a3-1d) were concentrated from hybridoma supernatants by centrifuging in an Amicon Ultra-15 (M, 50,000 compound) centrifugal filter device (Millipore) and purified by protein A-Sepharose (Bio-Rad) as described by us (24). Either from the beginning or from different stages (onset of acute phase and onset of chronic phase) of EAE, groups of mice were treated with either recombinant mouse p40* (carrier free) or mAb a3-1d via i.p. injection. Controls received normal PBS. Statistical analysis was determined by the RS/1 multicomparison procedure using a one-way ANOVA and Dunnett’s test for multiple comparisons with a common control group. Differences between means were considered significant when p values were <0.05.

Histological analysis

On 17 dpt (peak of the acute phase), five mice from each of the following groups (HBSS-control, EAE, EAE mice receiving p40*, EAE mice receiving p40* mAb, and EAE mice receiving hamster IgG) were anesthetized. After perfusion with PBS (pH 7.4) and then with 4% (w/v) paraformaldehyde solution in PBS, cerebrum and whole cord were dissected out from each mouse. The tissues were further fixed and then divided into two halves: one-half was used for histology analysis whereas the other half was used for myelin staining as described earlier (25–27). For histological analysis, routine histology was performed to obtain perivascular cuffing and morphological details of spinal cord and cerebellar tissues of EAE mice. Paraformaldehyde-fixed tissues were embedded in paraffin and serial sections (4 μm) were cut. Sections were stained with the conventional H&E staining method. Digital images were collected under brightfield setting using a ×40 objective. Slides were assessed in a blinded fashion for inflammation by three examiners in different anatomical compartments (meninges and parenchyma). Inflammation was scored using the following scale as described: for meninges and parenchyma: 0, no infiltrating cells; 1, few infiltrating cells; 2, numerous infiltrating cells; and 3, widespread infiltration. For vessels, scoring was as follows: 0, no cuffed vessel; 1, one or two cuffed vessels per section; 2, three to five cuffed vessels per section and 3, more than five cuffed vessels per section. At least six serial sections of each spinal cord and cerebellar tissues from each of five mice per group were scored and statistically analyzed by ANOVA.

Assessment of BBB and blood-splinal cord barrier (BSB) permeability

HBSS-treated control mice and different groups of EAE mice (n = 4 in each group) received 200 μl of 20 μM Alexa Fluor 680-SE-NIR dye (Invitrogen) via tail vein on 15 dpt (acute phase). After 2 h, mice were scanned in an Odyssey (ODY-0854; Licor) infrared scanner at 700- and 800-nm channels followed by perfusion with 4% paraformaldehyde. Spinal cord and different regions of brain were scanned in an Odyssey infrared scanner. The red background came from an 800-nm filter, whereas the green signal was from Alexa Fluor 680 dye at the 700-nm channel. The density of the Alexa Fluor 680 signal was quantified with the help of Quantity One version 4.6.2 software using the volume contour tool analysis module.

Staining for myelin

Serial longitudinal sections of paraformaldehyde-fixed spinal cords and coronal sections of paraformaldehyde-fixed cerebellum were stained with Luxol fast blue for myelin as described earlier (27, 28). Slides were assessed in a blinded fashion for demyelination by three examiners using the following scale: 0, normal white matter; 1, rare foci; 2, a few areas of demyelination; and 3, large areas of demyelination. At least six serial sections of each spinal cord and cerebellum from each of five mice per group were scored and statistically analyzed by ANOVA.

Semi-quantitative RT-PCR analysis

Total RNA was isolated from spinal cord and optic nerves by using a RNeasy mini kit (Qiagen) and from cerebellum by using Ultraseq-II RNA reagent (Biotecx Laboratories) according to the manufacturer’s protocol. To remove any contaminating genomic DNA, total RNA was digested with DNase. Semi-quantitative RT-PCR was conducted as described earlier (26, 27) using a RT-PCR kit from BD Clontech. Briefly, 1 μg of total RNA was reverse transcribed using oligo(dt)₁₂₋₁₈, as primer and Moloney murine leukemia virus reverse transcriptase (BD Clontech) in a 20-μl reaction mixture. The resulting cDNA was appropriately diluted and diluted cDNA...
was amplified using Titanium TaqDNA polymerase and the following primers: VCAM-1: sense, 5'-CAAGGGTACACCAGCTCATGA-3' and antisense, 5'-TGTGGGGACCTGAGATCC-3'; ICAM-1: sense, 5'-CTGGCTGGGACCTCAGTG-3' and antisense, 5'-GGCTGGGACCTCAGTG-3'; P-selectin: sense, 5'-ACGAGCTGGACGGCCCG-3' and antisense, 5'-GGCTGGGACCTCAGTG-3'; iNOS: sense, 5'-CCCTTCCGAGTTTCTGGGACA-3' and antisense, 5'-GGCTGGGACCTCAGTG-3'; GAPDH: sense, 5'-TGGTGAAGTGCCTTTGAG-3' and antisense, 5'-TCGCTGTACAGCTGTTG-3'; myelin oligodendrocyte glycoprotein (MOG): sense, 5'-CTCTCTCTCTTCCTCTCTCT-3' and antisense, 5'-AGATGTACAGCACACACCGGGTT-3'; MBP: sense, 5'-TGGAGAGATTCACCGGAGA-3' and antisense, 5'-TGAAGCTGCTGGGACCTCAGTG-3'; PLP: sense, 5'-CTTTTGGGCTCTGGAACCCCTT-3' and antisense, 5'-CTATCCATCCAGCCGCTTGCTC-3'; GAPDH: sense, 5'-CTACCTCCACGAGGAGA-3' and antisense, 5'-AGTCTAGTCGCCACGCTGTCT-3'.

Amplified products were electrophoresed on a 1.8% agarose gel and visualized with ethidium bromide staining. The relative expression of each gene with respect to GAPDH was measured after scanning the bands with a Fluor Chem 8800 Imaging System (Alpha Innotech).

Real-time PCR analysis

It was performed using the Applied Biosystems Prism 7700 Sequence Detection System as described earlier (27, 29) using TaqMan Universal Master mix and optimized concentrations of FAM-labeled probe and forward and reverse primers according to the manufacturer’s protocol. All primers and FAM-labeled probes for mouse genes and GAPDH were obtained from Applied Biosystems. The mRNA expression of respective genes was normalized to the level of GAPDH mRNA. Data were processed by the Applied Biosystems Sequence Detection System 1.6 software and analyzed by ANOVA.

Sandwich ELISA for p402

The concentration of p402 was measured in serum and homogenates of cerebellum, spinal cord, and spleen by a sandwich ELISA as described by us recently using p402 mAb a3-1d as the coating Ab and p401, mAb d7-12c as the detection Ab (24). Briefly, for coating, a3-1d mAb (1.3 mg/ml) was diluted in 1/3000 and added to each well (100 ml/well) of a 96-well ELISA plate. The biotinylated p402 mAb d7-12c (2 mg/ml) was diluted 1/3000 and used as detection Ab.

Results

Level of p402 in serum, spleen, cerebellum, and spinal cord of mice with relapsing-remitting EAE (RR-EAE)

Although it is well documented that the mRNA of p40 is overexpressed in CNS tissues of EAE animals, the production of p40 has not been demonstrated in EAE animals. It is also not known whether the level of p40 correlates with the disease process of EAE. Recently, we have generated mAbs against mouse p40, and demonstrated that Abs produced from clones a3-1d and d7-12c specifically recognize p40, but not p40, IL-12, and IL-23 (24). Therefore, we examined the level of p40, in serum, spleen, spinal cord, and cerebellum of mice at different phases of RR-EAE by sandwich ELISA as described by us (24). The disease process of RR-EAE in our experiments shows the onset of acute phase within 8–10 dpt followed by the acute phase within 14–16 dpt followed by the remission phase within 19–22 dpt (25–27). Therefore, samples (serum, spleen, spinal cord, and cerebellum) were collected from EAE mice on 8 dpt (onset of acute phase), 15 dpt (acute phase), and 21 dpt (remission phase) for analysis. Four mice (n = 4) were used for each time point. Control mice receiving only tail section of acute phase (Fig. 1A). Similarly, the level of p40 was also very high in spleen, cerebellum, and spinal cord during the acute phase of EAE (Fig. 1B). Although the level of p40 decreased considerably in serum and spleen during the remission phase, interestingly the cerebellar and spinal cord level of p40 did not decrease significantly during the same phase of EAE (Fig. 1B).

The p402 mAb a3-1d inhibits disease progression in adoptively transferred model of EAE

Because the level of p402 increased in serum and spleen of mice with RR-EAE, we examined whether neutralization of p402 modulates the progression of disease in adoptively transferred EAE mice. Because IFN-γ plays an important role in the disease process of EAE and MS, before examining mAbs in EAE animals, we investigated specificities of p402 mAbs (a3-1d and d7-12c) (24) in neutralizing IFN-γ production from mouse naive splenocytes. As expected, both IL-12p70 (Fig. 2A) and IL-23 (Fig. 2B) induced the expression of IFN-γ mRNA (upper panels) and the production of IFN-γ protein (lower panel) in naive splenocytes. Interestingly, p402, the so-called biologically inactive molecule, also induced the expression of IFN-γ mRNA and protein (Fig. 2C). In fact, p402 appeared to be a stronger inducer of IFN-γ than IL-12 and IL-23 (Fig. 2, A–C). In contrast, p40 monomer was unable to induce the expression of IFN-γ mRNA and protein in splenocytes (Fig. 2D).
FIGURE 2. Effect of p402 mAbs (a3-1d and d7-12c) on p402- p40-, IL12p70-, and IL-23-induced expression of IFN-γ in mouse naive splenocytes. Splenocytes isolated from naive female SJL/J mice received different concentrations (100 and 200 ng/ml) of either mAbs (a3-1d and d7-12c) or control IgG for 15 min followed by stimulation with 10 ng/ml mouse rIL-12p70 (A), IL-23 (B), p402 (C), or p40 (D). After 6 h of stimulation, the mRNA expression of IFN-γ was examined by semiquantitative RT-PCR (upper panel). Concentrations of IFN-γ were measured in supernatants by ELISA (lower panel). Results are mean ± SD of three different assays. *p < 0.001 vs control splenocytes and ^p < 0.001 vs p402-stimulated splenocytes.

A manuscript describing the IFN-γ-inducing property of p402 in detail is also under preparation (M. Jana and K. Pahan, unpublished observation).

Earlier we found that mAbs (a3-1d and d7-12c) against p402 block p402-, but not p70- and p40-, induced production of TNF-α and nitrite from microglia and macrophages (24). Consistently, mAbs (a3-1d and d7-12c) neutralized the ability of p402, but not IL-12p70 and IL-23, to induce the expression of IFN-γ mRNA (Fig. 2, A–C, upper panels) and protein (Fig. 2, A–C, lower panel) in mouse naive splenocytes. On the other hand, normal hamster IgG had no effect on p402-, p70-, and IL-23-induced expression of IFN-γ (Fig. 2). Again, mAbs (a3-1d and d7-12c) and normal IgG had no effect on the inability of p40 to induce the expression of IFN-γ (Fig. 2D). These results again demonstrate that mAbs a3-1d and d7-12c are specific for p402. Although in cell culture experiments these mAbs are specific for p402, in vivo at 37°C, these mAbs may exhibit cross-reactivity toward p40-containing cytokines. To address this possibility, we examined the effect of mAbs (a3-1d and d7-12c) on p402- and IL-23-induced expression of IFN-γ and iNOS in vivo in the spleen of female SJL/J mice. We used IL-23 because this heterodimer has been reported to play a more important role than IL-12 in the disease process of EAE (14). Within 8 h of i.p. injection, both p402 (Fig. 3A) and IL-23 (Fig. 3B) induced the mRNA expression of IFN-γ and iNOS in vivo in spleen. However, mice that received mAbs a3-1d and d7-12c before p402 treatment showed markedly reduced mRNA expression of IFN-γ and iNOS in spleen (Fig. 3A). In contrast, under similar treatment conditions, mAbs (a3-1d and d7-12c) had no effect on IL-23-induced splenic mRNA expression of IFN-γ and iNOS (Fig. 3B). To check specificity, a group of mice also received normal hamster IgG along with either p402 or IL-23 and it is evident from Fig. 3 that IgG had no effect on p402- and IL-23-induced expression of IFN-γ and iNOS in spleen. These mAbs (a3-1d and d7-12c) alone also had no effect on splenic expression of IFN-γ and iNOS (data not shown). Taken together, these results clearly demonstrate that mAbs (a3-1d and d7-12c) are specific for p402 both in cell culture and in vivo in animals.

Next, to examine the role of p402 in the disease process of EAE, we used p402 mAb a3-1d. Mice were treated with p402 mAb a3-1d in two different groups. In the first group, mice were treated with p402 mAb (100 μg/mouse) from the onset of acute phase (8 dpt). The results in Fig. 4A clearly show that the inhibitory effect of the p402 mAb on the clinical symptoms was observed within 4 days of treatment (from 12 dpt). There was further marked inhibition on subsequent days of treatment and this inhibition was maintained throughout the duration of the experiment (Fig. 4A). On the other hand, treatment of EAE mice with p402 from the onset of acute phase increased clinical symptoms (Fig. 4A). In the second group, p402 mAb treatment began from the onset of the relapsing phase (19 dpt) and was continued until 49 dpt. Fig. 4B clearly shows that the p402 mAb, in this instance, also halted the disease progression. Similar to the first instance, the inhibitory effect of p402 mAb was manifested only after 4 or 5 days of treatment (23 or 24 dpt). The EAE disease severity was always below or around stage 0.5 from 26 dpt until the end of the study (49 dpt; Fig. 4B). In contrast, p402 treatment from the onset of the relapsing phase increased disease severity (Fig. 4B). These results clearly suggest that p402 plays an important role in the disease process of EAE and that p402 mAb can control the ongoing RR-EAE when administered either early (at the onset of acute disease) or late (at the onset of relapsing disease).

Effect of recombinant p402 and p402 mAb a3-1d on the encephalitogenicity of MBP-primed T cells

Because MBP-primed T cells are encephalitogenic and adoptive transfer of these T cells induces EAE, we were interested in investigating whether p402 mAb was capable of inhibiting the
encephalitogenicity of MBP-primed T cells. T cells from lymph nodes of donor mice were treated with MBP in the presence of either recombinant p402 or p402 mAb and these p402-treated and p402 mAb-treated MBP-primed T cells were adoptively transferred to recipient mice. Another group of mice received only MBP-primed T cells as positive control. Our result showed that groups of mice that received p402 mAb-treated MBP-primed T cells exhibited significantly reduced clinical symptoms and disease severity (Fig. 5A) compared with mice receiving only MBP-primed T cells. In contrast, mice receiving recombinant p402-treated MBP-primed T cells displayed increased clinical symptoms and disease severity (Fig. 5A) compared with mice receiving only MBP-primed T cells. These results clearly suggest that p402 stimulates while p402 mAb inhibits the encephalitogenicity of MBP-primed T cells.

The p402 mAb a3-1d inhibits the generation of encephalitogenic T cells in vivo in donor mice

Because in vitro treatment of MBP-primed T cells with p402 mAb inhibited the encephalitogenicity of MBP-primed T cells, we next investigated whether p402 mAb treatment in donor mice is capable of inhibiting the generation of encephalitogenic T cells in vivo. Therefore, donor mice were treated with p402 mAb once on 2 days after injection and T cells from these donor mice were primed with MBP for 4 days and transferred adoptively to recipient mice. In the control group, EAE was induced by adoptive transfer of MBP-primed T cells from donor mice that received only PBS. Our results showed that mice that received T cells from p402 mAb-treated donor mice exhibited dramatically less clinical symptoms and disease severity compared with the control EAE group (Fig. 5B). On the other hand, mice receiving T cells from recombinant p402-treated donor mice displayed a greater degree of clinical symptoms and disease severity compared with the control EAE group (Fig. 5B). These results suggest that p402 plays an important role in the generation of encephalitogenic T cells and that neutralization of p402 by mAb suppresses the production of encephalitogenic T cells in donor mice.

The p402 mAb a3-1d inhibits infiltration of mononuclear cells into the CNS of mice with RR-EAE

Because p402 mAb inhibited the disease process of adoptively transferred EAE, we were prompted to investigate the mechanistic details by which p402 mAb is capable of doing so. It is believed that EAE as well as MS is caused by infiltration of autoreactive T cells and associated mononuclear cells, like macrophages, into the CNS followed by broad-spectrum inflammatory events. We investigated whether p402 mAb attenuated infiltration in adoptively transferred EAE mice. Mice receiving either recombinant p402 or
p402 mAb from 8 dpt (onset of the acute phase) were sacrificed on 15 dpt. H&E staining (Fig. 6A) in cerebellar sections of EAE mice showed widespread infiltration of inflammatory cells into the cerebellum. Treatment of EAE mice with p402 resulted in increased infiltration of inflammatory cells into the cerebellum. In contrast, p402 mAb treatment markedly inhibited the infiltration of inflammatory cells into the cerebellum of EAE mice (Fig. 6A). In contrast, control IgG had no effect on inflammatory infiltration into the cerebellum. Quantitation of relative level of inflammation in Fig. 6B shows that p402 mAb dramatically reduced infiltration and the appearance of cuffed vessels in both cerebellum and spinal cord sections of RR-EAE mice. On day 12 of immunization, mice were sacrificed, and total lymph node cells were further primed with MBP (50 µg/ml) for 4 days. A total of 2 × 10⁷ viable MBP-primed T cells was adoptively transferred to naive recipient mice. Six mice were used in each group. Mice were examined for clinical symptoms every day until 30 dpt.

Infiltration is mediated by adhesion molecules like ICAM-1, VCAM-1, P-selectin, and so forth, which are expressed in the endothelium of BBB as well as in glial cells in CNS parenchyma. As observed in patients with MS, the brain, spinal cord, and optic nerve are affected in RR-EAE in female SJL/J mice. Therefore, we examined the effect of p402 mAb on the expression of adhesion molecules in cerebellum, spinal cord, and optic nerve of EAE mice. Our semiquantitative RT-PCR data revealed marked expression of these adhesion molecules in the spinal cord (Fig. 7A), cerebellum (Fig. 7B), and optic nerve (Fig. 7C) of EAE mice compared with control mice. Consistent with the stimulation in infiltration, treatment of EAE mice with p402 resulted in increased expression of ICAM-1, VCAM-1, and P-selectin in the spinal cord, cerebellum, and optic nerve as compared with untreated EAE mice (Fig. 7). However, treatment of EAE mice with p402 mAb, but not control IgG, resulted in effective inhibition of these contact molecules in the spinal cord, cerebellum, and optic nerve of EAE mice (Fig. 7).

The p402 mAb a3-1d preserves the integrity of the BBB and BSB in mice with RR-EAE

BBB and BSB are membranic structures that act primarily to protect the brain and the spinal cord, respectively, from chemicals in the blood, while still allowing some essential molecules to enter. It is known that during active MS and EAE, BBB and BSB break down in a section of the brain and spinal cord, respectively, due to widespread inflammation, thereby allowing different blood molecules and toxins to enter into the CNS. We investigated whether
neutralization of p40₂ modulated the integrity of BBB and BSB. We injected an infrared dye (Alexa Fluor 680) via tail vein and, 2 h after the injection, live mice were scanned in an Odyssey infrared scanner. As evidenced from Fig. 8A (first lane), infrared signals were not visible on areas over the brain and the spinal cord in control HBSS-injected mice. In contrast, in EAE mice, some infrared signals were detected on areas over the brain and the spinal cord (Fig. 8A, second column), suggesting a possible breakdown of BBB and BSB. Treatment of EAE mice with recombinant p40₂ markedly increased the appearance of infrared signals over the brain and spinal cord (Fig. 8A, cf lane 3 with lane 2). On the other hand, p70 treatment did not increase the entry of infrared dye into the CNS of EAE mice as evident from infrared signals over the brain and spinal cord (Fig. 8A, cf the last lane with the second lane). These results suggest a greater breakdown of BBB and BSB in the presence of p40₂, but not p70. Consistently, p40₂ mAb a3-1d strongly inhibited the entry of infrared dye into the CNS (Fig. 8A,

FIGURE 7. Effect of p40₂ and p40₂ mAb on the expression of adhesion molecules in the spinal cord, cerebellum, and optic nerve of EAE mice. Spinal cord (A), cerebellum (B), and optic nerve (C) of HBSS-treated normal, control EAE (15 dpt), p40₂-treated EAE (15 dpt receiving p40₂ from 8 dpt), p40₂ mAb-treated EAE (15 dpt receiving p40₂ mAb from 8 dpt), and control IgG-treated EAE (15 dpt receiving control IgG from 8 dpt) mice were analyzed for mRNA expression of ICAM-1, VCAM-1, and P-selectin by semiquantitative RT-PCR. Four mice were used in each group. Results represent three independent experiments.

FIGURE 8. Effect of p40₂ and p40₂ mAb on the integrity of BBB and BSB in EAE mice.  A, HBSS-treated control mice and different groups of EAE mice (n = 4 in each group) received 200 μl of 20 μM Alexa Fluor 680-SE-NIR dye (Invitrogen) via the tail vein on 15 dpt (acute phase). After 2 h, mice were scanned in an Odyssey (ODY-0854; Licor) infrared scanner at the 700- and 800-nm channels. Mice were perfused with 4% paraformaldehyde. Spinal cord (B) and different regions of brain (C) were scanned in an Odyssey infrared scanner. The red background came from an 800-nm filter, whereas the green signal was from Alexa Fluor 680 dye at the 700-nm channel. D, The density of the Alexa Fluor 680 signal in different parts of the brain was quantified with the help of Quantity One, version 4.6.2 software, using the volume contour tool analysis module. Data are expressed as the mean ± SD of four different mice. *p < 0.001 vs EAE.
FIGURE 9. Effect of p402 and p402 mAb on the expression of proinflammatory molecules in the spinal cord, cerebellum, and optic nerve of EAE mice. Spinal cord (A and B), cerebellum (C and D), and optic nerve (E and F) of HBSS-treated normal, EAE (15 dpt), p402-treated EAE (15 dpt receiving p402 from 8 dpt), p402 mAb-treated EAE (15 dpt receiving p402, mAb from 8 dpt), and control IgG-treated EAE (15 dpt receiving control IgG from 8 dpt) mice were analyzed for mRNA expression of iNOS and IL-1β by semiquantitative RT-PCR (A, C, and E) and quantitative real-time PCR (B, D, and F). Data are expressed as the mean ± SD of four different mice. *p < 0.001 vs EAE.

cf lane 4 with lane 2). However, control IgG did not influence the entry of infrared dye into the CNS of EAE mice as evidenced by the aligning of infrared signals over the spinal cord and brain (Fig. 8A, cf lane 5 with lane 2).

To confirm these results further, mice were sacrificed and the spinal cord and different parts of the brain (frontal cortex, midbrain, and cerebellum) were scanned for infrared signals in an Odyssey infrared scanner. Consistent with live mice results, we did not notice much infrared signal in the spinal cord and different parts of the brain in control HBSS-treated mice (Fig. 8, B–D, lane 1), but a significant amount of infrared dye was visible in CNS tissues of EAE mice (Fig. 8, B–D, lane 2). Again, treatment of EAE mice by p402 markedly stimulated the entry of infrared dye into the spinal cord and different parts of the brain (Fig. 8, B–D, cf lane 3 with lane 2). Conversely, neutralization of p402 by mAb a3-1d markedly attenuated the entry of infrared dye into the spinal cord and different parts of the brain (Fig. 8, B–D, cf lane 4 with lane 2). As observed after scanning live mice (Fig. 8A, lane 6), p70 treatment of EAE mice did not increase the entry of infrared dye into the spinal cord and different parts of the brain (Fig. 8, B–D, cf lane 6 with lane 2). Taken together, these results suggest that p402 plays an important role in the breakdown of BBB and BSB and that its neutralization preserves the integrity of BBB and BSB in EAE mice.

Suppression of proinflammatory molecules in CNS tissues of mice with RR-EAE by p402 mAb a3-1d

Because the infiltration of mononuclear cells was inhibited and the integrity of BBB and BSB was maintained upon neutralization of p402, we next examined whether the p402 mAb a3-1d was capable of inhibiting the expression of proinflammatory molecules in the CNS of EAE mice. We used semiquantitative RT-PCR (Fig. 9, A, C, and E) and quantitative real-time PCR (Fig. 9, B, D, and F) to analyze the expression of proinflammatory genes in the spinal cord, cerebellum, and optic nerve. Marked expression of proinflammatory molecules like iNOS and IL-1β was observed in the spinal cord (Fig. 9, A and B), cerebellum (Fig. 9, C and D), as well as in the optic nerve (Fig. 9, E and F) of EAE mice as compared with control mice. However, treatment of EAE mice with p402 mAb a3-1d dramatically reduced the expression of these proinflammatory molecules in the spinal cord, cerebellum, and optic nerve of EAE mice (Fig. 9).

The p402 mAb a3-1d inhibits demyelination in mice with RR-EAE

It is believed that infiltration of blood mononuclear cells, breakdown of BBB and BSB, and associated neuroinflammation play an important role in CNS demyelination observed in MS patients and EAE animals. Therefore, we examined whether neutralization of p402 protected EAE mice from demyelination. Our semiquantitative RT-PCR data reveal marked loss of myelin genes like MBP, MOG, PLP, and CPAs in the spinal cord (Fig. 10A), cerebellum (Fig. 10B), and optic nerve (Fig. 10C) of EAE mice compared with HBSS-treated control mice. Again, treatment of EAE mice with p402 led to further loss of myelin gene expression in the spinal cord, cerebellum, and optic nerve (Fig. 10, cf lane 3 with lane 2). However, significant restoration (almost to the control level) of myelin gene expression was observed in the spinal cord (Fig. 10, cf lane 4 with lane 2). In contrast, control IgG was unable to restore the expression of myelin genes in EAE mice (Fig. 10, cf lane 5 with lane 2).

To confirm this finding further, we stained longitudinal sections of spinal cord and coronal sections of cerebellum by Luxol fast blue for myelin and observed widespread demyelination zones in the white matter of spinal cord (Fig. 11, A and D) and brain (Fig. 11, B and C) of EAE mice compared with that of HBSS-treated control mice. Again, treatment of RR-EAE mice with p402 led to further loss of myelin in spinal cord (Fig. 11, A and D) and brain (Fig. 11, B and C). However, treatment of RR-EAE mice with p402
mAb a3-1d remarkably restored the myelin level in both spinal cord (Fig. 11, A and D) and brain (Fig. 11, B and C). On the other hand, control IgG had no effect on the loss of myelin in spinal cord (Fig. 11, A and D) and brain (Fig. 11, B and C) of RR-EAE mice.

Taken together, these results suggest that neutralization of p402 inhibits demyelination in the CNS of RR-EAE mice.

**Discussion**

Although MS could be monophasic, the majority of MS patients (>80%) experience relapsing-remitting symptoms. The relapsing-remitting disease course begins with an acute phase peak followed by multiple relapses of chronic phases that gradually decrease in number and intensity. The relapsing-remitting model of EAE (RR-EAE) is a widely used model for studying the disease process of RR-MS and, in particular, is very much effective for examining novel therapeutic approaches against RR-MS. The role of IL-12 and IL-23, heterodimeric bioactive cytokines containing p40 as a subunit, in the disease process of EAE is becoming clear (9, 12, 30). However, dendritic cells, macrophages, and microglia, cells that are able to secrete heterodimeric IL-12 or IL-23, always produce an excess of p40 as p40. Again, several reports (9, 31, 32) indicate that the level of p40 mRNA in the CNS of patients with MS is much higher than in the CNS of control subjects, whereas the level of p35 mRNA is about the same or decreases compared with that of controls. Similarly, in mice with EAE, an animal model of MS, expression of p40, but not p35, mRNA increases in brain and spinal cord (33). These studies suggest that p40 may have a key function as a homodimer not just as part of either the p40:p35 heterodimer forming IL-12 or the p40:p19 heterodimer forming IL-23. However, it was known that p40 was biologically inactive until we demonstrated the induction of NO synthase (iNOS) and TNF-α by p402 in microglia and macrophages (20, 21). Recently, we have generated functional blocking mAbs against mouse p402 in the hamster (24) to understand biological functions of p402 further in vivo in animals. In the present study, we delineate the first evidence that functional blocking mAbs against p402, a so-called biologically inactive molecule, inhibits the disease process of RR-EAE. Our conclusion is based on the following observations. First, p402 mAb a3-1d inhibited the progression of adoptively transferred RR-EAE when administered either from the onset of the acute phase or from the onset of the relapsing phase. In contrast, normal hamster IgG had no effect on the progression of RR-EAE. Converse to the neutralization of p402, recombinant p402 increased disease severity when administered either in the early or late stage of the disease process. Second, adoptive transfer of MBP-primed T cells, but not p402 mAb-treated MBP-primed T cells, induced the clinical symptoms of EAE in female SJL/J mice. However, treatment of MBP-primed T cells with recombinant p402 augmented the encephalitogenicity of MBP-primed T cells. Third, treatment of donor mice with p402 mAb a3-1d also inhibited the generation of encephalitogenic T cells. In contrast, treatment of donor mice with recombinant p402 stimulated the generation of encephalitogenic T cells.

Although we have not tested whether p402 mAb entered into the CNS through leaky BBB and BSB during the acute phase of EAE, our results suggest that p402 is indeed produced within the CNS during the onset of acute phase and the acute phase itself. Quantification of p402 by a sandwich ELISA (24) shows that the level of p402 increased in the serum, spleen, spinal cord, and cerebellum during the onset of the acute phase and the acute phase of EAE. As expected, during the remission phase, the level of p402 decreased in serum and spleen of EAE mice almost close to the control level. However, the level of p402 did not decrease significantly in cerebellum and spinal cord during the remission phase of EAE. In the CNS, activated glial cells, mainly microglia, are supposed to produce p402. Our results suggest that although clinical symptoms were low during the remission phase, activated microglia in spinal cord and brain of EAE mice were still producing p402. Recently,
we have found that p40_2, but not IL-12p70, the bioactive cytokine, is capable of inducing the expression of IL-16, a T cell chemotactic factor, in microglia and astroglia (22). Therefore, it is possible that during the remission phase, microglial p40_2 may play a very important role in transducing the chemotactic signal via IL-16 for the next relapse.

Infiltration of leukocytes into the CNS is a key neuroinflammatory event in EAE as well as MS. Our results demonstrate that the functional blocking mAb against p40_2 markedly inhibits the infiltration of mononuclear cells into the CNS of EAE mice. Although the exact mechanism of leukocyte invasion is not completely understood, this process most likely involves the expression of the endothelial VCAM-1 and ICAM-1 (34), which is temporally correlated with the onset of clinical signs. Up-regulation of ICAM-1 and VCAM-1 was reported on cerebral vessels during EAE preceding the perivascular infiltration by lymphocytes and the onset of disease (35). Our studies have firmly shown that p40_2 mAb down-regulates the expression of ICAM-1 and VCAM-1 in the CNS of EAE mice. Importance of selectins in the extravasation of T lymphocytes into the CNS is also evident when complete inhibition of rolling and arrest of Th1 cells in the inflamed brain of EAE mice was observed with anti-E- and P-selectin Abs (36). Interestingly, neutralization of p40_2 was also found to suppress the expression of P-selectin in the spinal cord, cerebellum, and optic nerve of EAE mice.

In addition to the infiltration of mononuclear cells, we have also found that the BBB and BSB are compromised in EAE and that an infrared dye enters into the spinal cord and different parts of the brain during the acute phase. Interestingly, the permeability through BBB and BSB increased upon treatment with p40_2 and decreased upon neutralization of p40_2, suggesting an important role of p40_2 in the breakdown of BBB and BSB integrity. NO, a major mediator in immune and autoimmune functions, has been also shown to increase the permeability of BBB, allowing substances to enter into the brain passively, leading to vasogenic edema and secondary brain damage (37). Although the precise molecular mechanisms for NO-induced breakdown of the BBB are not completely understood, in a cell culture model of the BBB, NO leads to a rapid breakdown in model barrier integrity and results in a reduction in endothelial cell ATP content and GAPDH activity (38). It is important to note that p40_2 alone is capable of inducing the expression of iNOS in cultured microglia via the activation of NF-κB (21). Consistently, the expression of iNOS in vivo in spinal cord, cerebellum, and optic nerve of EAE mice was stimulated by p40_2 and inhibited by p40_2 mAb (Fig. 9). Additionally, p40_2 could also increase the expression of adhesion molecules (ICAM-1 and VCAM-1) in glial cells and endothelial cells via the activation of NF-κB. Therefore, these proinflammatory molecules being induced by p40_2 may guide inflammatory leukocytes into and through the CNS, thus contributing to their multiplication and finally to BBB disruption. Therefore, the protective effect of p40_2 mAb against the leukocyte invasion into the CNS and the breakdown of BBB and BSB could be mediated through the neutralization of p40_2 and thereby the inhibition of iNOS and proinflammatory molecules.

Current MS treatments include IFNs, corticosteroids, and cytotoxic immunosuppressive agents which are often associated with a number of side effects and unsatisfactory outcomes (39). Our results demonstrate that p40_2 mAb attenuates the clinical symptoms of RR-EAE, inhibits the encephalitogenicity of MBP-primed T cells, blocks the infiltration of mononuclear cells into the CNS, suppresses the expression of adhesion molecules and proinflammatory molecules in the CNS, restores the integrity of BBB and BSB, normalizes the expression of myelin genes in the CNS, and stops demyelination. Although the disease process of MS is not exactly the same as EAE, the local concentration of p40_2 present in peripheral and CNS microimmune organs of MS patients may differ from what we have observed in peripheral and CNS tissues of RR-EAE mice and the in vivo condition of the cerebellum, spinal cord, and optic nerve of RR-EAE mice may not truly resemble the in vivo situation of these organs in MS patients, our results suggest that neutralization of p40_2 by the functional blocking mAb may be a therapeutic strategy against MS.

Disclosures
The authors have no financial conflict of interest.

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