

**The role of  $\alpha 4$  integrin in Theiler's murine encephalomyelitis virus (TMEV)-induced demyelinating disease: an infectious animal model for multiple sclerosis (MS)**

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**Abstract**

Natalizumab, which is an antibody against  $\alpha 4$  integrin, has been used for the treatment of multiple sclerosis (MS). In the present study, we investigated both the role of  $\alpha 4$  integrin and the therapeutic effect of HCA3551, a newly synthesized orally active small-molecule  $\alpha 4$  integrin antagonist, in the development of TMEV-induced demyelinating disease (TMEV-IDD). The mRNA levels of  $\alpha 4$  integrins were significantly up-regulated in the CNS of mice with TMEV-IDD as compared with naïve mice ( $*p < 0.05$ ). HCA3551 treatment in the effector phase significantly suppressed both the clinical and histological development of TMEV-IDD. The number of infiltrating mononuclear inflammatory cells in the CNS was significantly decreased in the mice treated with HCA3551 ( $**p < 0.01$ ). The labeling indices for CD68 antigen and the absolute cell numbers of TNF- $\alpha$ -producing CD4<sup>+</sup> T cells and IFN- $\gamma$ -producing CD8<sup>+</sup> T cells were significantly decreased in the CNS of mice treated with HCA3551 ( $*p < 0.05$ ). HCA3551 treatment in the effector phase might inhibit the binding of  $\alpha 4$  integrin to VCAM-1, thereby decreasing the number of MNCs in the CNS.

*Keywords:*  $\alpha 4$  integrin,  $\alpha 4$  integrin inhibitor, multiple sclerosis, Theiler's murine encephalomyelitis virus (TMEV), TMEV-induced demyelinating disease (TMEV-IDD)

*Running Head:* A small molecule  $\alpha 4$  integrin antagonist in TMEV-IDD

## Introduction

Multiple sclerosis (MS) is an immune-mediated chronic demyelinating disease that is associated with inflammation in the white matter of the human central nervous system (CNS). Histologically, MS is characterized by CNS lesions displaying inflammation, demyelination and axonal damage. Although its etiology remains unclear, MS is generally considered to be an autoimmune disease mediated by T helper (Th) 1 and Th 17 cells (1,2). The morphology of the acute lesion and the susceptibility to MS that is apparently conferred through certain MHC class II-restricted antigens suggest that autoreactive CD4<sup>+</sup> T cells play a crucial role in the disease process. Moreover, CD8<sup>+</sup> T cells might participate in the pathogenesis of MS based on animal models of this disease (3,4). Epidemiological evidence suggests that one or more infectious agents might be involved in the initial tissue damage, leading to autoimmunity. Evidence indicates that infection with the Epstein–Barr virus plays a major role in the pathogenesis of MS, although precise role for this virus remains incompletely understood (5,6). Several virus-induced and autoimmune models have been used to examine the underlying mechanisms of this disease (7-10). In particular, Theiler's murine encephalomyelitis virus (TMEV)–IDD provides an excellent infectious model for several reasons (9,11).

Theiler's murine encephalomyelitis virus (TMEV) is a positive sense single-stranded RNA (ssRNA) virus of the Picornaviridae family (12). The infection of TMEV in CNS induces immune-mediated demyelinating disease in susceptible mouse strains. TMEV-induced

demyelinating disease (TMEV-IDD) serves as a relevant infectious animal model for human MS, because this disease displays histopathological, genetic and clinical similarities to human MS. In addition, the development and progression of TMEV-IDD well correlated with the level of Th 1 responses specific for viral epitopes (13-15).

The  $\alpha 4$  integrins impact hematopoiesis, leukocyte trafficking in immune surveillance and inflammation, and leukocyte activation and survival (16).  $\alpha 4$  integrin, which is expressed on the surface of leukocytes, pairs with one of two  $\beta$  subunits, :  $\beta 1$  and  $\beta 7$  (17).  $\alpha 4\beta 1$  integrin primarily binds to vascular cell adhesion molecule-1 (VCAM-1) and the CS1 fragment of fibronectin (18). Specifically, the interaction of  $\alpha 4\beta 1$ /VCAM-1 plays a major role in the recruitment of mononuclear leukocytes to inflammatory sites in vivo (19). Anti- $\alpha 4$  integrin antibody significantly suppressed the development of paralysis and the infiltration of leukocytes into the CNS in rat experimental autoimmune encephalomyelitis (EAE) (20). Anti- $\alpha 4$  integrin antibody suppressed the clinical score and pathological features of EAE in the guinea pigs by inhibiting leukocyte infiltration into the CNS (21). In addition, magnetic resonance imaging revealed that an antibody against  $\alpha 4$  integrin significantly decreased the percentage of pixels as a result of the leakage of contrast material and CNS abnormalities associated with cerebral edema and inflammation compared with control animals of guinea pig EAE (22). In a placebo-controlled trial involving 213 patients with relapsing-remitting or relapsing secondary progressive MS, treatment with natalizumab, an antibody against  $\alpha 4$  integrin, showed a marked suppression of the mean number of new lesions and relapses compared with the placebo-group (23). However, progressive multifocal leukoencephalopathy (PML) has been reported

in MS and Crohn's disease patients who are treated with natalizumab (24-26). In the present study, we investigated the role of  $\alpha 4$  integrin and the effects of orally active small molecules  $\alpha 4$  integrin antagonists in the development of TMEV-IDD, which is an infectious animal model for MS.

Here, we demonstrated that the mRNA levels of  $\alpha 4$  integrins were significantly up-regulated in the development and treatment of TMEV-IDD with the newly synthesized orally active small molecule  $\alpha 4$  integrin antagonist, HCA3551, which markedly suppressed the development of TMEV-IDD.

## **Methods**

### *Mice*

Female SJL/J mice, at 6 weeks old, were purchased from Charles River Laboratories, Inc. (Ibaraki, Japan) and, housed and maintained for in an approved facility, in accordance with the Shinshu University Guide for Laboratory. The animals were housed in aluminum cages containing pine chips and fed food and water ad libitum. The protocol for animal experiments was approved through the Animal Care Committee of Shinshu University.

### *Virus*

The BeAn strain of TMEV was expanded in baby hamster kidney (BHK) cells in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 7% donor calf serum (Life Technologies,

Gaithersburg, MD, USA). Cell lysates with known plaque forming units (PFU) were used as viral stocks for animal experiments. Partially purified virus was prepared following centrifugation through 30% sucrose as previously described and used for *in vitro* assays (27). The viral titer was determined using a standard plaque assay on BHK cells.

#### *Infection of mice with TMEV*

The mice were intracerebrally (i.c.) infected with 30  $\mu$ l ( $0.67 \times 10^6$  PFU) of the BeAn strain of TMEV on day 0, and clinically observed and scored from day 0 to the end of the experiments. The mice were examined daily for clinical neurological signs, scored and recorded using the following grading scale: grade 0 = no clinical signs; grade 1 = mild waddling gait; grade 2 = moderate waddling gait and hindlimb paresis; grade 3 = severe hindlimb paralysis; grade 4 = severe hindlimb paralysis and loss of righting reflex; and grade 5 = moribund or death. Two independent investigators, who were blinded to the treatment of each animal, assessed the clinical score. The cumulative clinical score was calculated for the individual mice in each group.

#### *In vivo blockade of $\alpha 4$ integrins*

Six-week-old female SJL/J mice were separated into groups (Table 1). TMEV was injected (i.c.) into SJL/J mice i.c. on day 0. To block  $\alpha 4$  integrins, we used HCA3551 (Ajinomoto Pharmaceuticals Co., Ltd, Tokyo, Japan) an orally active small molecule antagonist that inhibits the binding of  $\alpha 4 \beta 1$  integrin to VCAM-1, but not  $\alpha L \beta 2$  integrin to ICAM-1. The IC50 values were 8.2

and >5,000 nM, respectively. In addition, the inhibitory activity of HCA3551 was not reduced after adding 50% human serum (IC<sub>50</sub>: 11nM), although the inhibitory activity of the  $\alpha 4$  integrin antagonist, afirategrast (GlaxoSmithKline Co., UK) was dramatically reduced (28). HCA3551 was dissolved in 0.5% hydroxypropyl methyl cellulose (methylcellulose) (Sigma-Aldrich, St Louis, MO, USA) as vehicle. The BeAn strain of TMEV induces a clinically undetectable level of early-phase disease, whereas the DA strain of TMEV induces significant polio-like symptoms (29). We used the BeAn strain of TMEV in previous studies, specifically a recent batch of the BeAn strain, which causes early onset TMEV-IDD. The onset of TMEV-IDD occurred at approximately 14 days post-infection using this batch of the BeAn strain of TMEV (30). Therefore, the mice were treated with 100 mg/kg HCA3551 twice a day via oral gavage from 11 to 40 days post infection (dpi) or at 41 dpi in the effector phase (group B and E, respectively) and from -3 to 14 dpi in the induction phase (group D). In a preliminary study, to evaluate the efficacy of HCA3551, HCA3551 was dissolved in 0.5% methylcellulose and orally administered at doses of 30, 100, and 300 mg/kg BID twice a day to female SCID mice to generate a mouse model of colitis through the adoptive transfer of CD4<sup>+</sup> T cells from diseased IL-10<sup>-/-</sup> mice at Ajinomoto pharmaceuticals. HCA3551 suppressed the severity of the mouse model of colitis in a dose-dependent manner. The inhibition of the severity of mouse colitis was statically significant in experimental groups treated with HCA3551 at doses of 30, 100 and 300 mg/kg. However, there was no significant difference between the 100 and 300 mg/kg doses (data not shown). Therefore, we assessed the effects of the 100 mg/kg dosage of HCA3551. Control groups (group A and C) were treated with 0.5% methylcellulose/H<sub>2</sub>O as a vehicle during the same

interval. The mice were sacrificed at 40 dpi in experiment 1 and at 41 dpi in the experiment 2. The details of the experimental design are shown in Table 1 and all mice were subjected to essentially identical manipulations.

#### *Gene expression measurements*

Total RNA was extracted from the CNS (brain and spinal cord) using TRIzol (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. Prior to the reverse transcription (RT) reaction, potentially contaminating genomic DNA was eliminated using DNase I (Roche, Basel, Switzerland). RNA was reverse-transcribed using SuperScript III (Life Technologies). The mRNA was quantified through real-time RT-PCR and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster, USA) or TaqMan Fast Universal Master Mix (Applied Biosystems) using the StepOnePlus real-time PCR System (Applied Biosystems). The details of the primer design are shown in Table 2. The primers for  $\beta$ -actin (Mm01205647\_g1) were obtained from Applied Biosystems. Amplification was conducted in a total volume of 10  $\mu$ l for 40 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C for  $\alpha$ 4 integrin and  $\beta$ -actin, and for 40 cycles of 1 s at 95°C and 20 s at 60°C for TMEV and  $\beta$ -actin. The mRNA levels were determined after normalizing the RNA concentration against  $\beta$ -actin.

#### *CNS mononuclear cell isolation*

Infiltrating mononuclear cells (MNCs) in the CNS (brain and spinal cord) were isolated

according to the method of Michael A. Lyman (31). Briefly, sterile Hanks' balanced salt solution (30 ml) was injected through the left ventricle of the heart to perfuse the CNS. The CNS was subsequently removed, forced through a cell strainer, and single-cell suspensions were prepared according to standard methods. Briefly, a Percoll-NaCl solution was prepared by the addition of 9 parts (v/v) Percoll (GE Healthcare, Little Chalfont, UK) to 1 part (v/v) 1.5 M NaCl solution. The CNS cell suspensions were centrifuged at  $300\times g$  for 5 min and resuspended in 5 ml of 37% Percoll-NaCl solution diluted with culture medium and overlaid onto 2.5 ml of 70% Percoll-NaCl solution diluted with culture medium. The gradient was centrifuged at  $2,000\times g$  for 20 min and the MNCs were harvested from the 37%-70% interface, followed by washing and counting.

#### *Flow cytometry*

For intracellular staining, MNCs isolated from the CNS were stimulated for 6 hours at  $37^{\circ}\text{C}$  in culture medium containing VP<sub>270-86</sub> (MHC class II-restricted immunodominant peptide for CD4<sup>+</sup> T cells: WTSQEAFSHIRIPLP; Operon, Tokyo, Japan) or VP<sub>3159-166</sub> (MHC class I-restricted immunodominant peptide for CD8<sup>+</sup> T cells: FNFTAPFI; Operon) of TMEV (5  $\mu\text{M}$  for each peptide) and brefeldin A (10  $\mu\text{g}/\text{ml}$ ; Sigma, St. Louis, MO), in a humidified 5% CO<sub>2</sub> atmosphere. The MNCs were washed and triple- or double-labeled with FITC-conjugated anti-mouse IFN- $\gamma$  or TNF- $\alpha$  mAb, PE-conjugated anti-mouse IL-4, IL-10 or IL-17A mAb and PerCP-conjugated anti-mouse CD4 or CD8 mAb for 30 min at room temperature in the dark. All antibodies were obtained from BD Biosciences (San Diego, USA). After washing, the cells were analyzed on a FACSCanto II Flow

Cytometer (BD Biosciences).

### *Histology*

The mice were sacrificed for histological examination at 40 dpi (group A and B). Subsequently, the spinal cords were removed and fixed as previously described (32). Spinal cord sections from paraffin-embedded tissues were prepared at thickness of 3- or 6-  $\mu\text{m}$ . The sections were stained with hematoxylin-eosin (H&E) or Klüver-Barerra's (KB) staining. Immunohistochemical staining was performed using indirect immunoperoxidase techniques. The following primary antibodies were used: rabbit polyclonal anti-CD3 antibody (Abcam, Tokyo, Japan), rat monoclonal anti-mouse CD45R/B220 antibody (BD Biosciences) and rabbit polyclonal anti-CD68 antibody (Abcam). The tissue sections were pretreated with microwaves using 1 mM EDTA buffer solution (pH 8.0) for 30 min prior to staining with a primary antibody. Peroxidase-labeled Histofine simple stain mouse MAX-PO (R) or (Rat) (Nichirei, Tokyo, Japan) was used as secondary antibody and the peroxidase reaction was visualized through incubation in a 3'-3'-diaminobenzidinetetrachloride (DAB)/H<sub>2</sub>O<sub>2</sub> solution. At least three sections per mouse were read under a light microscope and evaluated in a blinded fashion by three independent investigators unaware of the treatment of each animal. Furthermore, we adopted labeling indices (LIs) to more quantitatively show the positive cells. The LIs for CD3, a T cells marker, CD45R, a B cells marker, and CD68, a macrophages markers, were examined in the same serial sections and photographed using a light microscope at a magnification of  $\times 200$  in three randomly selected fields. Using the photographs, we examined at

least 3000 spinal cord cells per mouse. The LIs for CD3, CD45R and CD68 antigens were expressed as the number of positive cells per 100 cells examined. Three independent observers calculated the LIs twice.

#### *Statistical analysis*

Comparisons were performed with the Mann-Whitney *U*-test using JMP software (SAS institute Inc., Tokyo, Japan). The differences were considered statistically significant when the two-tailed *p*-value was <0.05.

## **Results**

#### *The expression of the mRNA of $\alpha 4$ integrin in the CNS of mice with TMEV-IDD*

To investigate the mRNA levels of  $\alpha 4$  integrins during TMEV infection, we compared the mRNA levels of  $\alpha 4$  integrins in the CNS (brain and spinal cord) between naïve SJL/J and TMEV-IDD mice using real-time RT-PCR. The  $\alpha 4$  integrins mRNA levels were significantly increased in the CNS (brain and spinal cord) of mice with TMEV-IDD at 40 dpi (n=7) compared with naïve SJL/J mice (n=6) (\**p*<0.05) (1.0 and 2.5, respectively) (Fig. 1).

#### *Antagonist of $\alpha 4$ integrin ameliorates the development of TMEV-IDD*

To determine the effects of HCA3551, the orally active small molecule  $\alpha 4$  integrin antagonist,

on the development of TMEV-IDD, we treated mice with HCA3551 at the induction phase (from -3 to 14 dpi) or the effector phase (from 11 to 40 or 41 dpi) of the disease. The experimental design and the results of the effects of HCA3551 on the clinical course are summarized in Table 1 and Fig. 2, respectively, including two independent experiments. TMEV-infected control animals (groups A and C treated with 0.5% methylcellulose as a vehicle at the effector phase) showed the typical course of TMEV-IDD. There were no significant differences in the clinical signs between control groups C and group D, which was treated with HCA3551 at the induction phase. However, the clinical signs of groups B and E, which were treated with HCA3551 at the effector phase, were significantly suppressed compared with the control groups ( $*p < 0.05$ ; from 14 to 40 dpi versus group A, at 21 dpi and from 32 dpi to 41 dpi versus group C) ( $**p < 0.01$ ; from 22 to 31 dpi versus group C), respectively. The mean maximum clinical scores (MMS) for the control groups were 2.5 in group A and 3.4 in group C, respectively. The MMS for group D mice treated with HCA3551 at the induction phase was 3.0. In contrast, the MMSs of group B and E mice, treated with HCA3551 at the effector phase, significantly decreased to 1.7 and 1.9 ( $**p < 0.01$  versus group A) ( $*p < 0.05$  versus group C), respectively. Assessments based on the mean cumulative clinical scores (MCSs) showed similar results. The MCSs of the control groups were 42.5 for group A and 61.0 for group C. The MCSs of the mice for group D were 52.1. However, the MCSs for groups B and E were significantly decreased to 28.0 and 37.2, respectively compared with methylcellulose-treated control mice ( $**p < 0.01$  versus group A) ( $*p < 0.05$  versus group C) respectively.

### *Histological findings*

The representative mice were blindly selected from groups A and B (Table 1) for histological examination. The severe perivascular and parenchymal MNC infiltration and extensive demyelination, characteristics of TMEV-IDD, were observed in the white matter of the spinal cords obtained from mice treated with methylcellulose (Fig. 3. 1A, 1C, 1E and 1G). In contrast, less severe perivascular and parenchymal MNC infiltration and demyelination were observed in the white matter of the spinal cords obtained from TMEV-infected mice treated with HCA3551 during the effector phase (Fig. 3. 1B, 1D, 1F and 1H). Furthermore, we performed immunohistochemical staining to evaluate the subpopulation of infiltrating MNC in the spinal cord. The sections of spinal cord from TMEV-infected control mice treated with methylcellulose revealed the marked infiltration of CD3+ T cells, CD45R+ B cells and CD68+ macrophages into perivascular and parenchymal areas (Fig. 3. 1I, 1K and 1M), whereas a slightly reduced infiltration of CD3+ T cells, CD45R+ B cells and CD68+ macrophages was observed in the spinal cords obtained from TMEV-infected mice treated with the orally active small molecules  $\alpha 4$  integrin antagonist, HCA3551 (Fig. 3. 1J, 1L and 1H). The LIs for CD3, CD45R and CD68 antigens in the spinal cord sections obtained from methylcellulose-treated control mice were 21.9, 10.4 and 29.5, respectively. In contrast, the LIs for CD3, CD45R and CD68 antigens in the spinal cord sections obtained from HCA3551-treated mice significantly decreased to 7.0, 5.4 and 9.0 ( $*p < 0.05$  versus methylcellulose-treated mice) (Fig. 3. 2A-2C) respectively. There was no significant intraobserver variation in the assessment of CD3, CD45R or CD68.

*Treatment of mice with the orally active small molecule  $\alpha 4$  integrin antagonist, HCA3551, in the effector phase decreases MNC infiltration into the CNS*

The neuropathological changes in TMEV-IDD and MS are associated with the blood-brain barrier breakdown and infiltrates of MNCs (33). Clinical disease in TMEV-IDD is correlated with the invasion of CNS through MNCs. To compare the levels of cellular infiltrations between the methylcellulose-treated control group and HCA3551-treated groups, the animals were sacrificed at 40 dpi in experiment 1 and 41 dpi in experiment 2. The infiltrating MNCs were isolated, using Percoll, from the CNS of TMEV-infected mice treated with HCA3551 or methylcellulose (n=17 and n=14, respectively). The isolated cells were calculated. The mean number of MNCs isolated from the CNS of the mice treated with methylcellulose was  $1.89 \pm 0.15 \times 10^6$ . In contrast, the mean number of MNCs isolated from the CNS of HCA3551-treated mice in the effector phase was significantly decreased to  $1.03 \pm 0.18 \times 10^6$  (\*\* $p < 0.01$ ) (Fig. 3. 3A). These results suggest that oral treatment with HCA3551, an antagonist against  $\alpha 4$  integrin, suppressed disease development by inhibiting cellular infiltration to the CNS of TMEV-infected mice.

*Administration of the orally active small molecule antagonist to  $\alpha 4$  integrin, HCA3551, in the effector phase suppresses TNF- $\alpha$ -producing CD4<sup>+</sup> cells and IFN- $\gamma$ -producing CD8<sup>+</sup> cells.*

To examine the effect of HCA3551 in the effector phase on the number of pro-inflammatory cytokine-producing cells, we assessed the intracellular levels of IFN- $\gamma$ -, TNF- $\alpha$ -, IL-4-, IL-10- and

IL-17A-producing MNCs in the CNS at 40 dpi using flow cytometry after stimulation with VP2<sub>70-86</sub> or VP3<sub>159-166</sub> peptides. The number of TNF- $\alpha$ -producing CD4<sup>+</sup> T cells and IFN- $\gamma$ -producing CD8<sup>+</sup> T cells in the CNS was significantly decreased in mice treated with HCA3551 compared with the control mice (\* $p$ <0.05 and \*\* $p$ <0.01, respectively) (Fig. 4B). Notably, CD4<sup>+</sup> T cells producing TNF- $\alpha$  and VP3<sub>159-166</sub>-specific CD8<sup>+</sup> T cells were particularly reduced among T cells in the CNS of HCA3551-treated mice, as these T cells have been associated with the pathogenesis of TMEV-IDD (34,35).

*Effects of the orally active small molecule  $\alpha$ 4 integrin antagonist, HCA3551, on viral persistence*

To determine the effect of the orally active small molecule  $\alpha$ 4 integrin antagonist, HCA3551, on TMEV replication in the CNS, the viral mRNA levels in the CNS at 40 dpi were assessed by real-time RT-PCR (Fig. 5). There was no significant difference in the TMEV mRNA levels between methylcellulose-treated control mice and mice treated with the orally active small molecule  $\alpha$ 4 integrin antagonist. These results suggest that the administration of orally active small molecule  $\alpha$ 4 integrin antagonist, HCA3551, during the effector phase does not affect TMEV replication. These results suggest that the inhibition of  $\alpha$ 4 integrin function might also affect the migration of protective T cells involved in the clearance of TMEV persistence in the CNS, in addition to the pathogenic T cells.

## Discussion

The importance of  $\alpha 4$  integrin in EAE is well established (20,36,37), but there are no reports on the importance and the role of  $\alpha 4$  integrin in TMEV-IDD. Therefore, in the present study, we investigated the role of  $\alpha 4$  integrin and the effects of the orally active small molecule  $\alpha 4$  integrin antagonist, HCA3551, in the development of TMEV-IDD. HCA3551 is an orally active small molecule  $\alpha 4$  integrin antagonist with an  $IC_{50}$  value for VCAM-1 cell adhesion of 8.2 nM. Previous studies have suggested that T cells in inflamed CNS express high levels of active  $\alpha 4\beta 1$  integrin, and that the general expression of  $\alpha 4$  integrin is increased on the surface of circulating monocytes in EAE (36,38). Moreover, compared with remission, the expression of  $\alpha 4$  integrin was significantly up-regulated during relapse in relapsing-remitting MS patients (39). In the present study, we demonstrated that the mRNA levels of  $\alpha 4$  integrins were significantly increased in the CNS (brain and spinal cord) of mice with TMEV-IDD at 40 dpi compared with naïve SJL/J mice ( $*p < 0.05$ ) (Fig. 1).  $\alpha 4$  integrin is generally expressed on the surface of T cells, macrophages and circulating monocytes. Infiltrating cells to the CNS in TMEV-IDD are primarily macrophages, T cells and monocytes. Therefore, the up-regulated  $\alpha 4$  integrin mRNA of primarily reflects infiltrated macrophages, T cells and monocytes. These findings indicate that  $\alpha 4$  integrin might be closely associated with the development of TMEV-IDD.

Anti- $\alpha 4$  integrin antibodies or small molecule  $\alpha 4$  integrin antagonists have been demonstrated as effective for the treatment of EAE (20-22,38). However, to our knowledge, the effects of  $\alpha 4$

integrin and its antagonist on the development of TMEV-IDD, which one of only a few infectious animal models of MS, have not yet been studied. The results of present study revealed that HCA3551 treatment significantly suppressed the development of TMEV-IDD (Fig. 2). Furthermore, HCA3551 treatment dramatically decreased demyelination and the infiltration of MNCs into the CNS, consistent with a decrease in the number of TNF- $\alpha$ -producing CD4<sup>+</sup> T cells and IFN- $\gamma$ -producing CD8<sup>+</sup> T cells (\*\* $p$ <0.01 and \* $p$ <0.05, respectively) (Fig. 4B). This study is the first to demonstrate the efficacy of orally active small molecule  $\alpha$ 4 integrin antagonists in TMEV-IDD. However HCA3551 is not effective in TMEV-IDD when administered during the induction phase. After TMEV inoculation (i.c.), initial viremia followed by persistent low-level CNS infection is observed (40). TMEV infects neurons, glial cells and macrophages in the spinal cord (41). This stage has been referred to as the ‘induction phase’. During this phase, infiltration of macrophage and T cells into the CNS is low, and  $\alpha$ 4 integrin may not be important in this phase, thereby HCA3551 does not suppress TMEV-IDD when in the induction phase.

To further clarify the effects of the treatment of orally active small molecule  $\alpha$ 4 integrin antagonist, HCA3551, in TMEV-IDD, we measured the number of infiltrating cells, and the LIs for CD3, CD45R and CD68 antigen and assessed Th1, Th2 and Th17 responses in the CNS. The VCAM-1/ $\alpha$ 4 $\beta$ 1 integrin ligand-receptor pair plays major roles in the recruitment of mononuclear leukocytes to inflammatory sites in vivo (19). We showed that the administration of HCA3551 significantly suppressed the number of CNS inflammatory MNCs compared with administration of control vehicle methylcellulose (\*\* $p$ <0.01) (Fig. 3. 3A), suggesting that HCA3551 inhibits the

infiltration of MNCs into the CNS through the inhibition of  $\alpha 4$  integrin binding to VCAM-1. Furthermore, the LIs for CD3, CD45R and CD68 antigen were significantly decreased in the CNS of mice treated with HCA3551 compared with mice treated with methylcellulose ( $*p < 0.05$ ) (Fig. 3. 2A-2C). The numbers of TNF- $\alpha$ -producing CD4<sup>+</sup> T cells and IFN- $\gamma$ -producing CD8<sup>+</sup> T cells in the CNS were significantly decreased and the numbers of IL-17A-producing CD4<sup>+</sup> T cells in the CNS were decreased in mice treated with HCA3551 compared with control mice treated with methylcellulose (Fig. 4B). Previous studies have suggested that Th17 cells express lower amounts of  $\alpha 4$  integrin than Th1 cells and Th1 cells preferentially infiltrate the spinal cord via an  $\alpha 4$  integrin-mediated mechanism, whereas the entry of Th17 cells into the brain parenchyma occurs in the absence of  $\alpha 4$  integrins but is dependent on  $\alpha L\beta 2$  expression (42). Therefore, the differential expression of integrin molecules might result in the significant decrease in TNF- $\alpha$ -producing CD4<sup>+</sup> T cells and IFN- $\gamma$ -producing CD8<sup>+</sup> T cells, but unaltered level of IL-17-producing CD4<sup>+</sup> T cells compared with control mice. Thus, the significant suppression of TMEV-IDD was accompanied with the reduction of the LIs for the CD68 antigen, macrophages markers, and the reduced numbers of TNF- $\alpha$ -producing CD4<sup>+</sup> cells and IFN- $\gamma$ -producing CD8<sup>+</sup> cells in the CNS of mice treated with HCA3551, because as these cell types have been implicated to play pathogenic roles in the disease development (13,34,35). These results suggest that treatment with HCA3551 decreased the number of MNCs infiltrated into the CNS of TMEV-infected mice, they suppressed the development of TMEV-IDD with decreasing the number of MNCs infiltrated into the CNS of TMEV-infected mice.

We examined whether the suppressive effect of HCA3551 treatment on TMEV-IDD might

reflect the elimination of viral persistence. We assessed the levels of replication in the CNS of HCA3551-treated mice or methylcellulose-treated mice using real-time RT-PCR. The results showed that there were no significant differences in the mRNA levels of TMEV between methylcellulose-treated control mice and HCA3551-treated mice (Fig. 5). Thus, it seems that HCA3551 treatment reduced the development of TMEV-IDD without interfering viral persistence. Consistently, viral levels might not necessarily be associated with the development of demyelinating disease in this model (43,44).

The results of the present study suggested that  $\alpha 4$  integrin might play a critical role in the development of TMEV-IDD. Treatment with HCA3551 might ameliorate TMEV-IDD through the inhibition of  $\alpha 4$  integrin binding to VCAM-1 accompanied a decrease in the number of MNCs in the CNS. HCA3551 also has a potential risk for PML. However, the pharmacological half-life of HCA3551 is 12 hours, which is much shorter than that of natalizumab (approximately 11 days) (28). The potential advantage is that the early removal of this drug from the body after the onset of PML could lead to more favorable outcomes, but this is a hypothesis which needs further validation (45).

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Fig. 1.

The expression of mRNA of  $\alpha 4$  integrin in the CNS of mice with TMEV-IDD. The mRNA levels of  $\alpha 4$  integrins in the whole CNS were measured using real-time RT-PCR. The data represent the relative expression levels normalized to  $\beta$ -actin levels. The mRNA levels of  $\alpha 4$  integrins were significantly increased in the CNS of TMEV-IDD at 40 dpi (n=7) compared with naïve SJL/J mice (n=6) (\* $p$ <0.05) (1.0 and  $2.5 \pm 0.93$ , respectively). The data are presented as the means  $\pm$  standard errors.

Fig. 2.

This figure summarizes the clinical scores of mice treated with methylcellulose or 100 mg/kg HCA3551, an orally active small molecule  $\alpha 4$  integrin antagonist. All mice were injected (i.c.) with  $0.67 \times 10^6$  PFU of TMEV in 30  $\mu$ l on day 0.

(Exp. 1) The mice were treated with 0.5% methylcellulose/H<sub>2</sub>O as a vehicle (group A) or 100 mg/kg of HCA3551 in methylcellulose (group B) twice a day via oral gavage per mouse from 11 to 40 dpi in the effector phase. The treatment design is shown in Table 1. Clinical signs of TMEV-IDD were significantly suppressed in mice treated with HCA3551 in the effector phase from 14 to 40 dpi compared with control mice treated with methylcellulose (\* $p$ <0.05). Thus significant suppression of TMEV-IDD was observed only when HCA3551 was administered to mice with TMEV-IDD in the effector phase. We performed Experiment 2 to confirm these results.

(Exp.2) Mice were treated with 0.5% methylcellulose/H<sub>2</sub>O as a vehicle (group C) or 100 mg/kg of

HCA3551 in methylcellulose twice a day via oral gavage per mouse from -3 to 14 dpi in the induction phase (group D) and from 11 to 41 dpi in the effector phase (group E). Clinical signs of TMEV-IDD were significantly suppressed in mice treated with HCA3551 in the effector phase (group E) from 21 to 41 dpi compared with control mice administered methylcellulose ( $*p < 0.05$ ; at 21 dpi and from 32 dpi to 41 dpi,  $**p < 0.01$ ; from 22 dpi to 31dpi). HCA3551 did not suppress TMEV-IDD when it was administered in the induction phase. Thus, the significant suppression of TMEV-IDD was observed only when HCA3551 was administered to mice with TMEV-IDD in the effector phase. The data are presented as the means  $\pm$  SD.

Fig. 3.

Histological findings. Hematoxylin-eosin (H&E) staining (1A - D); Klüver-Barerra's (KB) staining (1E - H); and immunohistochemical staining for CD3 as a marker of T cells (1I and 1J), CD45R as a marker of B cells (1K and 1L), and CD68 as a marker of macrophages (1M and 1N). The mice were blindly selected from groups A and B (Table 1) prior to histological examination and sacrificed at 40 dpi. Longitudinal sections of spinal cords from the control mice administered with methylcellulose (1A, 1C, 1E, 1G, 1I, 1K and 1M), or from mice treated with orally active small molecule  $\alpha 4$  integrin antagonist, HCA3551, in the effector phase (1B, 1D, 1F, 1H, 1J, 1L and 1N). At 40 dpi, spinal cord sections from control mice administered methylcellulose showed severe perivascular and parenchymal MNC infiltration and demyelination (1A and 1G). However, the sections of spinal cords from mice treated with HCA3551 in the effector phase showed less severe perivascular and

parenchymal MNC infiltration and demyelination (1B and 1H). Furthermore, less infiltration of CD3<sup>+</sup> T cells (1J), CD45R<sup>+</sup> B cells (1L) and CD68<sup>+</sup> macrophages (1N) were observed in HCA3551 treated-mice compared with methylcellulose-treated control mice (1I, 1K and 1M). Original magnification, ×100 (1A, 1B, 1E and 1F), and ×200 (1C, 1D, 1G, 1H and 1I-1N). LIs for CD3, CD45R and CD68 in the CNS. The data are shown for CD3 (2A), CD45R (2B) and CD68 (2C). The LIs for CD3, CD45R and CD68 antigen were significantly decreased in the CNS of mice treated with HCA3551 compared with methylcellulose-treated mice (\**p*<0.05) (2A - 2C). The data are presented as the means ± SD. To examine the effects of HCA3551 in the effector phase on the infiltration of inflammatory MNCs into the CNS, the brains and spinal cords from mice administered with methylcellulose or HCA3551 in the effector phase were obtained at 40 dpi in Exp. 1 and 41 dpi in Exp. 2. The numbers of infiltrated MNCs isolated from CNS of mice were counted. The numbers of MNCs infiltrated into the CNS of mice administered HCA3551 in the effector phase (n=14) were significantly decreased compared with mice administered methylcellulose (n=17) ( $1.03 \pm 0.18 \times 10^6$  and  $1.89 \pm 0.15 \times 10^6$ , respectively) (\*\**p*<0.01) (3A). The data are presented as the means ± SD.

Fig. 4.

CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses to viral epitopes at 40 dpi. Intracellular cytokine staining was performed on MNCs isolated from the CNS of mice administered methylcellulose or HCA3551 in the effector phase at 40 dpi. After stimulation with CD4<sup>+</sup> (VP2<sub>70-86</sub>) or CD8<sup>+</sup> (VP3<sub>159-166</sub>) T cell epitopes of TMEV (2 μM for each peptide) for 6 hours, the cells were triple-labeled with

CD4/IFN- $\gamma$ /IL-4 or CD4/TNF- $\alpha$ /IL-10, or double-labeled with CD4/IL-17A or CD8/IFN- $\gamma$  for flow cytometry on a FACS Canto II. (A) Representative FACS histograms of infiltrating CD4<sup>+</sup> or CD8<sup>+</sup> T cells into the CNS were shown. Numbers in the FACS histograms represent % of IFN- $\gamma$ -, TNF- $\alpha$ -, IL-4-, IL-10- or IL-17A-producing CD4<sup>+</sup> T cells among total CD4<sup>+</sup> T cells and % of IFN- $\gamma$ -producing CD8<sup>+</sup> T cells among total CD8<sup>+</sup> T cells. (B) The total numbers of cytokine-producing CD4<sup>+</sup> or CD8<sup>+</sup> T cells in the CNS are shown. The numbers of TNF- $\alpha$ -producing CD4<sup>+</sup> and IFN- $\gamma$ -producing CD8<sup>+</sup> T cells were significantly decreased in mice treated with HCA3551 in the effector phase (n=13) compared with the control mice administered methylcellulose (n=9) (\* $p$ <0.05 and \*\* $p$ <0.01, respectively). The data are presented as the means  $\pm$  SD.

Fig. 5.

Viral replication levels in the CNS of TMEV-infected mice administered HCA3551 or methylcellulose in the effector phase. Relative expression levels of TMEV-mRNA in the CNS of each group at 40 dpi. The transcriptional levels of TMEV in the CNS were measured using real-time RT-PCR. The data represent the expression levels normalized to  $\beta$ -actin. There was no significant difference in the mRNA level of TMEV between methylcellulose-treated control mice and HCA3551-treated mice. The data are represented as the means  $\pm$  SD.

Table 1.

Summary of effects of administration of HCA3551, orally active small antagonist molecules against  $\alpha 4$  integrin, on TMEV-IDD.

	Group	Treatment	Number of mice	Day of treatment	Incidence	Mortality	MDO $\pm$ S.D. (Day)	MMS $\pm$ S.D. (Day)	MCS $\pm$ S.D. (Day)
Experiment 1.	A	Methylcellulose (positive control) 100 mg/kg	25	Days 11 to 40	25/25 (100%)	0/25 (0%)	18.4 $\pm$ 3.24	2.46 $\pm$ 0.61	42.53 $\pm$ 11.8
	B	HCA3551 (effector phase)	25	Days 11 to 40	25/25 (100%)	0/25 (0%)	22.0 $\pm$ 3.71**	1.67 $\pm$ 0.34**	27.97 $\pm$ 5.04**
Experiment 2.	C	Methylcellulose (positive control) 100 mg/kg	6	Days -1 to 14	6/6 (100%)	0/6 (0%)	18.0 $\pm$ 1.76	3.42 $\pm$ 0.67	61.08 $\pm$ 8.43
	D	HCA3551 (induction phase)	7	Days 11 to 41	16/16 (100%)	0/16 (0%)	20.1 $\pm$ 1.23	3.04 $\pm$ 0.46	52.11 $\pm$ 9.86
	E	100 mg/kg HCA3551 (effector phase)	7	Days 11 to 41	22/22 (100%)	0/22 (0%)	20.6 $\pm$ 3.13	1.93 $\pm$ 1.05*	37.21 $\pm$ 17.07*

MDO: Mean Day of Onset.

MMS: Mean maximum Clinical Score.

MCS: Mean Cumulative Clinical Score. \*Significant difference between Group A and B or E and F ( $p < 0.05$ )\*\*Significant difference between Group A and B ( $p < 0.01$ )

Table 2.

List of forward and reverse primers.

Primer name		Sequence 5'-3'
$\alpha$ 4 integrin	forward	CCCAGGCTACATCGTTTTTGT
	reverse	CATGAATGGGGGTAAGGATG
$\beta$ -actin	forward	TCTAGACTTCGAGCAGGAGATG
	reverse	GTTGGCATAGAGGTCTTTACGG
TMEV	probe	AM-CGCGCGCCAAAAGCAAGC-TAMRA
	forward	TTGAGCTCTCTGAGGGTGAACA
	reverse	AGAACCTTCCCGCCTCCTTTC

Fig. 1.

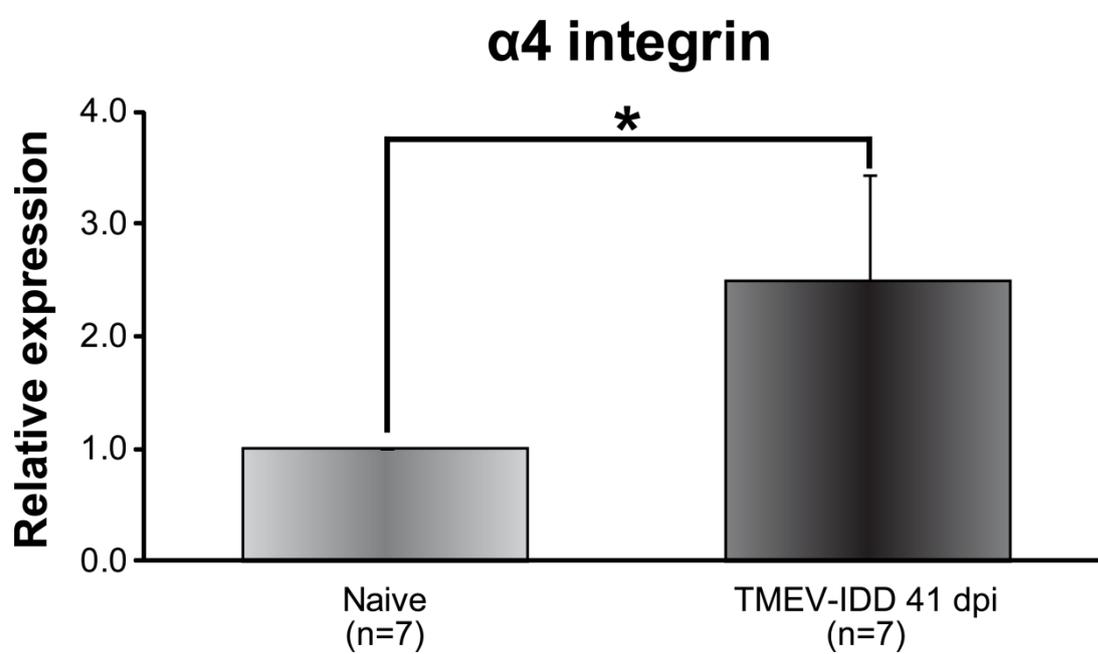
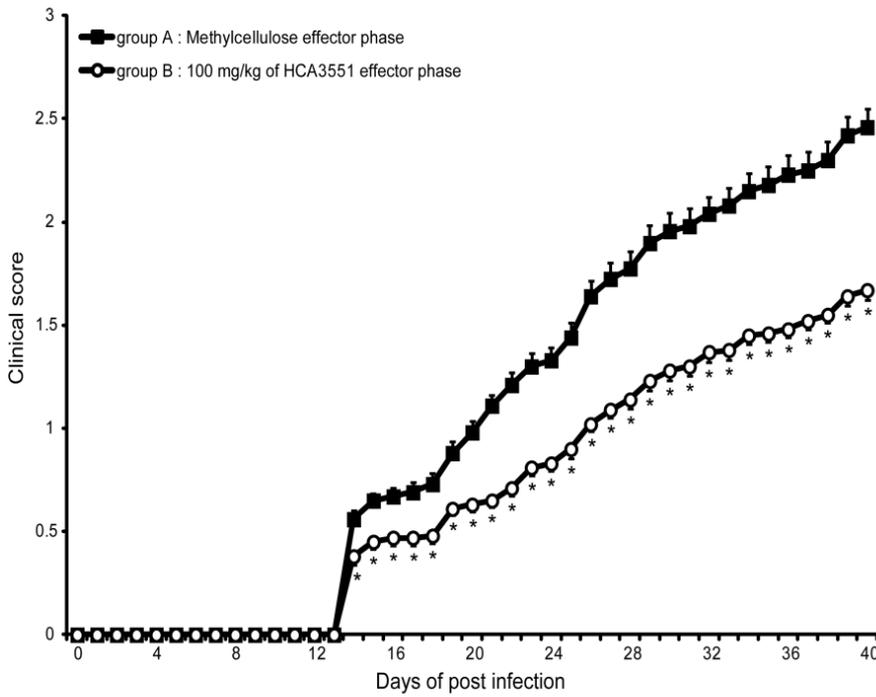
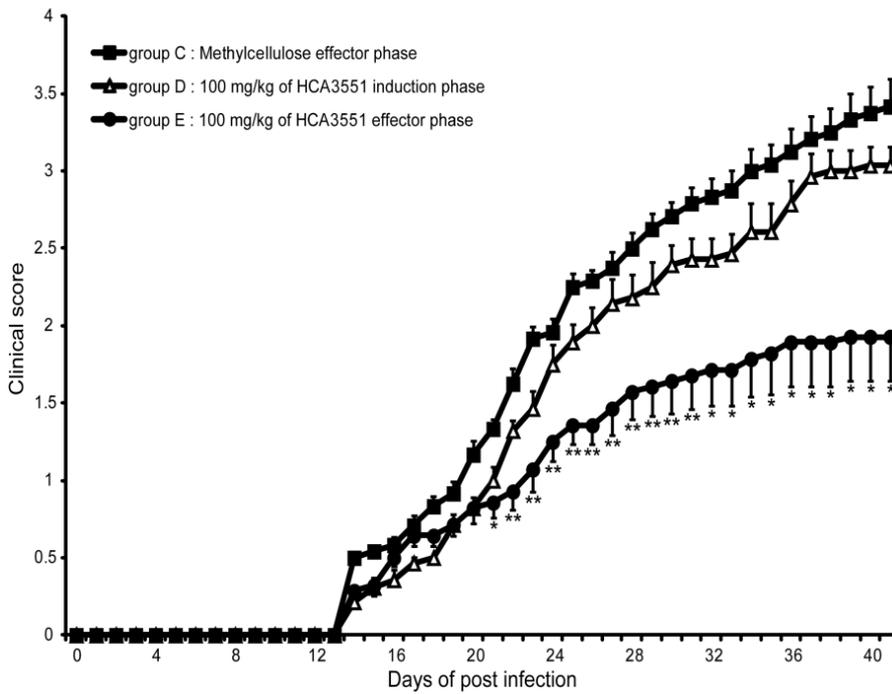


Fig. 2.

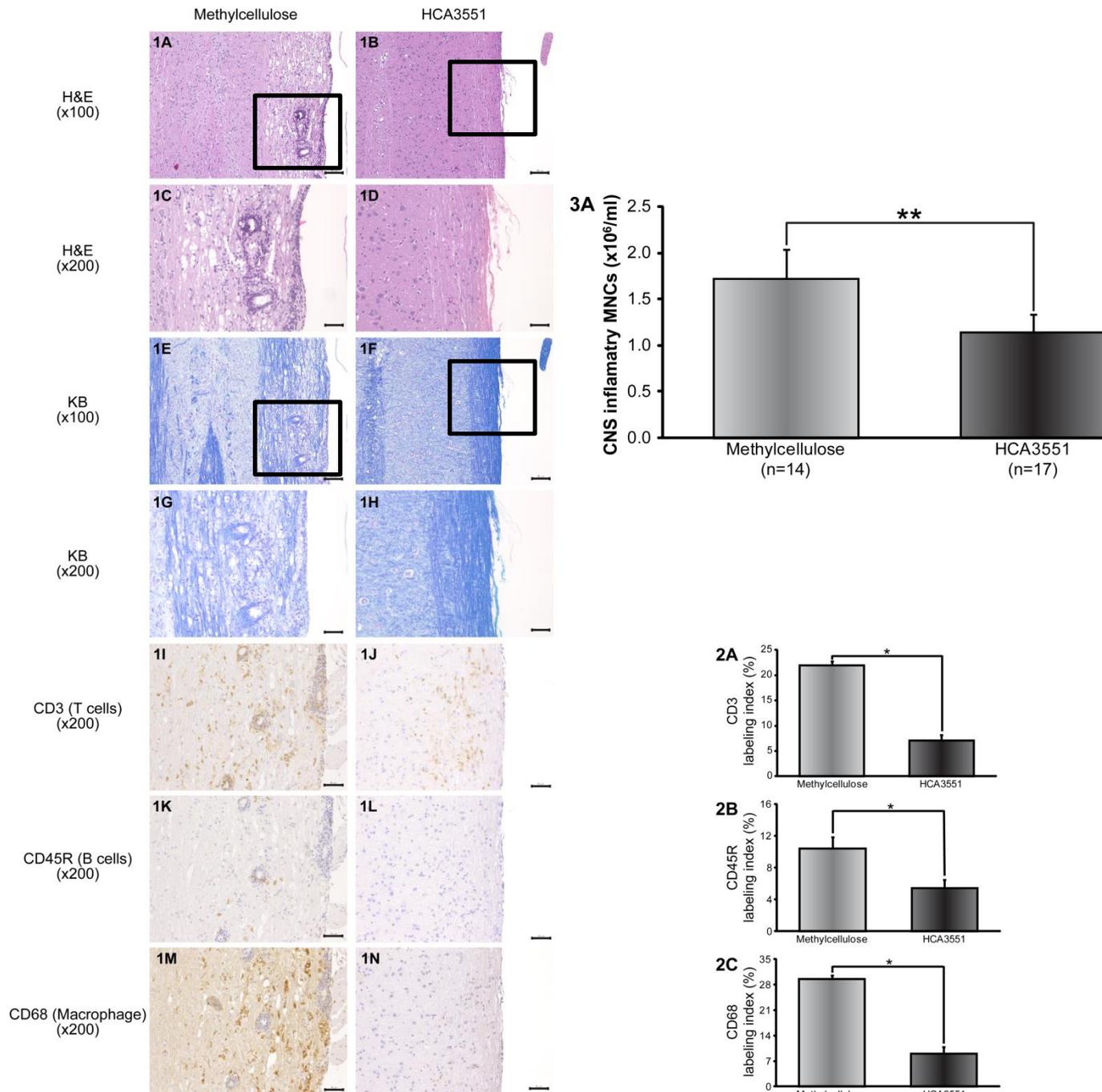
## Exp. 1



## Exp. 2



revised Fig. 3.



revised Fig. 4.

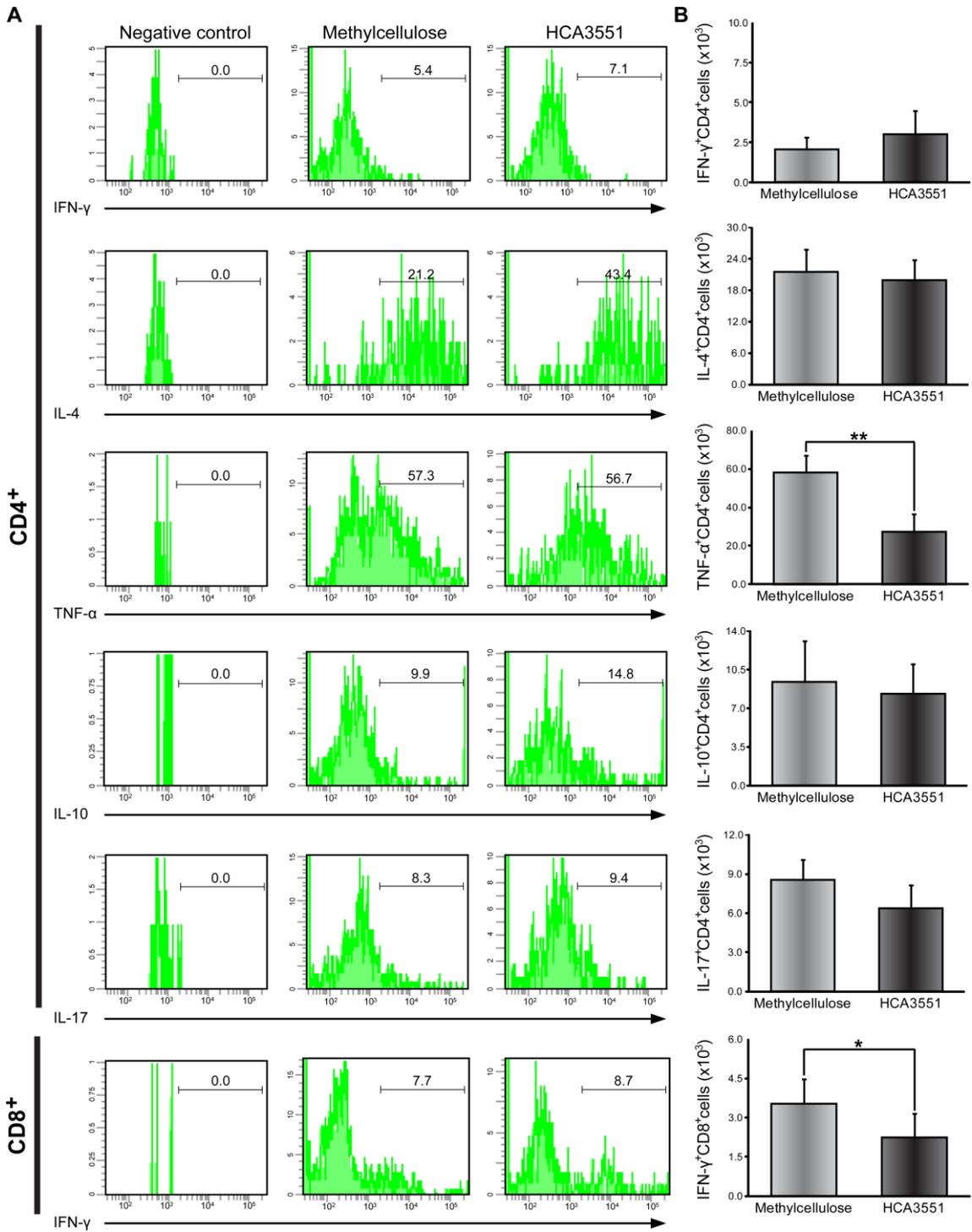


Fig. 5.

