**Supplementary Materials**

**Brief Report**

# Immunoregulation of Theiler's virus-induced demyelinating disease by Glatiramer Acetate without Suppression of Anti-Viral Immune Responses

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## Materials and Methods

### Animal experiments

Female 5-week-old SJL/J mice (Jackson Laboratory, Bar Harbor, ME) were infected intracerebrally with $2 \times 10^5$ plaque forming units (PFUs) of the Daniels (DA) strain of Theiler's murine encephalomyelitis virus (TMEV), as described previously [1]. TMEV-infected mice received intraperitoneal injections daily with a low (0.15 mg/mouse) or high dose (2 mg/mouse) of glatiramer acetate (GA, Teva Pharmaceuticals, Petach Tikva, Israel) for 4 weeks (days 0 to 27, Whole) or during the acute (days 0 to 6, Early) or chronic phase (days 21 to 27, Late) of TMEV infection. All TMEV-infected (4-6 mice per group) mice were monitored daily and euthanized 5 to 6 weeks post infection (p.i.). The two doses of GA used in this study, 0.15 mg/mouse and 2 mg/mouse, were two standard doses that have been widely used in previously published reports investigating the efficacy of GA in experimental autoimmune encephalomyelitis (EAE) [2]. The treatment schedule and the timing of euthanasia were based on our previous manuscripts on TMEV-induced demyelinating disease (TMEV-IDD) with various immunomodulatory treatments [3]. The control groups included TMEV-infected mice without GA treatment (TMEV alone group) and GA-treated mice without TMEV infection. Mice were maintained under specific pathogen-free conditions in our animal care facility.

Since TMEV-infected mice do not show oblivious clinical signs until the late chronic phase, we evaluated the clinical signs of TMEV-infected mice using impaired righting reflex scores; the proximal end of the mouse's tail was grasped and twisted to the right and then to the left (0, a healthy mouse resists being turn over; 1, the mouse is flipped onto its back but immediately rights itself on one side; 1.5, the mouse is flipped onto its back but immediately rights itself on both sides; 2, the mouse rights itself in 1 to 5 seconds; 3, righting takes more than 5 seconds; and 4, the mouse cannot right itself) [4]. Positive impaired righting scores reflect polioencephalitis in the brain during the acute phase and inflammatory demyelination in the spinal cord during the chronic phase.

### Immunohistochemistry

TMEV-infected mice were perfused with phosphate-buffered saline (PBS) followed by a 4% paraformaldehyde (PFA, Sigma-Aldrich, St. Louis, MO) solution in PBS. After the PFA fixation, the spinal cord was harvested, divided into 10 to 12 transversal segments, and embedded in paraffin. The spinal cord tissues were sliced at 4 μm-thick using an HM 325 Rotary Microtome (Thermo Fisher Scientific Inc., Waltham, MA). TMEV antigens in spinal cord sections were visualized by immunohistochemistry with hyperimmune serum against TMEV [4] using a Histofine MAX-PO kit (Nichirei Biosciences, Tokyo, Japan) [5]. The numbers of TMEV antigen-positive cells were counted under a light microscope [6].

### Lymphoproliferative assays

Mononuclear cells (MNCs) were isolated from the spleen of TMEV-infected mice using Histopaque®-1083 (Sigma-Aldrich) [7]. MNCs were cultured in RPMI 1640 medium (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Mediatech), 2 mM L-glutamine (Mediatech), 50 μM β-mercaptoethanol (Sigma-Aldrich), and 1% antibiotic-antimycotic solution (Mediatech), at $2 \times 10^5$ cells/well in 96-well plates (Corning, Inc., Corning, NY). We stimulated MNCs with $2 \times 10^5$ cells/well of TMEV-infected antigen presenting cells (TMEV-APCs) or mock-infected antigen presenting cells (mock-APCs), or 50 μg/ml of GA in the presence or absence of anti-CD4 [GK1.5, American Type Culture Collection, (ATCC), Manassas,

VA] or anti-CD8 (Lyt2.43, ATCC) antibody for 5 days [7]. The concentration of GA was based on previously published manuscripts on GA as well as our standard antigen-specific lymphoproliferation assay protocol [8]. TMEV-APCs were made from whole spleen cells infected *in vitro* with TMEV at a multiplicity of infection (MOI) of 1, while mock-APCs were made from mock-infected spleen cells. Both TMEV-APCs and mock-APCs were incubated overnight and irradiated with 2,000 rads using a $^{137}$Cs irradiator (J.L. Shepherd & Associates, San Fernando, CA). To assess the levels of lymphoproliferative responses, [$^{3}$H]thymidine (PerkinElmer, Inc., Waltham, MA) was added to the culture at the concentration of 1 μCi/well for the last 24 hours. MNCs were harvested on Reeves Angel 934AH filters (Brandel, Gaithersburg, MD) using a PHD™ Harvester 200A (Brandel). The incorporated radioactivity was measured using a Wallac 1409 Liquid Scintillation Counter (PerkinElmer). All cultures were performed in triplicate and the data were expressed as Δcpm (experimental cpm in TMEV-APCs or GA stimulation – control cpm in mock-APCs or no stimulation).

**Enzyme-linked immunosorbent assays (ELISAs)**

We collected blood from the heart of TMEV-infected mice 5 to 6 weeks p.i. The levels of serum anti-TMEV or anti-GA antibodies were assessed by ELISAs, as described previously [9, 10]. We coated 96-well flat-bottom Nunc-Immuno plates (Thermo Fisher Scientific) with 10 μg/ml of TMEV antigen or GA. Serial dilutions of sera were added to the plates followed by a peroxidase-conjugated anti-mouse IgG1 or IgG2c antibody (Thermo Fisher Scientific). Immunoreactive complexes were detected with *o*-phenylendiamine dihydrochloride (Sigma-Aldrich) and absorbances were read at 492 nm on a Multiskan MCC/340 Microplate Reader (Thermo Fisher Scientific).

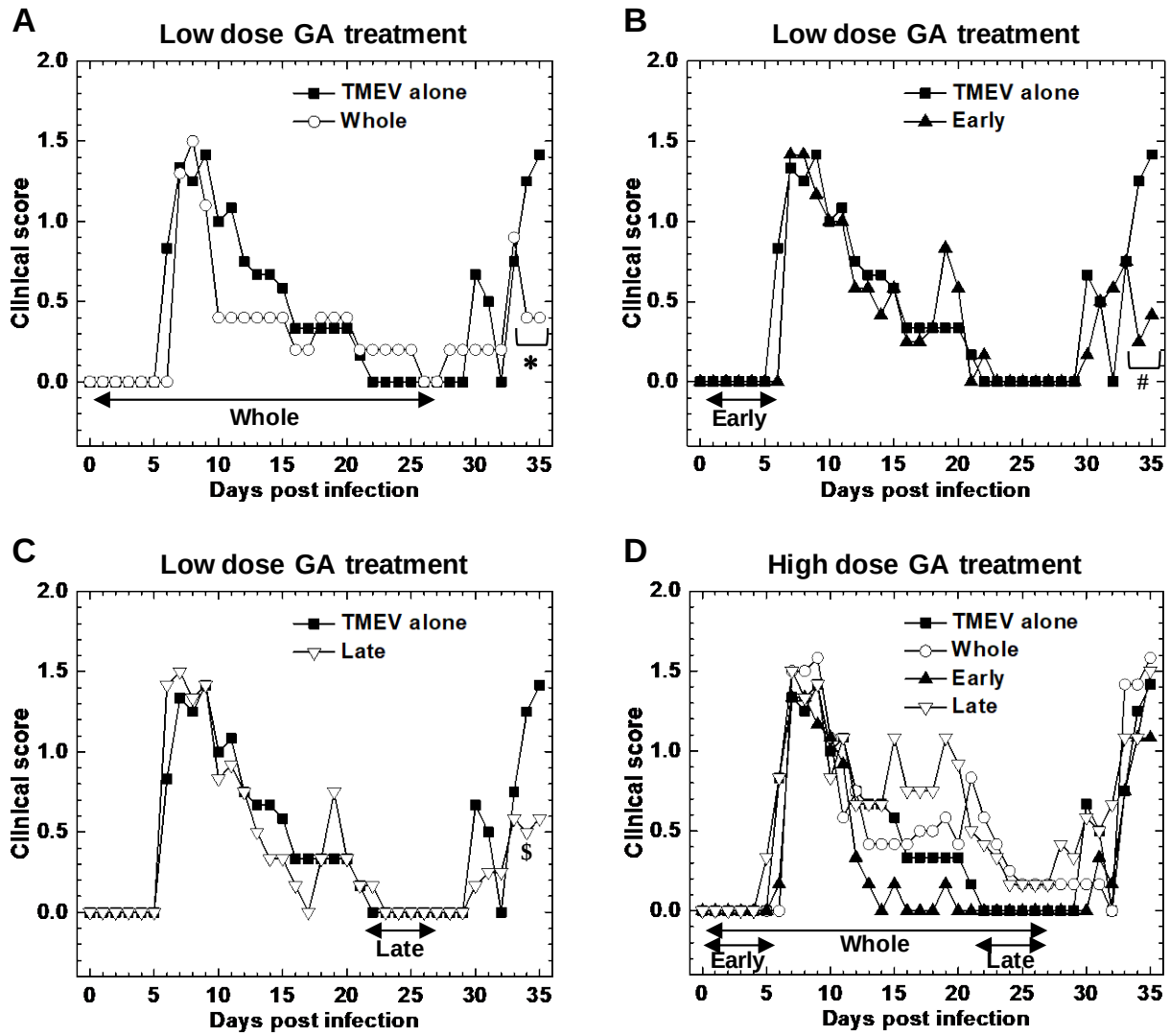
For cytokine assays, MNCs isolated from the spleen of TMEV-infected mice were cultured at $8 \times 10^6$ cells/well in 6-well-plates (Corning) and stimulated with 5 μg/ml of concanavalin A (ConA) or 50 μg/ml of GA for 2 days. The concentrations of interleukin (IL)-10 (BD Biosciences, San Diego, CA), IL-4 (BD Biosciences), interferon (IFN)-γ (BD Biosciences), and IL-17A (BioLegend, San Diego, CA) in the culture supernatants were quantified using ELISA kits, according to the manufacturer's instructions [7].

**Real-time polymerase chain reaction (PCR)**

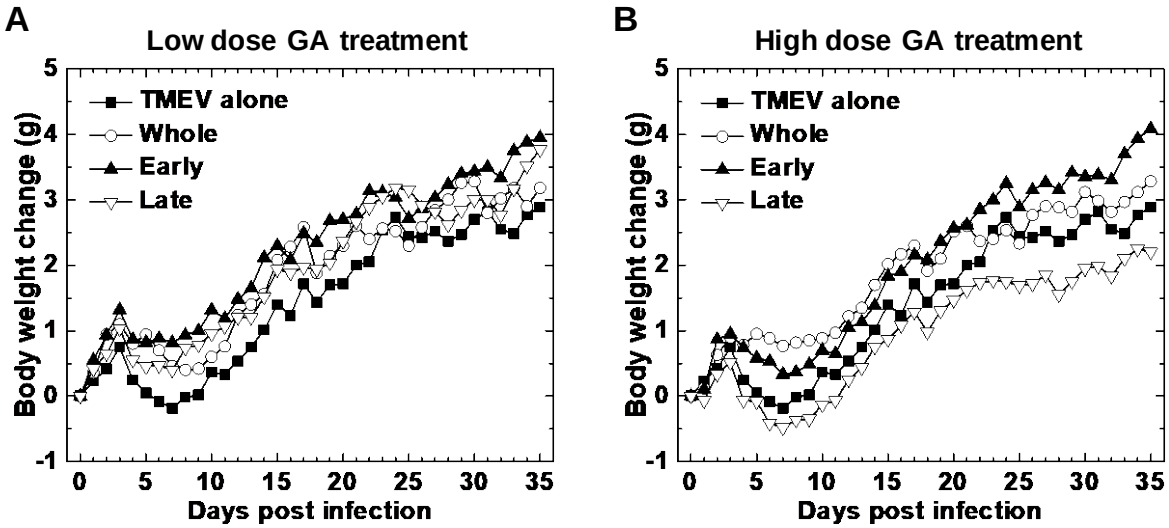
TMEV-infected mice were perfused with PBS. The spinal cord was harvested, frozen with liquid nitrogen, and homogenized in TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH) [11]. The total RNA was isolated from the homogenates using RNeasy® Mini Kits (Qiagen, Inc., Valencia, CA), according to the manufacturer's instruction. We reverse-transcribed 1 μg of the total RNA into cDNA using the ImProm-II™ Reverse Transcription System (Promega, Corp., Madison, WI), and then conducted real-time PCR with 50 ng of the cDNA using an RT$^2$ Fast SYBR® Green qPCR Master Mix (SABiosciences, Valencia, CA) and the MyiQ2 Two-Color Real-time PCR Detection System (Bio-Rad, Hercules, CA). To determine gene expression related to regulatory T cells (Tregs) and T helper (Th) 17 cells, we used the following primer pairs (Real Time Primers, LLC, Elkins Park, PA): *Foxp3*, forward (5′-GCTGGAGCTGGAAAAGGAGA-3′) and reverse (5′-GTGGCTACGATGCAGCAAGA-3′); and *Il17a*, forward (5′-CGCAAACATGAGTCCAGGGAGAGC-3′) and reverse (5′-TCAGGGTCTTCATTGCGGTGGAG-3′) [12]. We also used the following primer pair as a housekeeping gene for normalization; phosphoglycerate kinase 1 (*Pgk1*), forward (5′-GCAGATTGTTTGGAATGGTC-3′) and reverse (5′-TGCTCACATGGCTGACTTTA-3′).

**Statistical analyses**

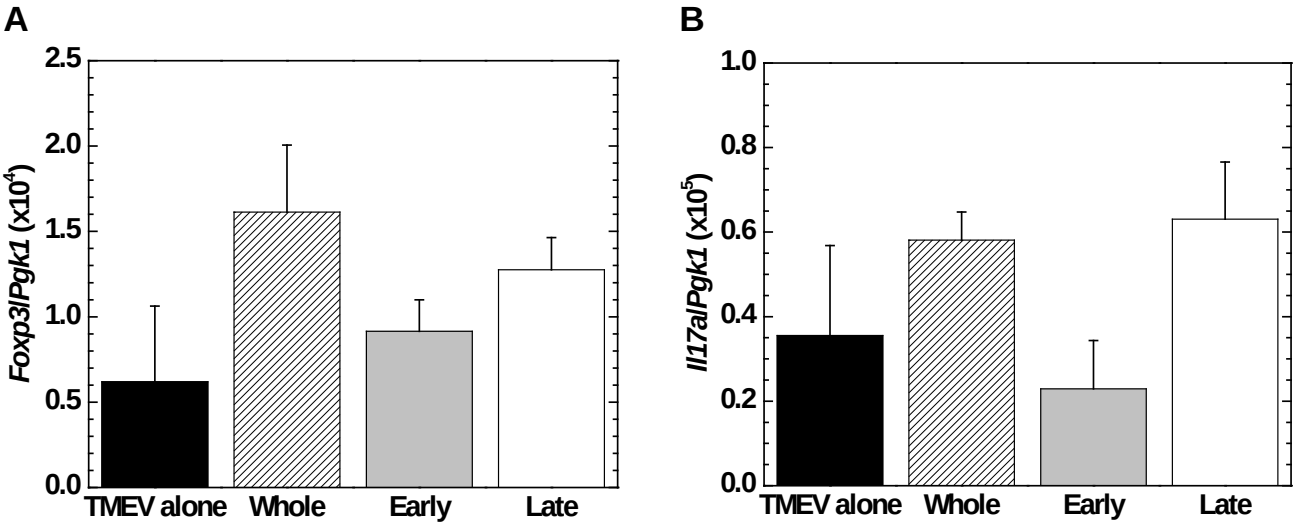
To determine statistical differences, the Kruskal-Wallis test and analysis of variance (ANOVA) were conducted for nonparametric data and parametric data using the OriginPro 8.1 (OriginLab Corporation, Northampton, MA). Data are shown as mean + standard error of the mean (SEM).



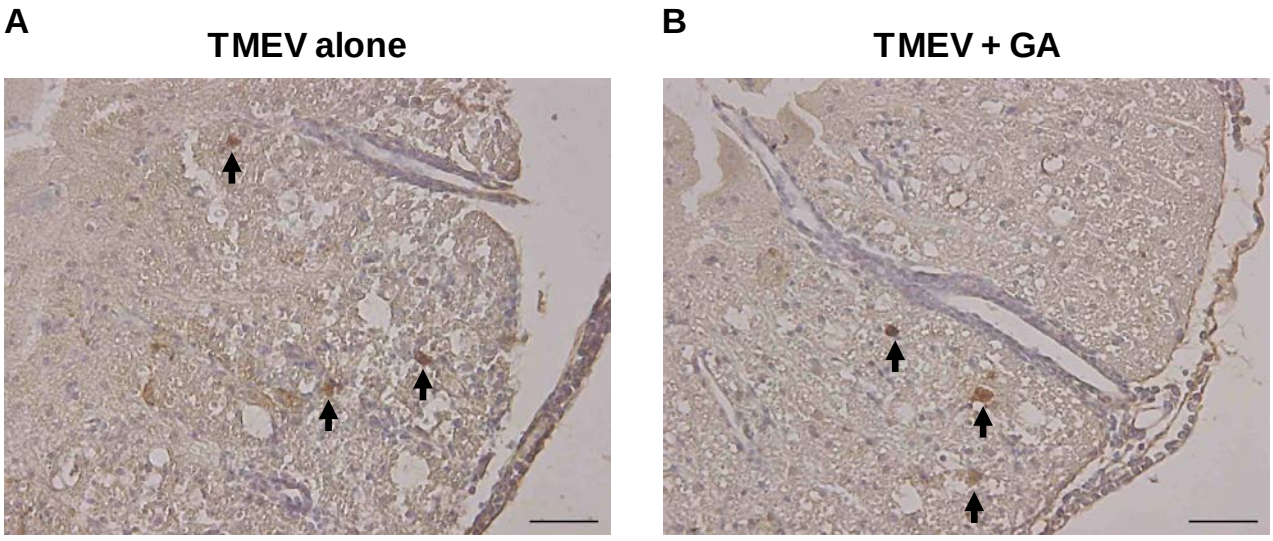

**Supplementary Fig. 1.** Modulation of clinical signs by glatiramer acetate (GA) treatment in a viral model of multiple sclerosis (MS), Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD). (**A-D**) Mice were infected with TMEV on day 0 and treated daily with GA for 4 weeks (days 0 to 27, Whole, ○), during the acute phase (days 0 to 6, Early, ▲), or during the chronic phase (days 21 to 27, Late, ▽) of TMEV infection. Control mice had TMEV infection without GA treatment (TMEV alone, ■). The clinical scores of TMEV-IDD were evaluated by impaired righting reflex scores. Results are representative of two independent experiments, which were conducted in a blind fashion, and expressed as mean clinical scores. Each experiment was composed of five to six mice per group. (**A-C**) Low dose treatment (0.15 mg/mouse) of GA. $P < 0.05$, Mann-Whitney $U$ test. (**D**) High dose GA treatment (2 mg/mouse). Note: since the Late group did not have GA treatment until day 21 post infection (p.i.), the Late group received only TMEV till day 20 p.i. Here, the clinical scores in the Late group should be similar to those in the TMEV alone group; the clinical differences between these two groups till day 20 p.i. must be interpreted as variation within mice receiving TMEV alone. Till day 20 p.i., the Whole group showed a similar clinical course to the control TMEV alone and Late groups. In contrast, the Early group tended to recover earlier than the other groups



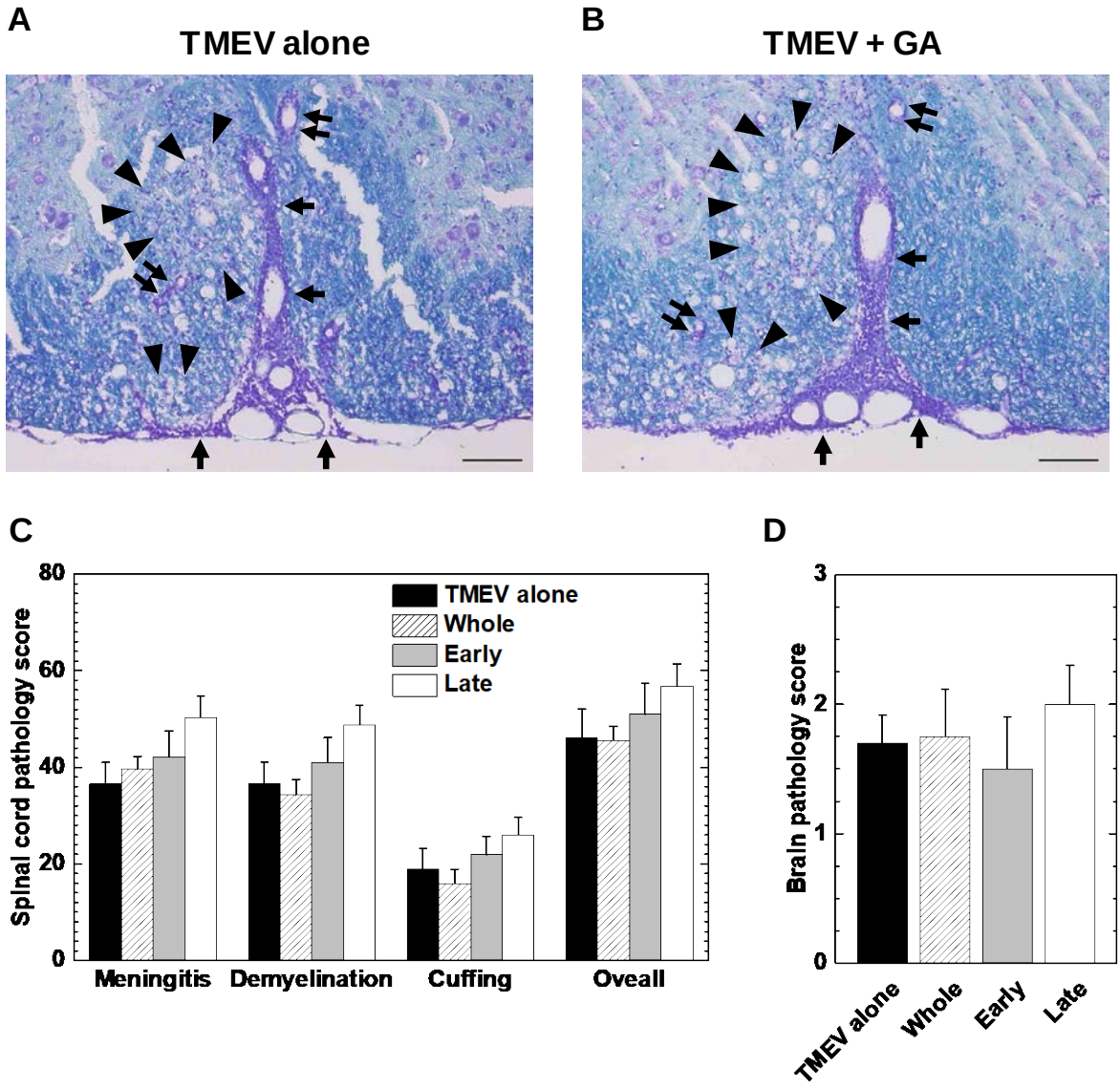

**Supplementary Fig. 2.** Effects of GA treatment on body weight changes after TMEV infection. Low dose treatment (**A**) and high dose treatment (**B**) of GA. TMEV alone (■), Whole (○), Early (▲), and Late (▽). Body weight changes were monitored daily. Results are representative of two independent experiments and expressed as mean body weight changes. Each experiment was composed of five to six mice per group




**Supplementary Fig. 3.** Effects of GA on forkhead box P3 (*Foxp3*) and interleukin (IL)-17A (*Il17a*) expression in the central nervous system (CNS). Real-time PCR analyses of *Foxp3* [regulatory T cell (Treg) marker, **A**] and *Il17a* [T helper (Th) 17 cell marker, **B**] in the spinal cord from control and GA-treated mice, 5 to 6 weeks p.i. Phosphoglycerate kinase 1 (*Pgk1*) expression was used as a housekeeping gene for normalization. Results are the averages expressed as mean expression levels + standard error of the mean (SEM). Each group was composed of three to four mice




**Supplementary Fig. 4.** Effects of GA on viral persistence in the CNS. Immunohistochemistry with hyperimmune serum against TMEV in the spinal cord from control (TMEV alone, **A**) and GA-treated (TMEV + GA, **B**) mice, 5 to 6 weeks p.i. Arrows indicate TMEV antigen-positive cells. Tissue sections are from the TMEV alone and Late groups, whose location of viral antigen was similar to those in the Early and Whole groups, and representative of two independent experiments. Scale bar = 50 μm




**Supplementary Fig. 5.** Effects of GA on neuropathology in control and GA-treated mice. (**A, B**) Luxol fast blue staining. Arrowheads, arrows, and paired arrows indicate demyelination, meningitis, and perivascular cuffing in the ventral funiculus of the spinal cord from control (TMEV alone, **A**) and GA-treated (TMEV + GA, **B**) mice, 5 to 6 weeks p.i. Tissue sections are from the TMEV alone and Late groups, whose neuropathology was similar to those in the Early and Whole groups, and representative of two independent experiments. Scale bar = 100 μm. (**C, D**) Spinal cord (**C**) and brain (**D**) pathology scores among the four groups. Results are the averages of two independent experiments expressed as the mean + SEM. Each experiment included four to six mice per group

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