



Immunoregulation of Theiler's virus-induced demyelinating disease by glatiramer acetate without suppression of antiviral immune responses

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Abstract

While most disease-modifying drugs (DMDs) regulate multiple sclerosis (MS) by suppressing inflammation, they can potentially suppress antiviral immunity, causing progressive multifocal leukoencephalopathy (PML). The DMD glatiramer acetate (GA) has been used for MS patients who are at high risk of PML. We investigated whether GA is safe for use in viral infections by using a model of MS induced by infection with Theiler's murine encephalomyelitis virus (TMEV). Treatment of TMEV-infected mice with GA neither enhanced viral loads nor suppressed antiviral immune responses, while it resulted in an increase in the *Foxp3/Il17a* ratio and IL-4/IL-10 production. This is the first study to suggest that GA could be safe for MS patients with a proven viral infection.

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Introduction

Viral infections are considered to trigger multiple sclerosis (MS), which is an inflammatory demyelinating disease of the central nervous system (CNS). Clinically, several viruses, including human herpesvirus 6, have been isolated from MS patients, and higher antiviral immune responses have been reported in MS patients than in healthy controls [1]. Experimentally, viral infections have been shown to induce demyelination due to direct lytic viral infection of the CNS (viral pathology) and/or recruitment of inflammatory cells into the CNS (immunopathology) [2, 3].

Theiler's murine encephalomyelitis virus (TMEV) is a non-enveloped, positive-sense, single-stranded RNA virus that belongs to the family *Picornaviridae*. TMEV is widely used as a viral model of MS, since TMEV infection induces chronic inflammatory demyelination in the CNS that resembles MS neuropathologically [4]. In the TMEV model, during the acute phase, around 1 week postinfection (p.i.), TMEV predominantly infects neurons in the brain and induces acute polioencephalomyelitis [5]. During the sub-clinical phase, 2–3 weeks p.i., although TMEV is largely cleared from the brain by antiviral T-cell and antibody responses, TMEV is axonally transported to the spinal cord [6]. Around 4 weeks p.i. (early chronic phase), mice begin to develop inflammatory demyelination in the spinal cord, where persistent viral infection in macrophages and glial

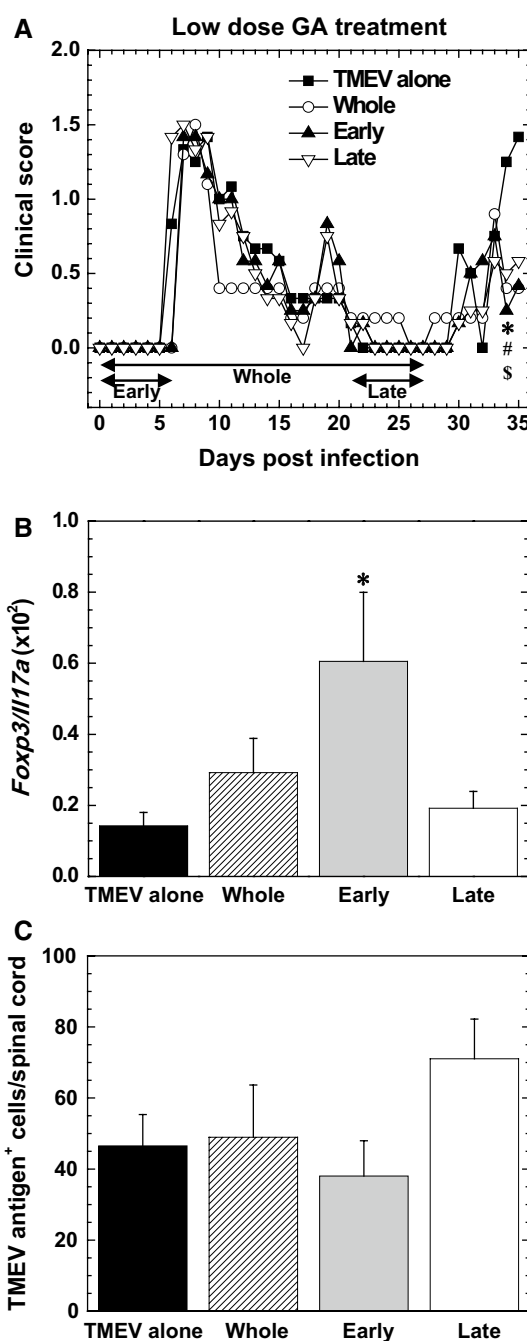
Fig. 1 Safety of glatiramer acetate (GA) treatment for Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD). **A** Clinical scores of TMEV-IDD. Mice were infected with TMEV on day 0 and treated daily with a low dose of GA for 4 weeks (days 0 to 27, Whole, unfilled circles), during the acute phase (days 0 to 6, Early, filled triangles), or during the chronic phase (days 21 to 27, Late, open inverted triangle) of TMEV infection. Control mice had TMEV infection without GA treatment (TMEV alone, filled squares). Clinical scores were evaluated by impaired righting reflex scores (Supplementary Methods). $P < 0.05$, TMEV alone versus *Whole, #Early, and \$Late, Kruskal-Wallis test. Results are representative of two independent experiments and expressed as mean clinical scores. Each experiment included five to six mice per group. **B** Ratios of *Foxp3* to *Il17a* levels in the spinal cord 5 to 6 weeks postinfection (p.i.). Levels of *Foxp3* and *Il17a* were semi-quantified by real-time PCR. *, $P < 0.05$, ANOVA. Results are the mean ratios + standard error of the mean (SEM). Each group was composed of three to four mice. **C** Numbers of viral antigen-positive cells visualized by immunohistochemistry in the spinal cord at 5 to 6 weeks p.i. Results are the averages of two independent experiments expressed as the mean + SEM. Each experiment included four to six mice per group

cells and antiviral immunity contribute to pathogenesis [7, 8].

Immunomodulatory drugs, including natalizumab and fingolimod, have been used as disease-modifying drugs (DMDs) for MS patients, since DMDs reduce their disease activity and progression [9]. While DMDs are beneficial in MS by regulating immunopathology, they sometimes cause CNS viral reactivation syndrome (e.g., progressive multifocal leukoencephalopathy [PML]) by suppressing antiviral immunity [10]. The DMD glatiramer acetate (GA) has never been linked to PML, but it has anti-inflammatory effects, resulting in enhanced interleukin (IL)-4 and IL-10 production and increased induction of regulatory T cells (Tregs) expressing the forkhead box P3 (Foxp3) transcription factor.

In a study of TMEV-induced demyelinating disease (TMEV-IDD), Ure et al. [11] tested the effects of GA, focusing mainly on remyelination during the late chronic phase, 27–52 weeks p.i., since a lack of remyelination has been proposed to explain a lack of recovery from TMEV-IDD during the late chronic phase, but not during the early chronic phase. The authors demonstrated that passive transfer of anti-GA antibodies to infected mice significantly enhanced remyelination without alteration of demyelination, although GA treatment itself did not affect remyelination or demyelination despite the induction of anti-GA antibodies. Thus, large gaps in our knowledge exist as to whether and how GA could be beneficial in the treatment of virus-induced demyelinating diseases and could suppress pro-inflammatory responses without suppressing antiviral immune responses.

We first determined whether GA treatment could affect TMEV-IDD during the early chronic phase. SJL/J mice were infected with TMEV on day 0 and injected daily with a low dose (0.15 mg/mouse) of GA on days 0 to 27 (Whole group), days 0 to 6 (Early group), or days 21 to 27 (Late group)



[12]. We monitored their clinical signs and body weight changes for 5 weeks (Supplementary Methods). During the acute phase, the infected mice, irrespective of GA treatment, had similar levels of impaired righting reflexes, indicating acute polioencephalomyelitis (Fig. 1A and Supplementary Fig. 1A–C). Around 3–4 weeks p.i., most of the mice in all groups recovered completely. During the chronic phase, the mice began to develop impaired righting reflexes, indicating the onset of TMEV-IDD. Mice from all GA-treated groups had significantly lower clinical scores than control mice without GA treatment (TMEV-alone group) ($P < 0.05$,

Kruskal-Wallis test). We repeated the experiment and confirmed the effects of low-dose GA treatment on TMEV-IDD. In the second experiment, we again observed significant differences among the four groups in their clinical scores during the early chronic phase (e.g., mean clinical score \pm standard error of the mean [SEM] on day 34: TMEV alone, 1.5 ± 0 ; Whole, 1.1 ± 0.3 ; Early, 0.8 ± 0.2 ; Late, 1.1 ± 0.2 [$P < 0.05$, Kruskal-Wallis test]), while there were no statistical differences in body weight changes among the groups (Supplementary Fig. 2).

Interestingly, treatment with a high dose of GA (2 mg/mouse) did not alter clinical signs significantly (Supplementary Fig. 1D). This suggests that the beneficial effects of GA treatment on TMEV-IDD depend on the dosage of the treatment. Although the precise mechanism is unclear, this could be due to the high-dose treatment being outside of the therapeutic window (note: the two standard ranges of doses that have been tested in mice, 0.15 mg and 2 mg per mouse, are much higher than the currently approved 20-mg daily dose used in humans [e.g., the 0.15 mg/20 g mouse dosage corresponds to 450 mg/60 kg in humans]) [13, 14]. Alternatively, some unknown adverse effects could counter the beneficial effects of GA. Thus, in subsequent studies, we treated mice with the low dose of GA to further evaluate the effects of GA treatment on TMEV-IDD.

In an autoimmune model of MS, experimental autoimmune encephalomyelitis (EAE), GA treatment has been shown to increase anti-inflammatory *Foxp3*⁺ Tregs and decrease pro-inflammatory IL-17-producing T helper (Th) 17 cells [15], resulting in amelioration of EAE. To determine whether GA treatment could also alter the ratio of Tregs to Th17 cells in TMEV-IDD, we performed a semiquantitative analysis of *Foxp3* and *Il17a* levels in the CNS by real-time PCR (Supplementary Methods). We found that the ratios of *Foxp3* to *Il17a* levels were higher in all GA-treated groups, particularly the Early group ($P < 0.05$, ANOVA), compared with the control group (Fig. 1B), while there was no statistical difference in the expression level of either mRNA among the groups (Supplementary Fig. 3). This suggests that GA treatment may regulate the clinical signs of TMEV-IDD by shifting T-cell responses from the pro-inflammatory to the anti-inflammatory pathway.

On the other hand, we demonstrated previously that Tregs can be a double-edged sword in TMEV infection [16], enhancing CNS viral loads while decreasing CNS inflammation due to the anti-inflammatory responses. To determine whether GA treatment could affect viral loads after TMEV infection, we counted viral-antigen-positive cells in the CNS by immunohistochemistry with hyperimmune serum against TMEV (Supplementary Methods). As reported previously [17], control mice from the TMEV-alone group had viral-antigen-positive cells in the white matter of the spinal cord, particularly in the ventral and lateral funiculi, during the

early chronic phase (Supplementary Fig. 4A). The number and location of viral-antigen-positive cells in all GA-treated groups were similar to those in the control group (Fig. 1C and Supplementary Fig. 4B). We also compared the levels of meningitis, parenchymal inflammation (perivascular cuffing), and demyelination in the CNS among the four groups and found no significant differences in CNS pathology (Supplementary Fig. 5). The discrepancies between CNS inflammation and clinical signs could be explained by increased migration of anti-inflammatory cells, including Tregs, which is consistent with the higher *Foxp3* expression in the CNS in all GA-treated groups than in the control group.

We next compared the levels of TMEV-specific lymphoproliferation among the groups by [³H]thymidine incorporation assays (Supplementary Methods) and found that all four groups underwent substantial lymphoproliferative responses to TMEV without statistical differences (Fig. 2). We also examined serum anti-TMEV antibody titers by enzyme-linked immunosorbent assays (ELISAs) for the IgG1 and IgG2c isotypes (Supplementary Methods) that are produced by SJL/J mice (SJL/J mice lack the IgG2a isotype). Mice from all four groups had high anti-TMEV IgG1 and IgG2c titers without statistical differences (Fig. 2). Thus, GA treatment neither increased viral loads nor suppressed antiviral immunity.

Clinically, GA treatment has been shown to induce cellular and humoral immune responses to GA in the periphery [18]. We quantified lymphoproliferative responses to GA by [³H]thymidine incorporation assays (Supplementary Methods) and detected high levels of GA-specific lymphoproliferation in all GA-treated groups, but not in the TMEV-alone group (Fig. 2). The lymphoproliferative responses to GA in all GA-treated groups were inhibited in the presence of anti-CD4 antibody, but not in the presence of anti-CD8 antibody. This was consistent with previous findings that GA binds efficiently to major histocompatibility (MHC) class II molecules, inducing GA-specific CD4⁺ T cells [14]. We also determined serum anti-GA antibody titers by ELISAs (Supplementary Methods) and found that all mice from the three GA-treated groups had significantly higher titers of anti-GA IgG1 than control mice ($P < 0.01$, ANOVA, Fig. 2). Interestingly, although all mice from the Whole group had substantial titers of anti-GA IgG2c ($P < 0.01$, ANOVA), only 6 of 14 mice (43%) in the Early group and 9 of 14 mice (64%) in the Late group had low anti-GA IgG2c titers. Thus, GA treatment induced the anti-GA IgG1 isotype (Th2 associated) more effectively than anti-GA IgG2c isotype (Th1 associated). Similarly, in GA-treated MS patients, anti-GA IgG1 titers were two- to threefold higher than anti-GA IgG2 titers [18].

Lastly, we determined whether GA treatment could alter the cytokine profile following TMEV infection. Splenic mononuclear cells (MNCs) were stimulated in vitro with

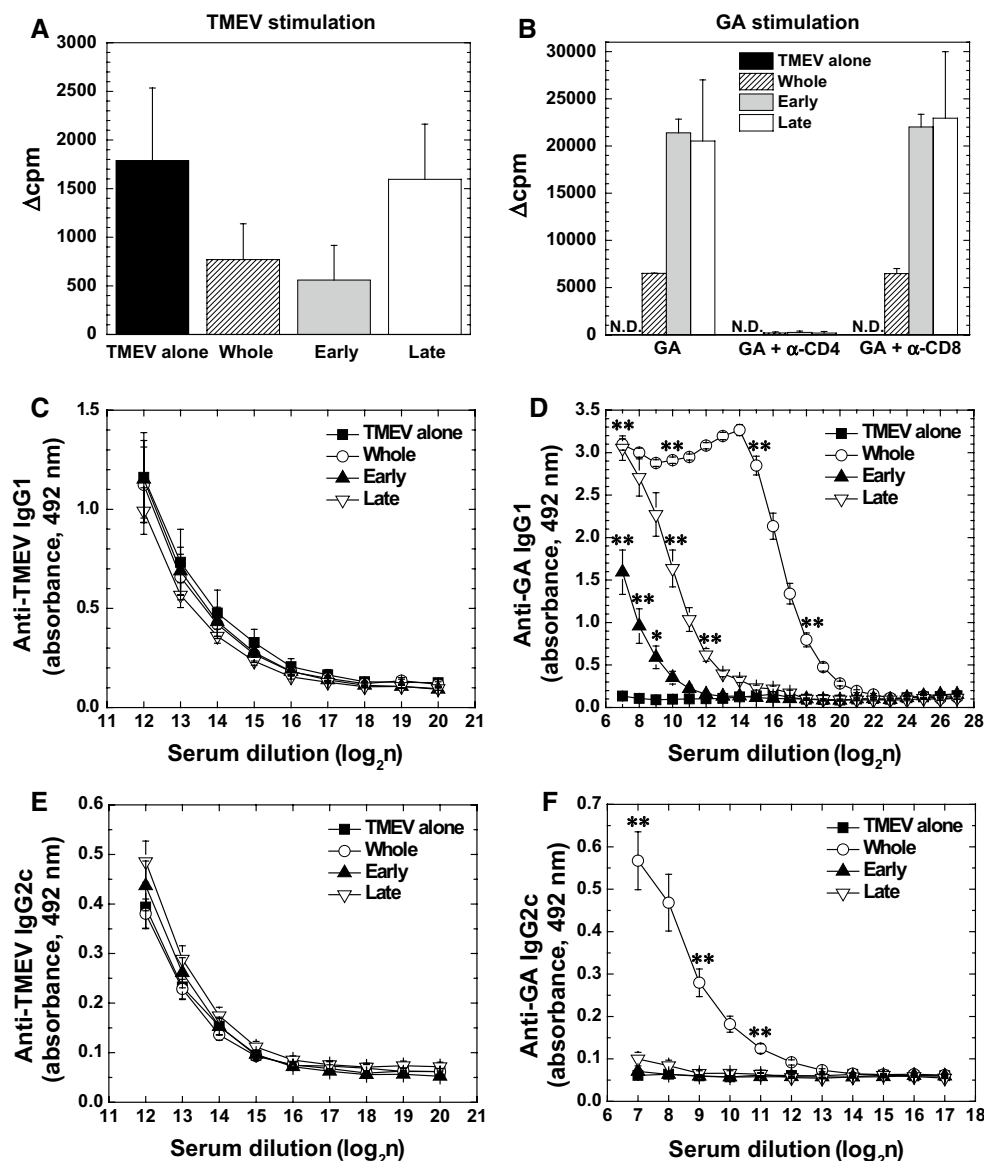


Fig. 2 GA-treatment-induced immune response to GA without alteration of the immune responses to TMEV. **A, B** Cellular immune responses to TMEV (**A**) and GA (**B**) 5 to 6 weeks p.i. Splenic mononuclear cells (MNCs) were stimulated with irradiated TMEV- or mock-infected antigen-presenting cells (APCs) or GA in the presence or absence of anti-CD4 or anti-CD8 antibody. Lymphoproliferative responses were quantified by [³H]thymidine incorporation assays and expressed as Δcpm (experimental cpm in TMEV-APCs or GA stimulation-control cpm in mock-APCs or no stimulation). Results are the average of two independent experiments expressed as the mean Δcpm ± SEM of two to three spleen pools. Each

spleen pool was composed of two to three spleens from four to six mice per group. All cultures were performed in triplicate. N.D.; not detectable. **C–F** Humoral immune responses to TMEV (**C, E**) and GA (**D, F**) 5 to 6 weeks p.i. TMEV alone, filled squares; Whole, unfilled circles; Early, filled triangles; Late, unfilled inverse triangles. Serum IgG1 and IgG2c titers for TMEV and GA were quantified by ELISA. *, $P < 0.05$; **, $P < 0.01$, ANOVA. Results are the average of two independent experiments expressed as the mean absorbance at 492 nm ± SEM. Each experiment included five to eight mice per group

a mitogen (concanavalin A [ConA]) or GA. The amounts of anti-inflammatory IL-10/IL-4 and pro-inflammatory interferon (IFN)-γ/IL-17 in the culture supernatants were quantified by ELISAs (Supplementary Methods). In ConA stimulation, the amounts of IL-10 were significantly higher in all GA-treated groups (Whole, $P < 0.01$; Early and Late,

$P < 0.05$, ANOVA) than in the TMEV-alone group (Fig. 3). The amounts of IL-4 were also higher in the Whole and Late groups (Late, $P < 0.05$, ANOVA), but not in the Early group, when compared with the TMEV-alone group. All GA-treated groups had lower amounts of IFN-γ than the TMEV-alone group, without statistical differences. The amounts of IL-17

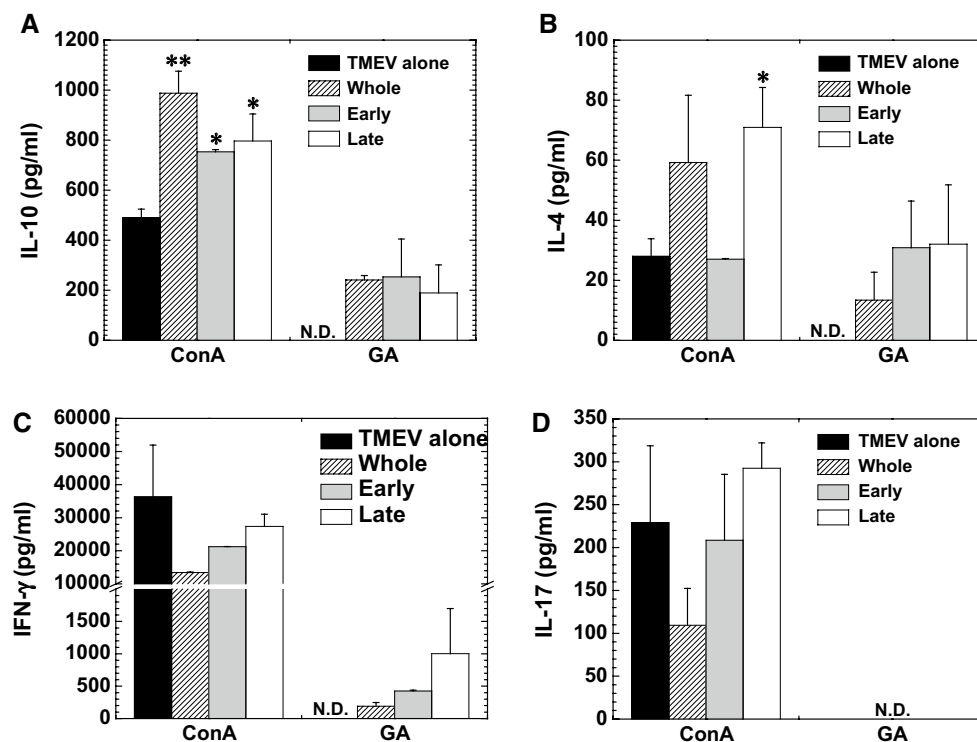


Fig. 3 Enhancement of anti-inflammatory cytokine production by GA treatment. **A–D** Cytokine profiles of splenic MNCs 5 to 6 weeks p.i. Splenic MNCs were stimulated with concanavalin A (ConA) or GA. Interleukin (IL)-10, IL-4, interferon (IFN)- γ , and IL-17 in the culture supernatant were quantified by ELISA. *, $P < 0.05$; **, $P < 0.01$;

ANOVA. Results are the average expressed as the mean concentration + SEM of two pools of spleens from two to three mice. Each spleen pool was composed of two to three spleens from four to six mice per group. N.D.; not detectable

were lower in the Whole and Early groups and higher in the Late group than in the TMEV-alone group, without statistical differences. In GA stimulation, while the GA-treated groups showed substantial IL-10, IL-4, and IFN- γ production, but not IL-17 production, these four cytokines were not detectable in the TMEV-alone group. These results suggest that GA-specific IL-10/IL-4 production can affect the clinical signs of TMEV-IDD and serum anti-GA IgG1 titers.

In summary, MS has been suggested to be an immune-mediated disease of the CNS associated with environmental factors, particularly viral infections [1]. Although the current FDA-approved DMDs have been effective in MS [9], most DMDs can potentially suppress not only anti-myelin but also antiviral immune responses; suppression of the latter has been reported to cause latent viral reactivation. For example, natalizumab treatment triggers PML, particularly in patients who are positive for antibody against JC virus [10]. Among DMDs, GA is one of the most widely prescribed first-line drugs for MS [19]. In this study using the TMEV model, in which viral persistence and antiviral immunity contribute to pathogenesis, we demonstrated that GA treatment neither increased viral loads nor decreased antiviral immunity. Furthermore, GA

treatment tended to be beneficial in the TMEV model by inducing anti-inflammatory immune responses. Thus, our findings suggest that GA treatment could be safe and effective for MS patients who are at risk of developing PML. However, we also found that *Foxp3* levels in the CNS tended to be higher in all GA-treated groups. Since Tregs play a pathogenic role in some viral infections by affecting antiviral immunity [16, 20], GA treatment might carry the risk of increasing viral replication by inducing Tregs. Thus, Treg levels, together with viral loads and antiviral immunity, will be worth monitoring in a therapeutic application of GA in patients with proven viral infections.

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Compliance with ethical standards

Conflict of interest The decision to publish this article was solely the responsibility of the authors. All statements, opinions, and content presented in this article are those of the authors and do not represent the opinions of Teva. Teva provided a medical accuracy review of the article.

Ethical approval All experimental procedures involving the use of animals were conducted according to the criteria outlined by the NIH and were approved by the IACUC of Louisiana State University.

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1 **Supplementary Materials**

2
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4 **Brief Report**

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7 **Immunoregulation of Theiler's virus-induced demyelinating disease by Glatiramer Acetate**
8 **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 2×10^5 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 obvious 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 turned 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 μ m-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 μ M β -mercaptoethanol (Sigma-Aldrich), and 1% antibiotic-antimycotic solution (Mediatech), at 2×10^5 cells/well in 96-well plates (Corning, Inc., Corning, NY). We stimulated MNCs with 2×10^5 cells/well of TMEV-infected antigen presenting cells (TMEV-APCs) or mock-infected antigen presenting cells (mock-APCs), or 50 μ g/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 ¹³⁷Cs irradiator (J.L. Shepherd & Associates, San Fernando, CA). To assess the levels of lymphoproliferative responses, [³H]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 PHDTM 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*-phenyldiamine 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 8×10^6 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].

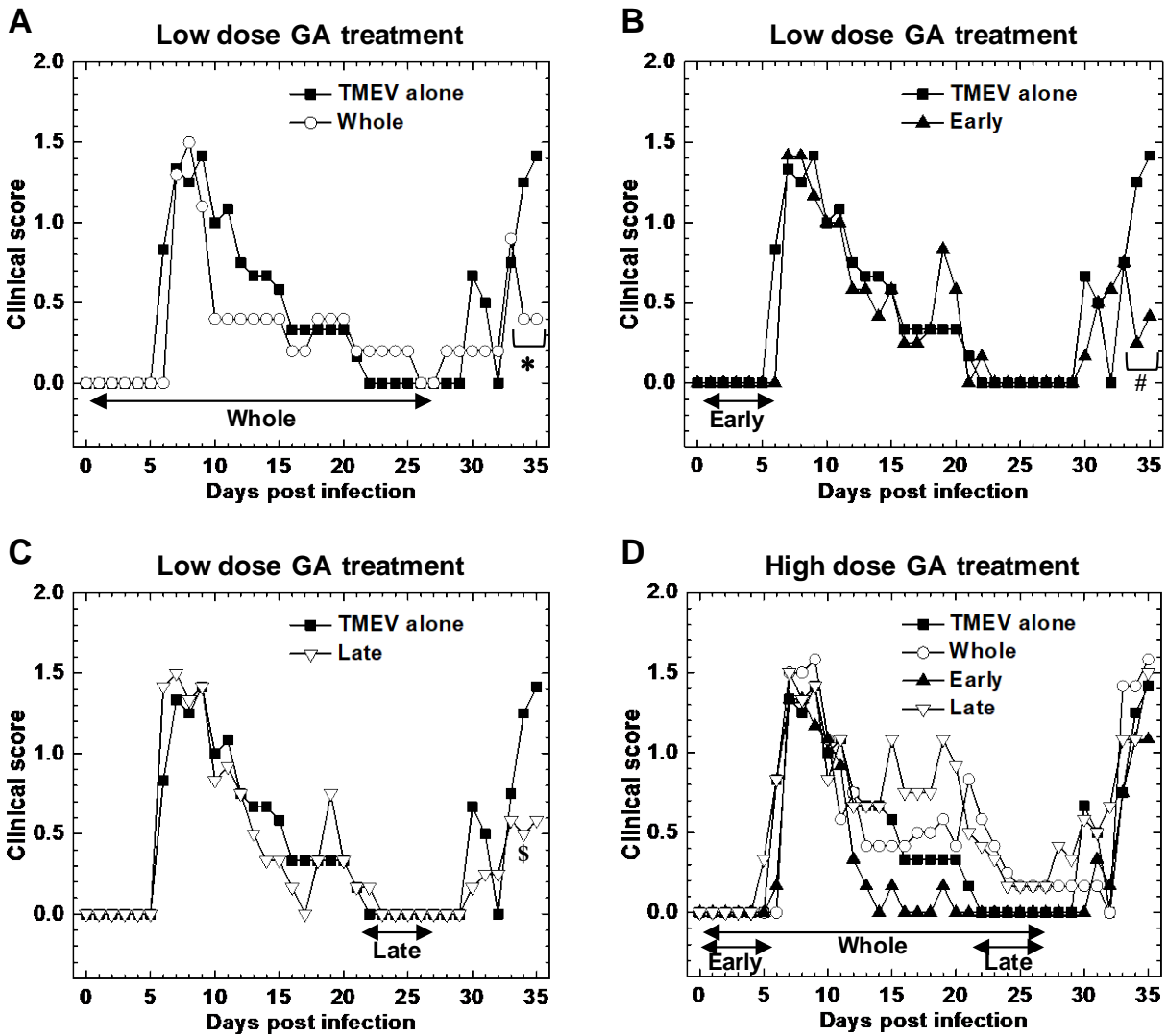
Real-time polymerase chain reaction (PCR)

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-IITM Reverse Transcription System (Promega, Corp., Madison, WI), and then conducted real-time PCR with 50 ng of the cDNA using an RT² 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'-GCAGATTGTTTGAATGGTC-3') and reverse (5'-TGCTCACATGGCTGACTTTA-3').

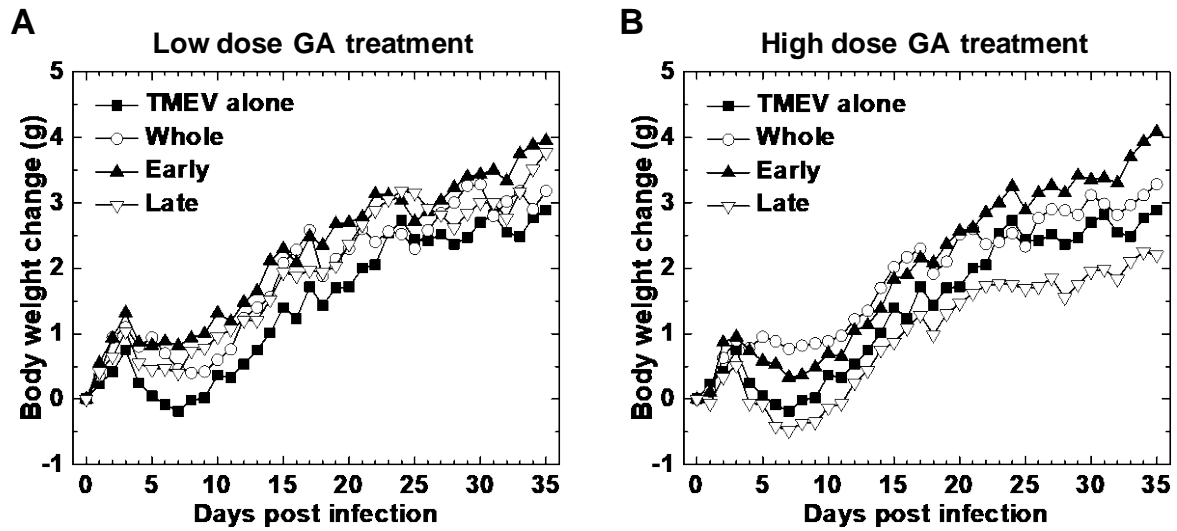
1 **Statistical analyses**

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

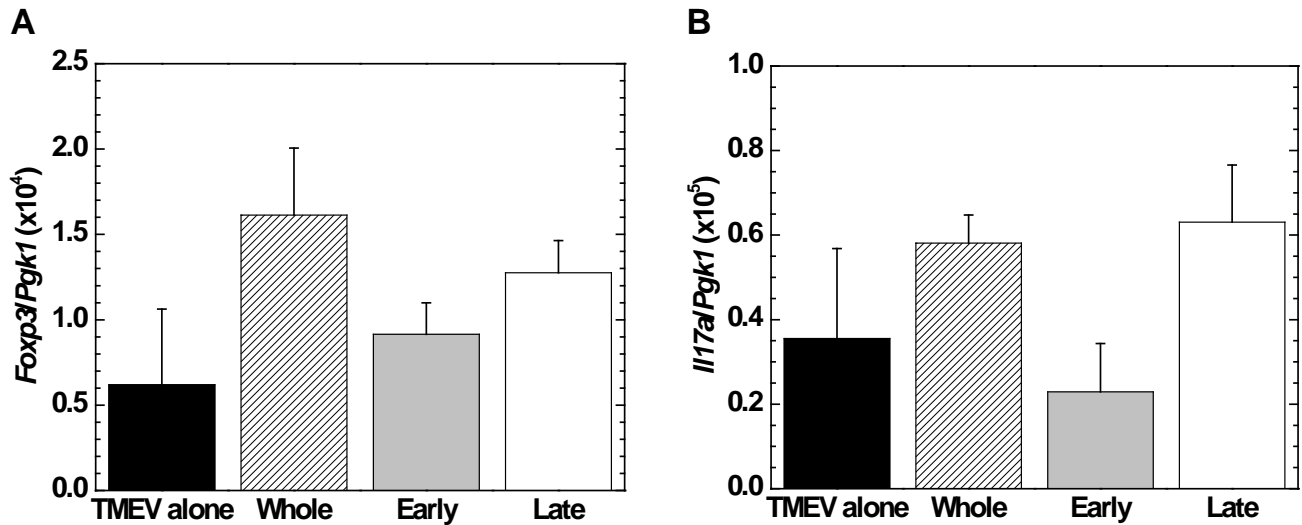
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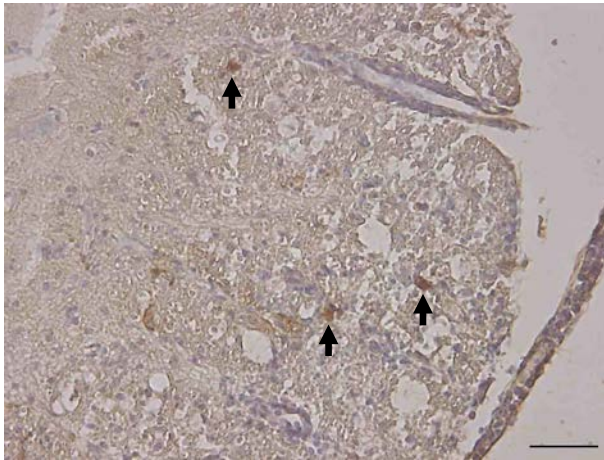
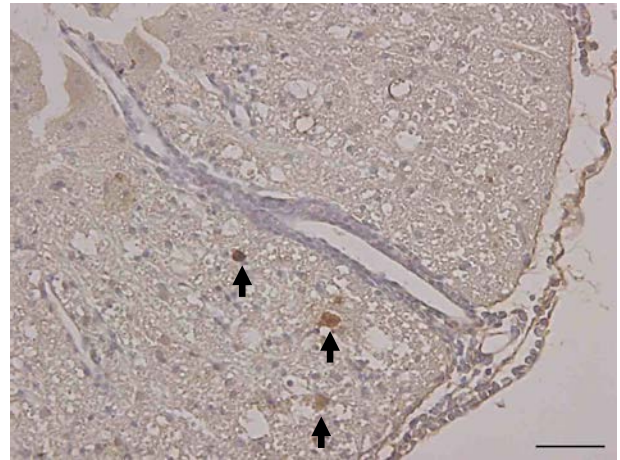
Supplementary Fig. 1. Modulation of clinical signs by glatiramer acetate (GA) treatment in a viral model of multiple sclerosis (MS), Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD). (A-D) Mice were infected with TMEV on day 0 and treated daily with GA for 4 weeks (days 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



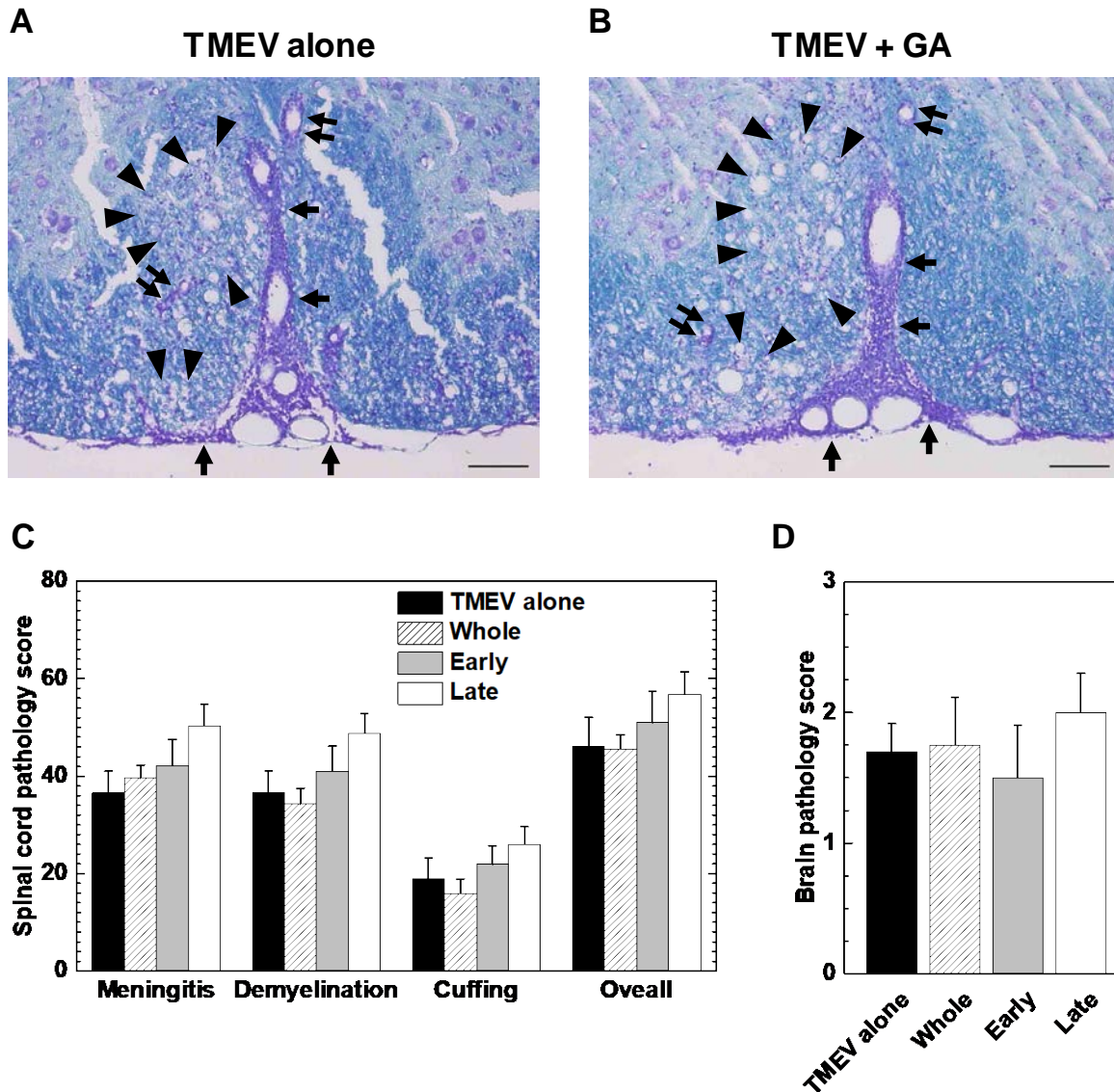
Supplementary Fig. 2. Effects of GA treatment on body weight changes after TMEV infection. Low dose treatment (**A**) and high dose treatment (**B**) of GA. TMEV alone (■), Whole (○), Early (▲), and Late (▽). Body weight changes were monitored daily. Results are representative of two independent experiments 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

A**TMEV alone****B****TMEV + GA**

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 μ m



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 μ m. (**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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