Supplementary Materials

Tonabersat Significantly Reduces Disease Progression in an Experimental Mouse Model of Multiple Sclerosis

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Supplementary Figures

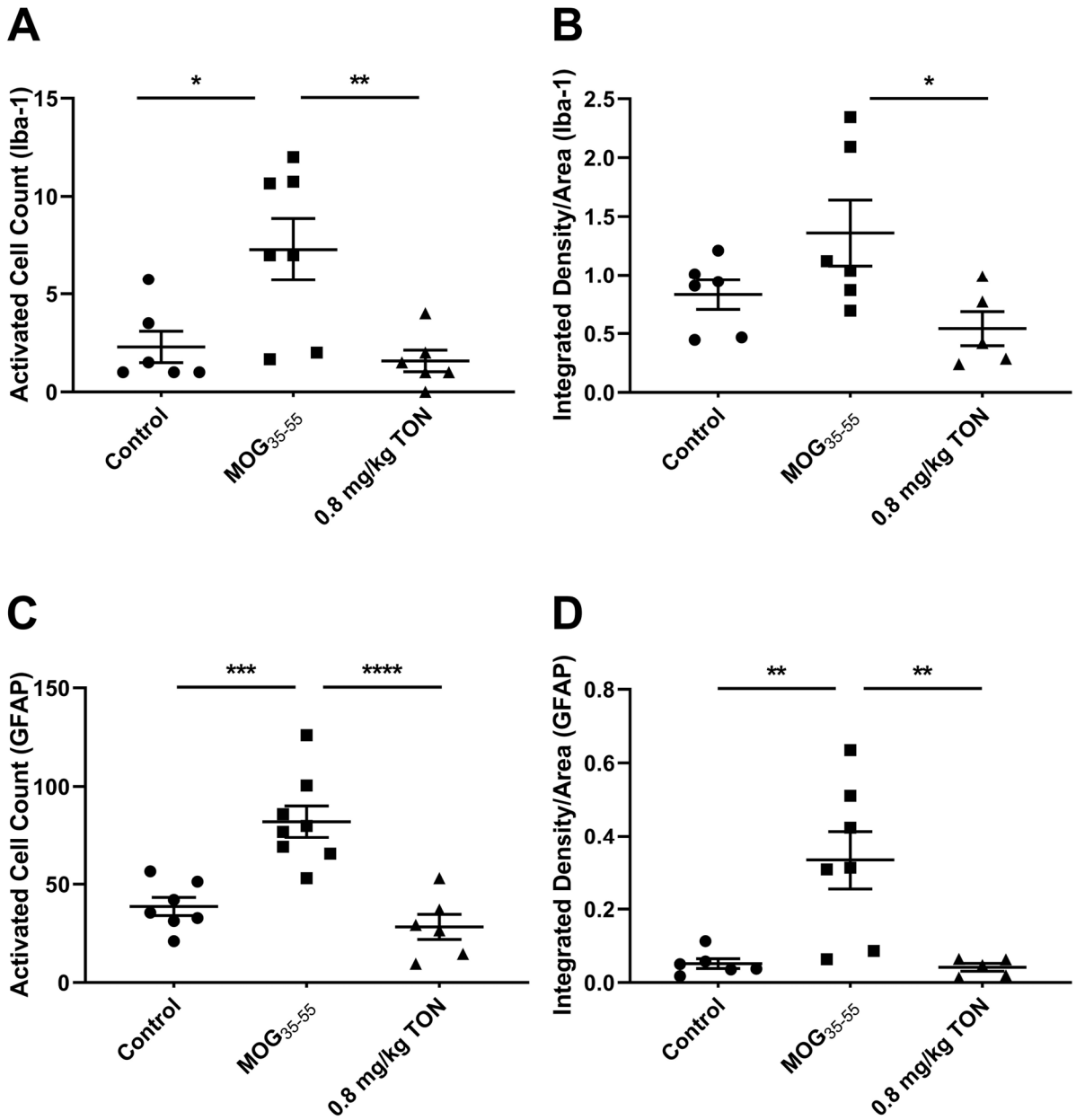

Figure S1. Mean activated cell count and mean integrated density of Iba-1 and GFAP in the motor cortex of control, $MOG_{35–55}$ EAE mice, and $MOG_{35–55}$ EAE mice with 0.8 mg/kg tonabersat treatment. The motor cortex of control mice, $MOG_{35–55}$ EAE ($MOG_{35–55}$) mice and $MOG_{35–55}$ EAE mice with 0.8 mg/kg tonabersat treatment (0.8 mg/kg TON) were stained with Iba-1 and GFAP. Activated microglia and astrocytic cell counts (**A**,**C**), and Iba-1 and GFAP integrated densities (**B**,**D**) of $MOG_{35–55}$ treated mice showed a significant increase compared to both control and 0.8 mg/kg TON mice. Control mice and 0.8 mg/kg TON mice shared similar means with no significant differences between the two groups for either inflammatory marker. Data points expressed as mean values ± SEM (one-way ANOVA and Tukey's post-hoc test (* $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$; control mice $n = 6–7$, $MOG_{35–55}$ treated mice $n = 7–8$ and 0.8 mg/kg TON mice $n = 5–6$)).

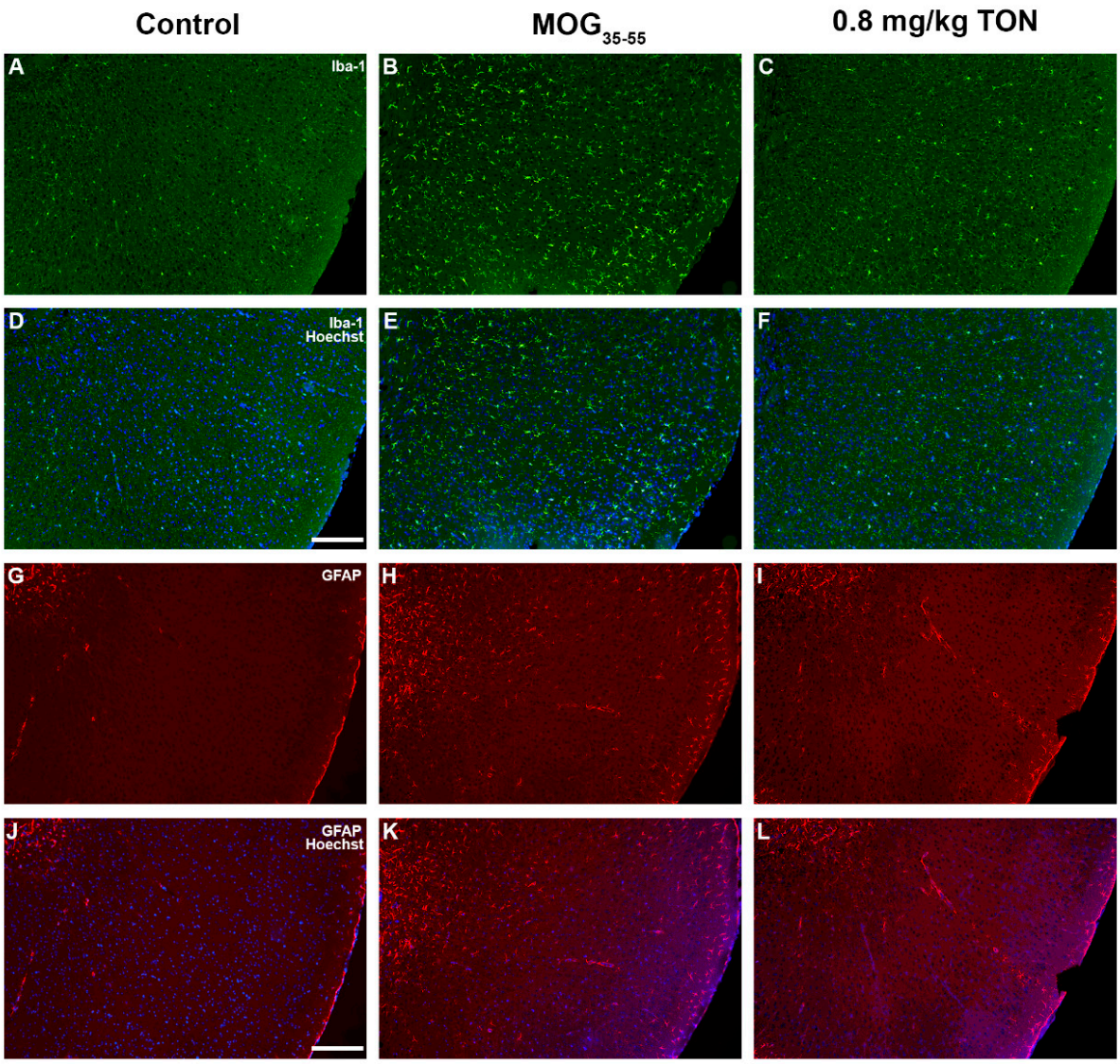

Figure S2. (**A**–**F**) Photomicrographs showing the effects of tonabersat on Iba-1 expression within the mouse motor cortex. Representative images depicting the immunolabeling of Iba-1 (green) and Hoechst (blue) within the motor cortex of the mouse brain in control (**A**,**D**), $MOG_{35–55}$ EAE ($MOG_{35–55}$) mice (**B**,**E**), and $MOG_{35–55}$ EAE mice with 0.8 mg/kg tonabersat treatment (0.8 mg/kg TON) (**C**,**F**). $MOG_{35–55}$ mice show the greatest level of Iba-1 expression, while the 0.8 mg/kg TON mice showed a significant reduction of inflammation that is parallel to control mice. Scale bar = 200 µm. (**G**–**L**) Photomicrographs showing the effects of tonabersat on GFAP expression within the mouse motor cortex. Representative images depicting the immunolabeling of GFAP (red) and Hoechst (blue) within the motor cortex of the mouse brain in control (**G**,**J**), $MOG_{35–55}$ mice (**H**,**K**), and 0.8 mg/kg TON mice (**I**,**L**). $MOG_{35–55}$ mice show the greatest level of GFAP expression, while the 0.8 mg/kg TON mice showed a significant reduction in inflammation analogous to control mice. Scale bar = 200 µm.

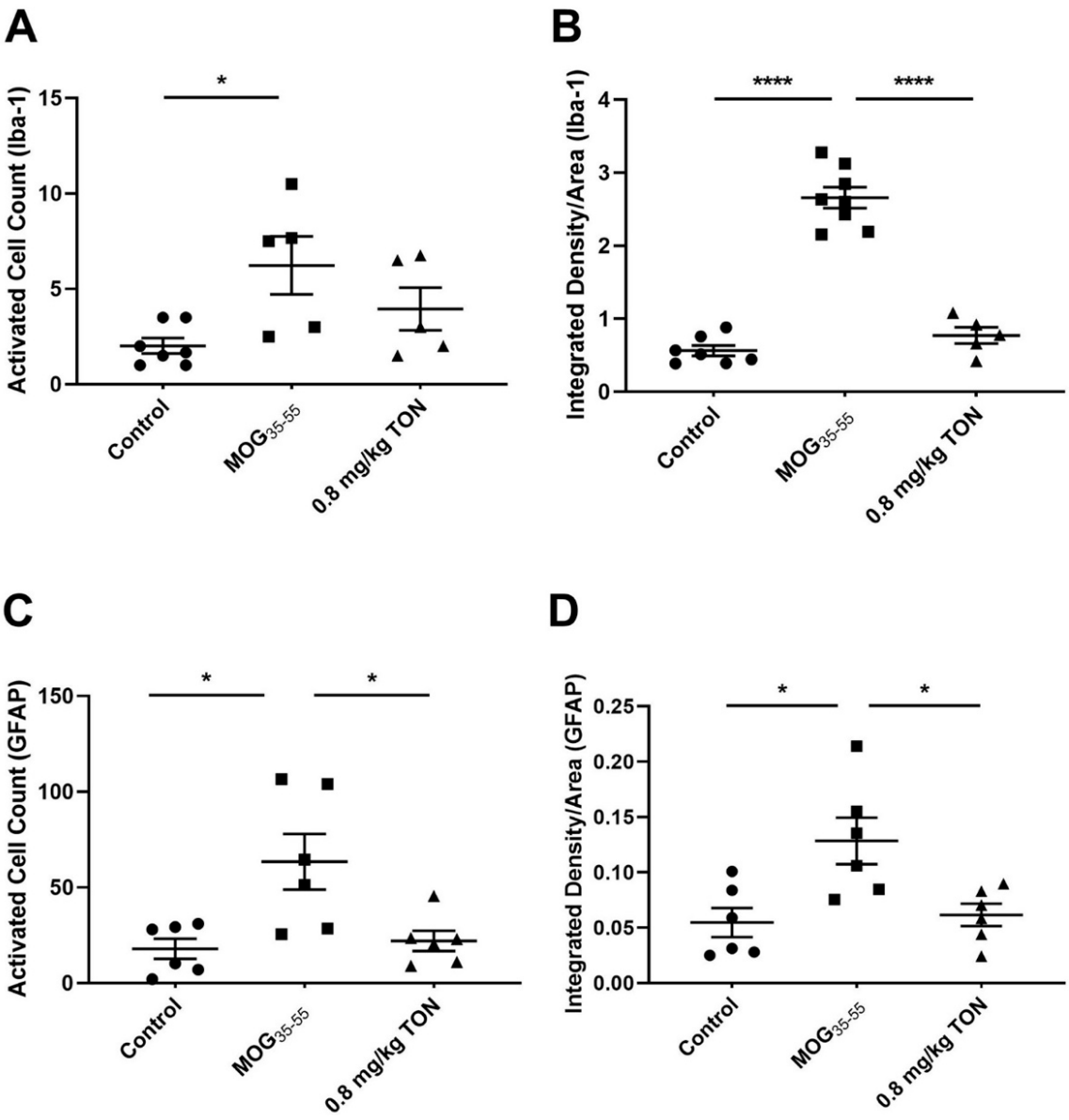

Figure S3. Mean activated cell count and mean integrated density of Iba-1 and GFAP in the striatum of control, $MOG_{35–55}$ EAE mice, and $MOG_{35–55}$ EAE mice with 0.8 mg/kg tonabersat treatment. The motor cortex of control mice, $MOG_{35–55}$ EAE ($MOG_{35–55}$) mice and $MOG_{35–55}$ EAE mice with 0.8 mg/kg tonabersat treatment (0.8 mg/kg TON) mice were stained with Iba-1 and GFAP. Activated microglia and astrocytic cell counts (**A**,**C**), and Iba-1 and GFAP integrated densities (**B**,**D**) of $MOG_{35–55}$ mice showed a significant increase compared to both control and 0.8 mg/kg TON mice. Control mice and 0.8 mg/kg TON mice shared similar means with no significant differences between the two groups for either inflammatory marker. Data points expressed as mean values ± SEM (one-way ANOVA and Tukey's post-hoc test (* $p \leq 0.05$, **** $p \leq 0.0001$; control mice n = 6–7, $MOG_{35–55}$ treated mice n = 7–8 and 0.8 mg/kg TON mice n = 5–6)).

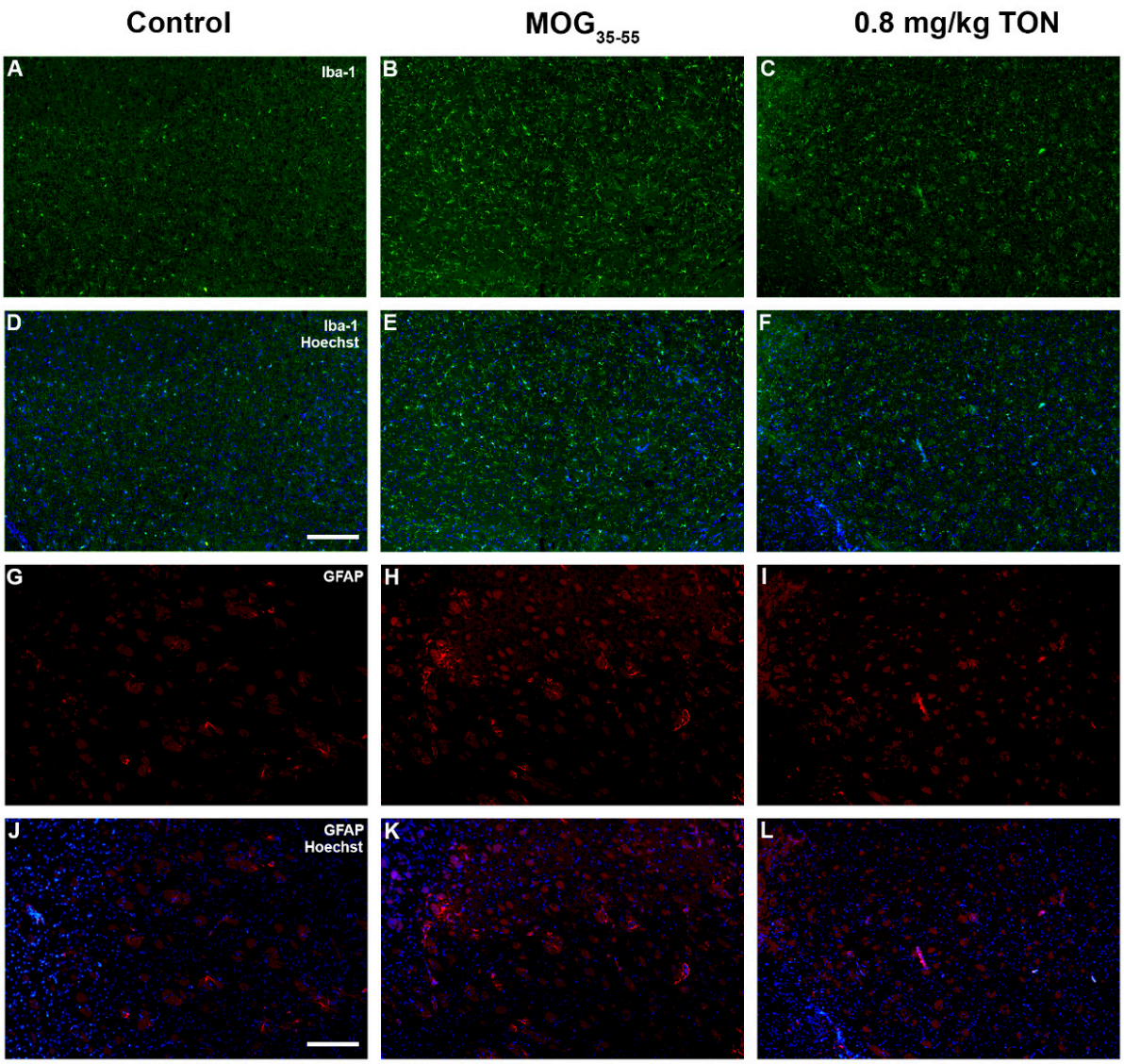

Figure S4. (**A**–**F**) Photomicrographs showing the effects of tonabersat on Iba-1 expression within the mouse striatum. Representative images depicting the immunolabeling of Iba-1 (green) and Hoechst (blue) within the striatum of the mouse brain in control (**A**,**D**), $MOG_{35–55}$ EAE ($MOG_{35–55}$) mice (**B**,**E**), and $MOG_{35–55}$ EAE mice with 0.8 mg/kg tonabersat treatment (0.8 mg/kg TON) (**C**,**F**). $MOG_{35–55}$ treated mice show the greatest level of Iba-1 expression, while the 0.8 mg/kg TON mice showed a significant reduction in inflammation that is comparable to control mice. Scale bar = 200 μm. (**G**–**L**) Photomicrographs showing the effects of tonabersat on GFAP expression within the mouse striatum. Representative images depicting the immunolabeling of GFAP (red) and Hoechst (blue) within the striatum of the mouse brain in control (**G**,**J**), $MOG_{35–55}$ mice (**H**,**K**), and 0.8 mg/kg TON mice (**I**,**L**). $MOG_{35–55}$ mice show the greatest level of GFAP expression, while the 0.8 mg/kg TON mice showed a significant reduction in inflammation that is analogous to control mice. Scale bar = 200 μm.

Supplementary Tables

Table S1. Clinical EAE Score Sheet.

Score	Scoring Method	Observation
0	Hold mouse by base of the tail	Unaffected; mouse can 'helicopter' tail
0.5	Hold mouse by base of the tail	Some loss of tail tone
1.0	Hold mouse by base of the tail	Complete tail limpness, with no evidence of limb weakness
1.5	Hold mouse at base of tail between index finger and thumb, resting the heel of your palm flat on a surface. Attempt to roll the mouse once by rolling its tail between your fingers.	Can roll mouse, but with some struggling
2.0	Hold mouse at base of tail between index finger and thumb, resting the heel of your palm flat on a surface. Attempt to roll the mouse once by rolling its tail between your fingers.	No hind limb paralysis upon ambulation, but mouse fails to remain upright when the examiner attempts to roll the mouse.
2.5	Hold mouse at base of tail and place its forepaws on edge of the cage.	Climbs into cage with difficulty; normal ambulation.
3.0	Hold mouse at base of tail and place its forepaws on edge of the cage.	Inability to climb over cage edge; partial paralysis of hind limbs; waddles upon ambulation (but does not drag limbs).
3.5	Observe ambulation	Partial paralysis of hind limbs as evidenced by dragging one limb upon ambulation.
4.0	Observe ambulation	Complete paralysis of both hind limbs; dragging body by forearms; still capable of moving around the cage.

Table S2. Treatment groups in this study.

Group	Treatment	Tonabersat Regime	Sample Size (*n*)
A	Control	N/A	9
B	$MOG_{35–55}$	N/A	9
C	$MOG_{35–55}$ + 0.2 mg/kg TON	Early	10
D	$MOG_{35–55}$ + 0.4 mg/kg TON	Early	10
E	$MOG_{35–55}$ + 0.8 mg/kg TON	Early	10
F	$MOG_{35–55}$ + 0.2 mg/kg TON	Late	10
G	$MOG_{35–55}$ + 0.4 mg/kg TON	Late	10
H	$MOG_{35–55}$ + 0.8 mg/kg TON	Late	10

Table S3. Primary antibodies used in the mouse immunohistochemistry experiments.

Antigen	Immunogen	Company, Host Species, Catalogue no.	Dilution	Detection
Casp-1 (Cleaved Asp210)	A synthetic peptide derived from human Caspase 1	Invitrogen, PA5-38099	1:100	Goat anti-rabbit Alexa 488 (2110499)
GFAP	Glial Fibrillary Acidic Protein from pig spinal cord	Sigma-Aldrich, Mouse, C9205	1:5000	Conjugated with Cy3
Iba-1	Synthetic peptide to recognize microglia	Abcam, Rabbit, Ab178846	1:20,000	Goat anti-rabbit Alexa 488 (2110499)
NLRP3	Synthetic peptide within human NLRP3 aa 1–100 conjugated to keyhole limpet haemocyanin	Abcam, Rabbit, Ab214185	1:100	Goat anti-rabbit Alexa 647 (Thermo Fisher, A21245)
MBP	Cytoplasmic side of myelin	Novus Biologicals, Rabbit, NB600-717	1:4000	Goat anti-rabbit Alexa 647 (Thermo Fisher, A21245)

Table S4. Day 18 mean clinical scores in the $MOG_{35–55}$ EAE mice and each early and late tonabersat drug dosing groups.

Dosing Group	Control Clinical Score	$MOG_{35–55}$ EAE Clinical Score	0.2 mg/kg TON Clinical Score	0.4 mg/kg TON Clinical Score	0.8 mg/kg TON Clinical Score
Early Dosing	0.0	3.7 ± 0.27	2.2 ± 0.15	1.8 ± 0.14	1.6 ± 0.10
Late Dosing			2.1 ± 0.16	1.8 ± 0.14	1.8 ± 0.15