IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Effects of Glatiramer Acetate in a Spontaneous Model of Autoimmune Neuroinflammation

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Glatiramer acetate (GA) (Copaxone), a well-established drug for the treatment of multiple sclerosis, is believed to modulate numerous pathways including antigen-presenting cells or cytokine responses. A new generation of spontaneous experimental autoimmune encephalomyelitis mouse models has been developed that mimic certain aspects of multiple sclerosis spectrum disorders. We assessed the effects of GA in the opticospinal encephalomyelitis model, which involves MOG_{35–55} peptide-specific T cells and B cells. A nonsignificant trend toward lower disease incidence was found for GA treatment (started on postnatal day 20). Immunohistochemical evaluations revealed no significant differences for inflammatory lesions and demyelination, cytokine production, proliferation, and cell surface markers of immune cells between GA-treated and PBS-treated (control) mice. Although a good correlation was found between the disease score of individual mice and some readout parameters (eg, immunohistochemical staining), this was not the case for others (eg, IFN- γ production). It seems plausible that a major effect of GA lies on alternative immunological pathways, such as initiating of an immune response that is not sufficiently reflected in this spontaneous experimental autoimmune encephalomyelitis model. Thus, the main advantage of the opticospinal encephalomyelitis model in our hands lies in the elucidation of factors influencing the onset of experimental autoimmune encephalomyelitis (eg, susceptibility factors). The model seems less suitable for investigation of disease severity modifications after therapeutic interventions. (*Am J Pathol* 2014, 184: 2056–2065; <http://dx.doi.org/10.1016/j.ajpath.2014.03.009>)

Glatiramer acetate (GA) (Copaxone) is an established drug for the treatment of relapsing–remitting multiple sclerosis (MS). This synthetic, random, basic copolymer consists of four amino acids capable of modulating immune responses resulting in a reduction of frequency and severity of relapses and disease progression.¹ A number of different pathways are believed to be influenced by GA. GA has been shown to bind to MHC class II molecules and compete for antigen-binding of myelin autoreactive antigens (eg, MOG, PLP, MBP).² In addition, GA can induce antigen-specific Th2 cells secreting anti-inflammatory cytokines such as IL-4 or IL-10 in response to antigen challenge while reducing proinflammatory Th1 and Th17 cells.³ GA probably also increases the frequency of FoxP3-expressing regulatory T cells, effects that are at least partly mediated by the generation of anti-inflammatory antigen-presenting cells that favor the differentiation of naïve T cells into Th2 or Th3 and regulatory

T cells.⁴ Nonetheless, the exact mode of GA action in MS is still unclear.

GA has been tested in a number of animal models of MS, in particular in variants of induced experimental autoimmune encephalomyelitis (EAE).^{5–7} Although classic EAE (active immunization with myelin antigens or passive transfer of myelin autoreactive cells) is a useful tool for study of some pathophysiological steps in MS, it does not cover the entirety of MS pathology. Notably, the spontaneous onset and certain disease symptoms of MS are not featured in classic EAE.⁸ Recently developed novel transgenic mouse models exhibit

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Table 1 Antibodies

Specificity	Label	Clone	Antibody class	Company
CD3	FITC	17A2	Rat IgG2b, κ	BioLegend (London, UK)
CD4	APC	RM4-5	Rat IgG2a, κ	BioLegend
CD8a	AF700	53–6.7	Rat IgG2a, κ	BioLegend
CD25	PE/Cy7	PC61	Rat IgG1, λ	BioLegend
CD69	PE	H1.2F3	Ar Ham IgG δ	Beckman Coulter
CD11b	PE	M1/70	Rat IgG2b, κ	BioLegend
CD11c	FITC	HL3	Ar Ham IgG1, λ 2	BD Biosciences (Heidelberg, Germany, or San Jose, CA)
CD86	PE/Cy7	GL-1	Rat IgG2a, κ	BioLegend
MHCII	APC	M5/114.15.2	Rat IgG2b, κ	eBioscience (Frankfurt, Germany, or San Diego, CA)
CD4	PB	GK1.5	Rat IgG2b, κ	BioLegend
CD152 (CTLA4)	PE	UC10-4B9	Ar Ham IgG	BioLegend
CD279 (PD-1)	APC	RMP1-30	Rat IgG2b, κ	BioLegend
CD62L	PE	MEL-14	Rat IgG2a, κ	BioLegend
CD44	PE/Cy7	IM7	Rat IgG2b, κ	BioLegend
CD3	PE	17A2	Rat IgG2b, κ	BD Biosciences
CD45R (B220)	APC	RA3-6B2	Rat IgG2a, κ	BioLegend
CD45	FITC	30-F11	Rat IgG2b, κ	eBioscience
CD11c	APC	N418	Ar Ham IgG	BioLegend
F4/80	eFluor 450	BM8	Rat IgG2a, κ	eBioscience
CD4	FITC	GK1.5	Rat IgG2b, κ	BD Biosciences
CD25	PE	7D4	Rat IgM, κ	BD Biosciences
FoxP3	APC	FJK-16s	Rat IgG2a, κ	eBioscience
CD86	PB	GL-1	Rat IgG2a, κ	BioLegend
CD3	AF700	17A2	Rat IgG2b, κ	eBioscience
CD8a	APC	53–6.7	Rat IgG2a, κ	BioLegend
CD44	FITC	KM201	Rat IgG1, κ	Beckman Coulter
CD25	FITC	PC61	Rat IgG1, λ	BioLegend
CD69	APC	H1.2F3	Ar Ham IgG1, λ 3	BD Biosciences

AF700, Alexa Fluor 700; APC, allophycocyanin; Ar Ham, Armenian hamster; Cy7, cyanine 7; eFluor 450, Pacific Blue replacement; FITC, fluorescein isothiocyanate; PB, Pacific Blue; PE, phycoerythrin.

spontaneous and heterogeneous disease onset. These models enable further investigations regarding GA-mediated mechanisms in neuroinflammatory disorders. Transgenic mice carrying a MOG_{35–55}-specific T-cell receptor (TCR) and MOG_{35–55}-specific B-cell receptor (MOG-IgH knock-in) were crossbred to produce TCR^{MOG} × IgH^{MOG} mice [also called Devic mice or opticospinal encephalomyelitis (OSE) mice]; 50% to 70% of these animals develop a spontaneous autoimmune disorder of the central nervous system (CNS).^{9,10} In the present study, we investigated the effects of GA in the OSE model. Clinical outcomes were correlated with histological and immunological evaluations to obtain further knowledge about GA-mediated mechanisms in autoimmune neuroinflammation.

Table 2 Weight-Adapted Injection Scheme

Weight (g)	GA (μ g)
≥ 15	200
10–14.9	133
5–9.9	66

Materials and Methods

Animals

Double transgenic animals (TCR^{MOG} × IgH^{MOG}) were generated by crossbreeding transgenic mice carrying either MOG_{35–55}-specific T-cell receptor (TCR^{MOG} knock-in, 2D2 mouse¹¹) or MOG_{35–55}-specific B-cell receptor (IgH^{MOG} knock-in, Th mouse¹²) as described previously.^{11,12} All animals used in this work were housed under specific pathogen-free conditions in the animal facility at the Institute for Chemical and Biochemical Sensors (ICB), Münster, Germany. All experiments were performed in

Table 3 Time Points for Experimental Evaluation

Time point*	Characterization	Postnatal age (days)
Preclinical	Healthy animals	25–28
Disease ₅₀	50% of animals show disease symptoms	33–37
P50	End of treatment	50

*Relative to start of experiment at P20.

Figure 1 Incidence and disease course in GA-treated (gray) and PBS-treated (control; black) OSE mice. **A:** Percentage of symptomatic animals in the control and GA groups, using a weight-adapted GA treatment (**left panel**) and a high-dose GA treatment (**right panel**). **B:** Individual EAE disease scores of the control and GA groups, evaluated for the entire 30-day duration of the experiment. Numbers of mice with identical EAE scores are indicated at the right. **C:** Mean weight development of all mice (**left panel**) and of symptomatic mice (**right panel**) normalized to the weight at treatment initiation (set to 1.0 for each mouse). Weight data are expressed as means $\pm$ SEM. $n = 19$ (**A**, weight-adapted control; **B**, control); $n = 18$ (**A**, weight-adapted GA; **B**, GA); $n = 10$ (**A**, high dose; **C**, all mice); $n = 6$ (**C**, symptomatic mice). * $P < 0.05$. Daggers indicate mice were sacrificed because of disease severity if the EAE score reached ≥ 7 . Con, control.

accordance with the guidelines of the prevailing animal protection act and with permission of the German animal protection authority (AZ 87-51.04.2010.A263).

Antibodies and Reagents

The primary anti-mouse antibodies used for flow cytometry are listed in Table 1. The respective isotype controls were purchased from BD Biosciences (Heidelberg, Germany; San Jose, CA).

Spontaneous EAE and GA Treatment

OSE mice were treated either with PBS (control) or with GA. GA is a synthetic copolymer that consists of four amino acids (L-lysine, L-glutamic acid, L-alanine, and L-tyrosine) in a fixed molar ratio. In the first set of experiments, mice were daily injected intraperitoneally, starting at postnatal day 20 (P20). In published studies, the typical dose is 150 to 250 μ g GA daily for adult mice (weighing approximately 18 to 20 g).^{13–15} We chose a weight-adapted injection scheme with 200, 133, or 66

Table 4 EAE Data for the Weight-Adapted Treatment Scheme in OSE Mice			
Characteristics	Control	GA	<i>P</i> value
Specificity			
T cells	MOG	MOG	
B cells	MOG	MOG	
Incidence [<i>n</i> / <i>N</i> (%)]	10/19 (53)	6/18 (33)	0.26
Mortality [<i>n</i> / <i>N</i> (%)]	2/19 (11)	1/18 (6)	0.58
Age at onset, means $\pm$ SEM (days)	33.8 $\pm$ 2.8	33.0 $\pm$ 2.5	0.56
EAE score, means $\pm$ SEM	6.1 $\pm$ 1.8	5.8 $\pm$ 1.9	0.79

μ g per dose (Table 2). GA at 20 mg/mL was diluted with PBS to the required concentration before injection. In a second set of experiments, mice were daily injected intraperitoneally with a fixed dose of 200 μ g GA as a high-dose treatment until P50. For experimental evaluation, three time points were determined: preclinical, Disease₅₀, and P50 (Table 3). Mice were randomized to control or GA treatment group. The groups were matched for age, weight, and sex. EAE scoring was performed daily by an observer (K.G.) masked to treatment group on an 11-point scale: 0, no abnormality; 1, limp tail tip; 2, limp tail; 3, moderate hindlimb weakness; 4, complete paralysis of one hindlimb; 5, mild paraparesis; 6, paraparesis; 7, paraplegia; 8, tetraparesis; 9, quadriplegia or premoribund state; and 10, death. If the EAE disease score reached ≥ 7 , the animal was immediately sacrificed and prepared for experimental evaluation.

Evaluation of Spontaneous EAE

Animals were sacrificed at the preclinical, Disease₅₀, and P50 time points after treatment initiation, or if the EAE disease score reached ≥ 7 . Animals were sacrificed and transcardially perfused with 20 mL PBS. The spleen was dissected and stored in 5 mL washing medium (Dulbecco's modified Eagle's medium containing 1% heat-inactivated fetal calf serum and 1% penicillin–streptomycin) on ice for subsequent isolation of splenocytes. For histological and immunohistochemical analyses, brain and spinal cord were dissected. The spinal cord was further divided into cervical, thoracic, and lumbar parts. The tissue was embedded in Tissue-Tek OCT optimal cutting temperature compound (Sakura Finetek, Torrance, CA), frozen with liquid nitrogen, and stored at -20°C . For isolation of CNS

Table 5 EAE Data for the High-dose Treatment Scheme in OSE Mice			
Characteristics	Control	GA	<i>P</i> value
Specificity			
T cells	MOG	MOG	
B cells	MOG	MOG	
Incidence [<i>n</i> / <i>N</i> (%)]	7/10 (70)	5/10 (50)	0.15
Mortality [<i>n</i> / <i>N</i> (%)]	2/10 (20)	1/10 (10)	0.53
Age at onset, means $\pm$ SEM (days)	34.3 $\pm$ 3.9	40.4 $\pm$ 5.0	0.054
EAE score, means $\pm$ SEM	5.6 $\pm$ 3.4	4.2 $\pm$ 2.4	0.68

Figure 2 Histopathology of GA and control OSE mice. The histopathological manifestations of EAE were evaluated at three time points: preclinical (**A**), Disease₅₀ (**B**), and P50 (**C**). Submeningeal and parenchymal inflammatory cell infiltrations (lesions) were visualized with H&E staining and demyelinated areas of the white matter were visualized with LFB staining. Representative images of the lumbar spinal cord of the mice are shown. Total lesion area (**B** and **C**, top row) and demyelination area (**B** and **C**, bottom row) were correlated with EAE score. Lesion areas and demyelinated areas were measured and set in proportion to total area of the tissue section. No changes were observed preclinically (**A** and data not shown). Data are expressed as individual data points and as means $\pm$ SEM for 10 slices per animal. $n = 4$ (**A**); $n = 8$ (**B** and **C**). Scale bars: 200 μ m (**A**, GA; **B**, H&E control; **C**, LFB control); 100 μ m (**A**, control; **B**, GA; **C**, H&E control and LFB GA); 50 μ m (**B**, LFB control; **C**, H&E GA). Dis50, Disease₅₀; pre, preclinical.

cells, the brain was dissected and the spinal cord was flushed out of the vertebral column with a PBS-filled syringe, and both were stored together in 2 mL Hanks' balanced salt solution medium for subsequent cell isolation steps.

Murine Cell Isolation and Culture

Spleens were isolated from the sacrificed mice. Tissue was homogenized and strained through a 40- μ m nylon filter. The

homogenates were rinsed with washing medium (Dulbecco's modified Eagle's medium containing 1% fetal calf serum, 1% glutamine, and 1% antibiotics) and resuspended in erythrocyte lysis buffer (150 mmol/L NH_4Cl , 10 mmol/L KHCO_3 , and 0.1 mmol/L EDTA; pH 7.3) for 2 minutes. Cell numbers were determined with a CASY Model TT cell counter (Innovatis, Reutlingen, Germany). Cells were cultured in Dulbecco's modified Eagle's medium containing 10 mmol/L HEPES, 25 μ g/mL gentamicin, 50 μ mol/L

Table 6 Evaluation of Lesions under H&E and LFB staining

Treatment by time point	Lesion area per slice, H&E stain (%)	Demyelinated area per slice, LFB stain (%)	<i>P</i> value
Preclinical			
Control	0	0	
GA	0	0	
Disease ₅₀			
Control	14 ± 6	12 ± 5	0.45
GA	8 ± 5	8 ± 4	0.53
P50			
Control	16 ± 4	17 ± 4	0.15
GA	7 ± 8	8 ± 5	0.17

β-mercaptoethanol, 5% fetal calf serum, 2 mmol/L glutamine, and 1% nonessential amino acids (Cambrex, Verviers, Belgium; East Rutherford, NJ).

Immunohistochemical Analysis

For histological evaluation of EAE, mice were transcardially perfused with PBS. Spinal cords were carefully excised and embedded in Tissue-Tek OCT optimal cutting temperature compound (Sakura Finetek). To ensure that the same lumbar region was analyzed for all mice, transverse cryosections (10 μm thick) were cut and stained with H&E or Luxol fast blue (LFB) according to standard protocols. Ten tissue sections separated by at least 40 μm were analyzed from five animals per group. Histological quantifications were assessed by an investigator (T.R.) masked to treatment group using an Axiophot 2 microscope (Carl Zeiss Microscopy, Jena, Germany) equipped with a charge-coupled device camera; images were analyzed with MetaVue research imaging software version

7.1.4 (Molecular Devices, Sunnyvale, CA). Lesion areas and demyelinated areas were measured and set in proportion to total area of the tissue section.

For fluorescence staining, cryosections were postfixed in 4% paraformaldehyde for 10 minutes and incubated in blocking solution (PBS containing 5% bovine serum albumin, 1% donkey serum, and 0.2% Triton X-100 surfactant). Slices were then incubated with antibodies against CD3 (1:200; KT3), CD4 (1:100; YTS191.1), or CD8 (1:100; YTS169.4) (all from AbD Serotec, Düsseldorf, Germany; Raleigh, NC) overnight at 4°C. Secondary antibody was Cy3 donkey anti-rat (Dianova, Hamburg, Germany). Next, after washing steps, tissue was mounted with 6 to 10 μL Prolong Gold antifade reagent with DAPI (Life Technologies, Carlsbad, CA) and covered. Negative controls were obtained by omitting either the primary or secondary antibody and revealed no detectable signal on subsequent analysis (data not shown).

Immunological Analysis

Splenocytes were restimulated with 10 μg/mL MOG_{35–55} peptide (the same stock as was used for immunization), and IL-17A, IFN-γ, IL-6, and IL-10 levels were assessed by enzyme-linked immunosorbent assay (ELISA) (Ready-SET-Go! ELISA kit; eBioscience, Frankfurt, Germany).

For evaluation of cell proliferation, the amount of ATP in the supernatant after cell lysis was assessed as an indicator of cell proliferation using an ATPlite luminescence ATP detection assay system (PerkinElmer, Waltham, MA) according to the manufacturer’s instructions and as described previously.¹⁶ Luminescence was measured on an Infinite 200 PRO multimode microplate reader (Tecan, Männedorf, Switzerland), and the splenocyte stimulation index was calculated as cell abundance with stimulation divided by cell abundance without stimulation.

Figure 3 Effect of GA on peripheral immune cell responses at Disease₅₀. Splenocytes from GA (gray bars) and control (black bars) OSE mice at Disease₅₀ were restimulated *in vitro* with MOG_{35–55} peptide. Production of cytokines IFN-γ (A), IL-17A (B), IL-10 (C), and IL-6 (D) was measured by ELISA, and cell proliferation (E) was assessed by ATP proliferation assay; each was correlated with EAE score. Data are expressed as means ± SEM or as individual data points. *n* = 14 (A, B, and D, control); *n* = 12 (A, B, and D, GA; C, control); *n* = 9 (C, GA); *n* = 7 (E, control); *n* = 5 (E, GA). *R*² = 0.03 (A); 0.0002 (B); 0.015 (C); 0.006 (D); 0.26 (E).

Figure 4 Effect of GA on peripheral immune cell responses at P50. Splenocytes from GA (gray bars) and control (black bars) OSE mice at P50 were restimulated *in vitro* with MOG_{35–55} peptide. Production of cytokines IFN- γ (A), IL-17A (B), IL-10 (C), and IL-6 (D) was measured by ELISA, and cell proliferation (E) was assessed by ATP proliferation assay; each was correlated with EAE score. Data are expressed as means $\pm$ SEM or as individual data points. $n = 15$ (A–D, GA); $n = 14$ (A–D, control); $n = 11$ (E, GA); $n = 10$ (E, control). $R^2 = 0.001$ (A); 0.08 (B); 0.30 (C); 0.13 (D); 0.03 (E).

For flow cytometric evaluation of CNS-invading cells, mice were perfused transcardially with PBS to diminish contamination by leukocytes located within blood vessels. CNS tissue (ie, brain and spinal cord) was dissociated mechanically, and mononuclear cells from the interface of a 30% to 50% Percoll (GE Healthcare, Little Chalfont, UK) density centrifugation gradient were counted with a CASY Model TT cell counter (Innovatis) and were stained with appropriate antibodies.

Flow Cytometry

Flow cytometric analysis of mouse leukocytes was performed as described previously.¹⁷ Cells were analyzed with a Gallios flow cytometer (Beckman Coulter).

For intracellular staining, a BD Cytofix/Cytoperm fixation and permeabilization solution kit with BD GolgiPlug was used (BD Biosciences) according to standard protocols. In one set of experiments, the APC anti-mouse/rat FoxP3 staining set (eBioscience) was used to stain CD4⁺CD25⁺FoxP3⁺ cells according to the manufacturer's instructions.

In some experiments, a standardized amount of BD Calibrite unlabeled beads (BD Biosciences) was added before staining. Cell numbers were normalized by definition of a cell/bead ratio as described previously.^{16,18,19}

Statistical Analysis

For incidence, group comparisons were analyzed by the Mantel–Cox test using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA). Mortality was compared by a χ^2 test. A two-way *t*-test or Kruskal–Wallis test was performed to detect statistically significant differences ($P < 0.05$). Except as otherwise noted, data are expressed as means $\pm$ SEM.

Results

In the first set of experiments, mice were treated with intraperitoneal GA, starting at P20. A weight-adapted injection scheme was chosen (Table 2). This treatment regime resulted in a trend toward lower disease incidence after 50 days (Figure 1A). In a second set of experiments, mice received 200 μ g GA intraperitoneally as a high-dose treatment scheme. GA-treated mice showed a trend toward later disease onset and lower disease incidence, but again the difference was not significant (Figure 1A). The EAE data are listed in Tables 4 and 5. All further results reported here refer to the weight-adapted injection scheme. The disease course of individual animals is presented in Figure 1B. The individual disease course of symptomatic mice was similar between control and GA treatment groups. This indicates that GA reduces disease incidence in OSE mice, but not disease severity. A temporary effect of GA was seen in body weight (Figure 1C).

We chose three time points for evaluation: preclinical (ie, before onset of disease symptoms), Disease₅₀ (ie, when 50% of mice in the control group showed clinical signs of disease), and the end of the experiment at P50 (Figure 1A). Before disease onset, no inflammatory infiltrates or demyelination were visible in either group (Figure 2A); in mice with clinical disease symptoms, submeningeal and parenchymal inflammatory lesions were found, as well as demyelinated areas of the white matter (Figure 2, B and C). No significant differences were observed between control and GA-treated mice (Table 6). The degree of histopathological alteration was shown to correlate with EAE disease score (Figure 2C).

At Disease₅₀ and at P50, splenocytes were isolated and stimulated *in vitro* with MOG_{35–55} peptide. No significant differences were observed for production of IFN- γ , IL-17, IL-10, and IL-6 or for cell proliferation between control

Figure 5 Immunophenotypic analysis of splenocytes by flow cytometry. **A** and **B**: Preclinical time point. Splenocytes isolated from GA (gray) and control (black) mice over 8 days (preclinical) were analyzed by flow cytometry for CD86 and MHCII (activation and costimulatory markers) in terms of MFI (**A**, left panel) and of the proportion of positive cells relative to all gated CD11b⁺CD11c⁺ cells (**A**, right panel), and for the proportion of CD4⁺CD25⁺FoxP3⁺ regulatory T cells (**B**) relative to all gated splenocytes. **C–G**: Disease₅₀ time point. Splenocytes were analyzed for immune cell subpopulations and for expression of activation marker molecules and coinhibitory markers on CD3⁺CD4⁺ and CD3⁺CD8⁺ cells. **C**: Mean CD4⁺/CD8⁺ T-cell ratio (left panel) was correlated with EAE score (right panel). **D**: Proportion of CD4⁺ or CD8⁺ naive cells (CD44⁺CD62L⁺), T_{CM} cells (CD44⁺CD62L⁺), and T_{EM} cells (CD44⁺CD62L⁺) relative to all gated cells. **E**: Expression of activation markers CD25 and CD69 on CD4⁺ and CD8⁺ T cells was determined (left panel), and the proportion of CD4⁺CD25⁺ cells was correlated with EAE score (right panel). **F**: Expression of coinhibitory marker molecules CD152 (alias CTLA-4) and PD1 on CD4⁺ and CD8⁺ T cells. **G**: Proportion of CD4⁺CD25⁺FoxP3⁺ cells relative to all gated splenocytes. Data are expressed as means ± SEM or as individual data points. *n* = 12 (**C**, **D**, and **F**, control); *n* = 11 (**C**, **D**, and **F**, GA); *n* = 8 (**E**, control; **G**, GA); *n* = 7 (**E**, GA; **G**, control); *n* = 4 (**A** and **B**). *R*² = 0.006 (**C**); 0.3 (**E**). APC, antigen-presenting cells; MFI, mean fluorescence intensity; T_{CM}, central memory T cells; T_{EM}, effector memory T cells.

and GA-treated mice, neither at Disease₅₀ (Figure 3) nor at P50 (Figure 4). Interestingly, only minor correlations were found between cytokine levels and EAE disease scores.

Flow cytometric analyses were performed to investigate changes in immune cell subtypes and expression profiles. No changes were observed preclinically for dendritic cells and regulatory T cells (Figure 5, A and B). All other investigations were performed both at Disease₅₀ (Figure 5, C–G) and at P50 (Figure 6). No significant differences were found for the CD4/CD8 ratio (as a marker of CD4⁺ T-cell expansion in the OSE model) (Figures 5C and 6A), memory T cells (Figures 5D and 6B), activation markers (Figures 5E and 6C), coinhibitory molecules (Figures 5F and 6D), and regulatory T cells (Figures 5G and 6E). At Disease₅₀, no differences were observed for immune cell subtypes in the

periphery and in the CNS between control and GA-treated mice (Figure 7, A–F).

Thus, GA treatment starting before development of spontaneous disease symptoms resulted in a nonsignificant trend toward lower disease incidence, and histological, immunological, and flow cytometric readouts revealed no significant alterations between control and GA-treated mice at the three time points.

Discussion

A number of research groups have performed studies making use of MOG_{35–55} T-cell receptor–specific 2D2 mice by, for example, crossbreeding these mice with knockout mice for a gene of interest.^{16,20,21} To our knowledge, however, the

Figure 6 Immunophenotypical analysis of splenocytes by flow cytometry at P50. Splenocytes isolated from GA (gray) and control (black) mice over 30 days were analyzed for immune cell subpopulations and for expression of activation and coinhibitory markers by flow cytometry. **A:** Mean $CD4^{+}/CD8^{+}$ T-cell ratio (left panel) was correlated with EAE score (right panel). **B:** Proportion of $CD4^{+}$ or $CD8^{+}$ naïve, T_{CM} , and T_{EM} cells relative to all gated cells. **C:** Expression of activation markers CD25 and CD69 on $CD4^{+}$ and $CD8^{+}$ T cells was determined (left panel), and the proportion of $CD4^{+}CD25^{+}$ cells was correlated with EAE score (right panel). **D:** Expression of coinhibitory marker molecules CD152 and PD1 on $CD4^{+}$ and $CD8^{+}$ T-cells. **E:** Proportion of $CD4^{+}CD25^{+}FoxP3^{+}$ cells relative to all gated splenocytes. Data are expressed as means $\pm$ SEM or as individual data points. $n = 11$ (A and C, control); $n = 8$ (A and C, GA); $n = 7$ (B, D, and E, control); $n = 5$ (B, D, and E, GA). $R^2 = 0.19$ (A); 0.05 (C).

present study is the first involving a therapeutic agent in OSE mice. Although a number of disease models (eg, toxic or viral demyelination models) have been developed to mimic aspects of MS in different animals, EAE models are most commonly used.^{8,22} In classical EAE models, the disease is induced by substances that heavily boost the immune system or by transfer of activated T cells.²³

Recently, spontaneous EAE models have been established that offer the possibility of studying disease aspects without some of the confounding factors (eg, adjuvants) present in classical EAE.^{9,24} Another advantage is that mice that develop spontaneous symptoms are exposed to less stress, and the disease course is not compromised by the immunization procedure. In the present study, we used the OSE mouse model characterized by MOG_{35–55} peptide-specific T cells and B cells. OSE mice develop demyelinating lesions in the spinal cord and optic nerve, but not in the brain or cerebellum.^{9,25}

Because we started treatment with GA at P20 (ie, before onset of disease symptoms), we consider our experimental procedure to be especially suited for evaluating potential prophylactic effects in the presymptomatic phase of the disease. A dose-dependent but nonsignificant trend toward lower disease incidence on GA treatment was found in our experimental setting.

Several explanations are available for the lack of a significant effect of GA in this model. Although current understanding of the variables controlling the outbreak of the disease in individual animals is still very limited, it seems plausible that, to affect disease incidence, therapeutic

interventions must be started very early (eg, treating pregnant dams with substances that can cross the placenta, or treating pups from P1). However, treatment of pups with an injectable drug such as GA can be begun only after weaning (eg, separation of dams and pups at P20). The spontaneous disease model might therefore be more suited to evaluating the possible influences of subtle environmental factors such as nutrition, environmental toxins, and gut microbiota,²⁶ rather than the effects of immunomodulating drugs.

Another limitation is that this model with transgenic B and T cells does not fully reflect the complexity of immune cell responses and interactions among other cell types (eg, initial priming with dendritic cells or the influence of regulatory T cells) in MS.²⁷ These critical steps in initiating immune responses are altered in this transgenic model, and it seems likely that the influence of GA on immune cell responses is therefore underestimated in the OSE model. A major mode of action of GA is the regulation of myeloid cells that define T-cell differentiation, resulting in a shift from a proinflammatory Th1- and Th17-biased cytokine profile toward an anti-inflammatory Th2 and T-regulatory cytokine profile.^{2,4} In B-cell and T-cell transgenic mice, these major mechanisms may not reach a critical threshold for affecting disease activity.

Another shortcoming of this model for conducting time-consuming and cost-intensive spontaneous EAE experiments, in contrast to classical EAE experiments, is the design of readouts. The capacity of the animal facility is critical; numbers

Figure 7 Flow cytometry of peripheral and CNS-invading immune cells and immunohistochemistry of representative lumbar spinal cord sections. **A–D:** Immune cell subpopulations of isolated CNS cells and splenocytes from GA (gray) and control (black) mice at Disease₅₀ were analyzed by flow cytometry and expressed as relative cell numbers (cell/bead ratio) (**A** and **C**) or as the total number of gated cells in each compartment (**B** and **D**). CD3⁺B220⁺ indicates B cells. **E:** Distribution of CD3⁺ and CD3⁺CD4⁺ cells in the CNS and the periphery was correlated with EAE score. Overlapping data points are marked by an oval. Please note that correlation is not shown here as EAE scores are mostly 0 and 7 and not incremental. **F:** Immunohistochemical staining for CD3⁺, CD4⁺, and CD8⁺ cells (red), with DAPI counterstain (blue), in representative lumbar spinal cord sections at the preclinical, Disease₅₀, and P50 time points. Data are expressed as means ± SEM or as individual data points. *n* = 6 (GA) or 5 (control). Scale bars, preclinical: 20 μm (CD3 control; CD4); 50 μm (CD3 GA; CD8). Scale bars, Disease₅₀: 10 μm (CD3 control; CD8); 20 μm (CD3 GA; CD4 control); 100 μm (CD4 GA). Scale bars, D50: 10 μm (CD8); 50 μm (CD3; CD4).

of breeding pairs must be sufficient to provide the required numbers of newborn mice at a given time, of which only 50% are double transgenic. Furthermore, only approximately 50% of OSE mice develop spontaneous EAE during the first 3

months after birth, with a variable onset of disease at approximately 6 ± 2 weeks of age.^{9,28} Individual EAE experiments necessarily consisted of relatively few animals. To compensate for these shortcomings, we chose three defined time points for

experimental evaluation (ie, preclinical, Disease₅₀, and P50). Furthermore, most data are shown for individual animals, not only to distinguish between untreated and treated, but also between asymptomatic and symptomatic animals.

In summary, the advantage of the OSE model in our hands lies in elucidating factors influencing the onset of EAE (eg, susceptibility factors). The models seem less suitable for investigating disease severity modifications after therapeutic interventions.

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