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5 **Beneficial Actions of Oleanolic Acid in an Experimental Model of Multiple**
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7 **Sclerosis: a Potential Therapeutic Role**
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Abstract

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4 Multiple sclerosis (MS) is a chronic autoimmune inflammatory disease for which there exist
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6 no therapies without undesired side effects. Thus, the establishment of less toxic treatments is
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8 an ongoing challenge. Nowadays, research on medicinal plants has been attracting much
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10 attention, since screening of its active principles could prove useful in identification of safe
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12 and innovative pharmaceutical molecules. In this study we investigated the therapeutic effect
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14 of oleanolic acid (OA) a plant-derived triterpene with potent anti-inflammatory and
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16 immunomodulatory activities, whose actions on CNS diseases remain far from completely
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18 characterized. We focussed on the potential therapeutic effect of oleanolic acid (OA) on an
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20 accepted experimental model of MS, the experimental autoimmune encephalomyelitis (EAE).
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22 We have found that OA treatment, before or at the early onset of EAE, ameliorates
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24 neurological signs of EAE-mice. These beneficial effects of OA seem to be associated with a
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26 reduction of blood-brain barrier leakage and lower infiltration of inflammatory cells within
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28 the CNS, as well as with its modulatory role in Th1/Th2 polarization: inhibition of
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30 proinflammatory cytokines and chemokines, and stimulation of anti-inflammatory ones.
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32 Moreover, EAE-animals that were treated with OA had lower levels of anti-MOG antibodies
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34 than untreated EAE-mice.
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44 Our findings show that the administration of the natural triterpenoid OA reduces and limits
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46 the severity and development of EAE. Therefore, OA therapy might be of clinical interest for
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48 human MS and other Th1 cell-mediated inflammatory diseases.
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56 **Keywords:** Encephalomyelitis; Neuroimmunology; Inflammation; Pharmacology;
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59 Triterpenes.
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1. Introduction

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3 Multiple sclerosis (MS) is an immuno-inflammatory, degenerative and often disabling disease
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5 of the central nervous system (CNS). The mechanisms by which this disease appears and
6
7 progresses are heterogeneous in nature and still unclear, but studies of patients with MS
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9 suggest that the observed demyelination in the CNS is a result of a T-cell-mediated
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11 autoimmune response [1]. Unfortunately, MS is a frustrating disease because there is no
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13 definitive therapy. Conventional therapeutic strategies, including immunosuppressive drugs
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15 and steroids, present undesirable side effects, particularly in long-term treatment. It thus
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17 becomes necessary to establish less toxic therapeutic options.
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22 Experimental allergic encephalomyelitis (EAE) is an experimental model widely used for the
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24 study of MS. The development and progression of clinical signs in EAE are associated with
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26 loss of blood-brain barrier (BBB) integrity and upregulation of adhesion molecules expression
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28 in CNS tissues, resulting in the migration of vascular inflammatory cells into the CNS
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30 compartment [2,3]. This inflammatory cascade triggers upregulation of various
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32 proinflammatory mediators produced by infiltrating leukocytes and resident glial cells in the
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34 CNS, which are determining factors in the tissue damage [4,5].
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39 In recent years, numerous studies describing the therapeutic properties of extracts from plants
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41 used in traditional medicine have been developed. Indeed, the use of such extracts as
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43 complementary and alternative medicine has lately increased, and also serves as an interesting
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45 source of drug candidates for the pharmaceutical industrial research. [6]. Oleanolic acid (OA),
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47 a natural pentacyclic triterpene widely found in a variety of plants, has been shown to display
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49 numerous biological properties with therapeutic potential [7,8]. It is present in the leaves and
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51 fruits of *Olea europaea*, among other vegetables. In fact, it is found in pomace olive oil, the
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53 principal source of fat in the Mediterranean diet [9] and supports the hypothesis that some
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55 components of olive oil may contribute to the beneficial properties of this diet for human
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1 health. It has been reported that OA has anti-inflammatory, anti-tumorigenic, anti-diabetic and
2 anti-viral properties, as well as cardioprotective, hepatoprotective and immunomodulatory
3 effects [10-12]. Recently, a number of synthetic oleanane triterpenoid derivatives based on
4 oleanolic acid has been synthesized, in an attempt to increase its potency; some of these are
5 currently undergoing clinical trials for the treatment of solid tumors [13].
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11 In addition, OA is clinically used in China for the treatment of hepatitis B, and as an adjunct
12 therapy for the prevention of hepatotoxicity [14]. In recent years, OA or herbal extracts
13 containing OA have been demonstrated to present immunomodulatory actions [12] and
14 promote improvement in brain function [15]. Synthetic analogs have also been revealed as
15 novel neuroprotective agents [16]. However, no information regarding its *in vivo* effect on
16 neuroinflammatory diseases is available. In this study, we demonstrate that OA significantly
17 reduces the clinical signs of EAE when administrated both before and after onset of the
18 disease. We outline its effects on inflammatory parameters such as cellular infiltration and
19 BBB dysfunction, as well as on the expression levels of cytokines, chemokines and adhesion
20 molecules.
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2. Materials and Methods

2.1. Reagents

CFA, *Pertussis* toxin, Evans Blue dye and all other chemicals not mentioned specifically were from Sigma-Aldrich (St Louis, MO, USA). Mac-1 (clone M1/70) and HRP-conjugated goat anti-mouse IgG1 and IgG2a polyclonal antibodies were from BD Pharmingen (San Jose, CA, USA). Goat anti-mouse IgG polyclonal antibody was from Amersham (Uppsala, Sweden). Ketamine was from Merial Laboratories (Barcelona, Spain) and Xylazine (Rompun) was from Bayer Healthcare (Monheim, Germany). ELISA Kits used for OPN were obtained from IBL (Hamburg, Germany); those for ICAM-1 were obtained from R&D Systems (Abingdon, UK), and those for TNF α and IL-10 were gotten from Axxora Platform (Lausen, Switzerland). Myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide (MEVGWYRSPFSRVVHLYRNGK) was generated in the peptide synthesis laboratory of Dr F. Barahona (CBM, Madrid, Spain). Oleanolic acid (molecular formula: C₃₀H₄₈O₃, molecular weight: 456.70) was obtained from pressed olive (*Olea europaea*) fruits as previously described [17] and was kindly provided in the powder form with a purity of >98%, by Dr. Ruiz-Gutierrez from the Instituto de la Grasa, Sevilla, Spain. The molecular structure of OA is shown in Fig 1.

2.2. Active Induction of EAE

C57BL/J6 mice (from Charles River Laboratories, Barcelona, Spain) were housed in the animal care facility at the Medical School of the University of Valladolid (UVa), Spain, and provided food and water *ad lib*. All experimental protocols were reviewed and approved by the Animal Ethics Committee of the UVa.

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Chronic progressive disease was induced in adult 8-10-wk-old female C57BL/J6 mice with myelin oligodendrocyte glycoprotein (MOG)₃₅₋₅₅ peptide. Briefly, mice were injected in the tail base bilaterally with 100 µl of an inoculum containing 100 µg of MOG peptide 35-55 emulsified in complete Freund's adjuvant (CFA) containing 0.4 mg *Mycobacterium tuberculosis* (H37Ra; Difco, Detroit, MI, USA). MOG-sensitized animals received a dose of 300 ng of *Pertussis* toxin intraperitoneal injection (i.p.) on the day of the immunization and two days later.

2.3. OA Treatment Procedure.

OA was first dissolved in 2% w/v dimethyl sulfoxide (DMSO) and then diluted with PBS for each experiment (the final concentration of DMSO is 0.2%, w/v). Mice were treated daily with 0.2% w/v DMSO or 50 mg/kg/d OA by i.p. injection from day 7 (before the onset of EAE: semiprophyllactic, OA2) or day 12 (after the onset of the symptoms: therapeutic, OA1) after immunization, until termination of the experiment: i) approximately 21-24 days post-immunization, when EAE mice showed hind limb paralysis, or ii), when severe symptoms of each animal group were apparent (tetraplegia), to assess the impact of oleanolic acid treatment on the evolution of disease. Control mice (without EAE induction) were also injected daily with OA for an equivalent period of time.

2.4. Clinical Evaluation.

Mice were examined, weighed and scored daily in a double-blind manner for signs of EAE for up to 20-24 days after immunization. The scores were graded according to the clinical severity of neurological symptoms on a scale of 0 to 5, with 0.5 points for intermediate clinical findings: grade 0, no abnormality; grade 0.5, partial loss/reduced tail tone, assessed by inability to curl the distal end of the tail; grade 1, tail atony; grade 1.5, slightly/moderately clumsy gait, impaired righting ability, or combination; grade 2, hind limb weakness; grade

1 2.5, partial hind limb paralysis; grade 3, complete hind limb paralysis; grade 3.5, complete
2 hind limb paralysis and fore limb weakness; grade 4, tetraplegic; grade 5, moribund state or
3 death. Scores of the two blinded investigators were average. Data were plotted as daily mean
4 clinical score for all animals in a particular treatment group. Scores of asymptomatic mice
5 (score = 0) were included in the calculation of the daily mean clinical score for each group.
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10 **2.5. Histological Studies.**

11 CNS tissues from five representative animals from the experimental groups were dissected
12 between 21-24 days after immunization. The brain, cerebellum and spinal cord were fixed and
13 embedded in paraffin. Paraffin-embedded tissues were cut on a microtome (5 μ m thickness)
14 and incubated sequentially with anti Mac1/CD11b (macrophages/microglia), horseradish
15 peroxidase-conjugated anti-goat antibody, and diaminobenzidine chromogen. Histological
16 examination was performed with a Nikon Eclipse 90i (Nikon Instruments Inc, (Amstelveen,
17 The Netherlands) connected to a DXM1200C digital camera (Nikon Instruments Inc,
18 Amstelveen, The Netherlands). Hematoxylin was used for background staining. Sections from
19 4–10 segments per mouse were examined blindly by one investigator.
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39 **2.6. Intravital Microscopy in Mouse Brain**

40 Intravital microscopy of the mouse cerebromicrovasculature was performed as previously
41 described [18]. Briefly, the mice were anesthetized by i.p. injection of a mixture of 100 mg/kg
42 Ketamine and 10 mg/kg Xylazine and the tail vein was cannulated for administration of
43 fluorescent dyes. A craniotomy was performed using a high-speed drill (Dremel, Madrid,
44 Spain) and the dura matter was removed to expose the underlying pial vasculature. The mouse
45 was maintained at 37 °C throughout the experiment and the exposed brain was continuously
46 superfused with artificial cerebrospinal fluid buffer (132 mM NaCl, 2.95 mM KCl, 1.71 mM
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1 CaCl₂, 0.64 mM MgCl₂, 24.6 mM NaHCO₃, 3.71 mM dextrose and 6.7 mM urea, pH 7.4) at
2 37 °C.
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5 In order to observe leukocyte/endothelium interactions, leukocytes were labelled *in vivo*
6 immediately before each recording, using rhodamine-6G (5 mg/kg body weight). Mice were
7 injected via the tail vein with 200 µl of a 0.05% rhodamine-6G solution: 5 mg of rhodamine-
8 6G were dissolved in 10 ml of 0.9% saline solution. Rhodamine 6G selectively stains
9 mitochondria and achieves 99.99% staining of leukocytes for up to 5 min after injection of the
10 concentration used in this study [18]. Then, leukocytes were examined using a Zeiss Axioplan
11 2 (Thornwood, NY, USA) imaging microscope connected to an AxioCam MR digital camera
12 using the AxioVision AC imaging software and an Acroplan 20x/0.50W Ph2 lens. Eight
13 different postcapillary venules of diameter between 30 and 70 µm were chosen for
14 observation. All experiments were recorded for later analysis. Rolling leukocytes were
15 defined as white cells moving at a velocity less than that of erythrocytes. Leukocytes were
16 considered adherent to the venular endothelium if they remained stationary for 30 s or longer.
17 Leukocyte adhesion was expressed as cells/mm² of venular surface area, as shown previously
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41 **2.7. Experimental Protocol for Intravital Microscopy**

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45 Leukocyte rolling and adhesion was observed by intravital microscopy at day 21-24 post
46 immunization with MOG₃₅₋₅₅. Animals were subjected to different protocols corresponding to
47 different treatment with OA as mention above: Mice were treated daily with 50 mg/kg/d OA
48 from either day 7 (OA2) or 12 (OA1) post immunization.
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2.8. BBB Permeability Measurement.

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4 In order to evaluate BBB disruption induced by EAE induction, we measured the
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6 extravasation of Evans Blue dye (EB) as a marker of albumin extravasation. At 21-24 days
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8 following EAE induction, mice were injected i.p. with 1 ml of 4% w/v Evans Blue. After 4 h,
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10 mice were sacrificed, perfused, and brain and spinal cord were removed. Dye was extracted
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12 from the CNS tissue for 2-3 days in formamide (4 ml/g of wet tissue) at room temperature.
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14 Extracted dye concentration was determined by measuring the absorbance at 650 nm. CNS
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16 tissue was dried 24 h at 60°C and weighed.
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22 Calculations were based on external standard readings, and extravasated dye was expressed as
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24 μg of Evans Blue/mg dried weight of brain tissue.
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2.9. Assays for Cytokines, Chemokines and Adhesion Molecules Levels Detection

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31 Mice (n=7, per group) were treated i.p with 50 mg/kg/d OA as mentioned above. Animals
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33 were sacrificed at day 20-24 post-immunization and CNS tissue samples (cerebral cortex,
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35 cerebellum and spinal cord) were prepared by homogenization in ice-cold PBS supplemented
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37 with protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA) using a tissue-tearor
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39 homogenizer (Cole-Parmer Instrument, IL, USA.). The samples were then centrifuged for 15
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41 min at 4000 rpm in order to separate extracellular supernatant from the cell pellet. Aliquots of
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43 supernatant were prepared and stored at -80 °C until cytokine assays were performed. OPN,
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46 ICAM-1, TNF α and IL-10 levels were examined by commercial ELISA kits following
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49 manufacturer's instructions. The concentration of the cytokines was determined by
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51 extrapolation from the appropriate standard curve, and was expressed as pg/mg total protein.
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57 Th1 cytokines and chemokines (TNF α , IFN γ , MCP-1 and MIP-1 α) were assayed from
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60 plasma using a multiplex assay kit (Lincoplex, Linco Research, St. Charles, MO, USA)
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1 according to the instructions of the manufacturer. Assay standard curves were created with the
2 stock provided by the kit, and the assay buffer served as the background (0 concentration).
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4 The concentrations of the cytokines were calculated by matching the optical density of the
5 samples against a regression equation established with the standard curve. The assay was
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7 performed in duplicate for the background, standards, controls, and samples.
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10 11 12 13 **2.10. Detection of Auto-antibodies**

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16 Serum was collected from animals on day 21 after immunization. Level of antibodies directed
17 against MOG was determined using the enzyme-linked immunoassay (ELISA) technique. 96-
18 well polystyrene microtiter plates were coated with 0.5 $\mu\text{g}/\text{well}$ of MOG₃₅₋₅₅ peptide by
19 overnight incubation in PBS at 4°C. After blocking with 5% BSA, the wells were incubated in
20 duplicate with the serum samples diluted 1:60 in PBS plus 10% FCS for 2 h at 20°C. After
21 washing, HRP-conjugated goat anti-mouse IgG, IgG1 or IgG2a polyclonal antibodies were
22 subsequently added for 90 min. After another washing, adding the substrate, and arresting the
23 reaction with 0.1 N HCl, absorbance was read at 450 nm. The results were expressed as mean
24 optical density at 450 nm.
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40 **2.11. Statistical Analyses**

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43 Statistical analysis was performed with the GraphPad Prism Version 4 software (San Diego,
44 CA, U.S.A.) by analysis of variance (ANOVA). Analyses were performed using two way
45 RM-ANOVA for comparison clinical parameters, and one-way ANOVA for comparison of
46 parameters (cytokines, extravasation, leukocytes and MOG antibodies) at day 21 post-
47 infection. A *post hoc* analysis was made by the Bonferroni's multiple comparison test.
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49 Results are expressed as mean \pm SD; *P* values < 0,05 were considered statistically significant.
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3. Results

3.1. Oleanolic acid reduces clinical signs

Oleanolic acid belongs to the oleanane-type triterpenoids, and although it has been shown to inhibit immuno-inflammatory responses, no effects of OA on MS have been reported. Therefore, we decided to evaluate the effect of OA on a murine experimental model of multiple sclerosis, EAE, at a dose previously proved to be both safe and therapeutically relevant in rodents [20,21].

To this end, we induced EAE in C57BL/6J mice as described previously by immunization with MOG₃₅₋₅₅ on day 0, and daily injected i.p. either vehicle or 50 mg/kg b.w. OA, when clinical symptoms were detected (OA1) or before the onset of the disease (OA2) until the end of the experiment. As seen in Fig 2A,B, signs of paralysis started to develop around days 11-14 (tail atony and clumsy gait) following immunization, pointing to EAE induction; such signs quickly became more apparent over the next 6–7 days and then stabilized temporarily (Fig. 2A,B). In addition, a progressive loss of body weight paralleled with a higher severity of the disease, in fact, on day 20 weight loss reached approximately 3 g for mice weighing 19 g on day 0 (Fig. 2C,D). By contrast, these two critical parameters were significantly less severe in both OA1 and OA2 groups: the treatment inhibited or delayed clinical manifestations ($p < 0.001$ versus control on day 20) and body weight loss ($p < 0.001$ versus control on day 20) associated with EAE. In these experiments, we demonstrated that a daily dose of OA initiated 7 days post-immunization delays the onset of EAE (Fig 2B) and body weight loss (Fig 2D). In addition, the clinical status of the mice whose treatment started at day 12 after immunization (OA1) (once symptoms arose) remained stable (middle score) during the following 12 days

1 (Fig. 2A), while untreated animals progressively deteriorated. This OA1 group also showed a
2 remarkable improvement in body weight (Fig. 2C). The effect observed on the weight
3 parameter is not the result of a direct effect of the compound on weight gain, because the
4 molecule itself does not affect the weight of healthy animals (data not shown).
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11 We next performed experiments to assess the impact of oleanolic acid treatment on the long
12 term evolution of clinical symptoms (endpoint: tetraplegic stage). In figure 3 we show both
13 evolution of clinical symptoms (Fig. 3A), and time to maximum score using Kaplan-Meier
14 survival analysis (Fig 3B). Although in all groups, mice continued to deteriorate over the
15 time, OA1- and OA2-treatments delayed the clinical disease. The average clinical score on
16 both OA-EAE groups was 2.5 (partial hind limb paralysis) at the time of maximum score of
17 untreated EAE-mice (score of 4) was achieved, and body weight loss by day 40 was
18 approximately 20% for OA1 and 16% for OA2, opposed to 50% for untreated EAE-animals
19 (data not shown), indicating a marked amelioration of symptoms by OA. Mice that developed
20 severe EAE (score of 4) were euthanized at this time. Differences in clinical evolution for the
21 treated EAE mice were not found significant between OA1 and OA2 groups.
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40 **3.2. Expression of cytokines and chemokines in the CNS and serum of OA-treated EAE** 41 **mice**

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43 To investigate whether the diminished EAE severity resulting from OA treatment could be
44 associated with a reduced immune-inflammatory reaction, we studied both cellular and
45 humoral responses. Thus, the modulation of some inflammatory events, such as accumulation
46 of cytokines or adhesion molecules, in the sera or CNS tissue of OA treated EAE mice was
47 first examined.
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1 We studied the levels of osteopontin (OPN), a proinflammatory cytokine, and ICAM-1, an
2 adhesion molecule, in CNS tissue extracts at day 21-24 after disease induction. In agreement
3 with clinical signs, the concentration of OPN and ICAM-1 (Fig.4A,B) in cerebral cortex,
4 cerebellum and spinal cord of EAE mice was significantly increased compared with control
5 animals. In contrast, both OA1 and OA2 treatment notably diminished the expression of both
6 proteins. As shown in Fig. 4A, OPN decreased up to 60% in the spinal cords of OA1-treated
7 EAE mice with a mean of (377 ± 48) pg/100mg tissue, and 63% in the spinal cords of OA2-
8 treated EAE-mice with a mean of (347 ± 65) pg/100mg tissue compared with (949 ± 68)
9 pg/100mg tissue in untreated EAE mice ($p < 0.001$). In cerebellum the levels of OPN in EAE
10 mice and controls were (507 ± 45) pg/100mg tissue and (165 ± 17) pg/100mg tissue,
11 respectively, and a decrease of 53.2% in OA1-treated EAE mice with a mean of (237 ± 36)
12 pg/100mg tissue, and 51,8% in OA2-treated EAE-mice with a mean of (244 ± 18) pg/100mg
13 tissue was observed, compared with untreated EAE mice ($p < 0.001$). In cerebral cortex the
14 levels of OPN in EAE mice and healthy controls were of (101.6 ± 6.2) pg/100mg tissue and
15 (33.9 ± 2.6) pg/100mg tissue, respectively, and a decrease of 49.3% in OA1-treated EAE
16 mice with a mean of (51.2 ± 10) pg/mg tissue and 51% in OA2-treated EAE-mice with a
17 mean of (49.4 ± 11) pg/mg tissue was observed as compared with untreated EAE mice
18 ($p < 0.001$).

19 In addition, as shown in Fig. 4B, ICAM-1, an important endothelial adhesion molecule
20 involved in recruiting cells into the brain, was highly expressed on the CNS tissues obtained
21 from EAE-mice: (117 ± 9.8) pg/100mg spinal cord, (59.9 ± 9.1) pg/100mg cerebellum and
22 (11.6 ± 1.2) pg/100mg cerebral cortex. In contrast, detected levels of ICAM-1 significantly
23 decreased in tissues obtained from OA-treated EAE-mice, as expected. In OA1- or OA2-
24 treated EAE-mice, ICAM-1 expression diminished in spinal cord by 80.2% and 79.7%

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2 respectively, in cerebellum by 81% and 82% respectively, and in cerebral cortex by 81% and
3 84.5%, respectively.
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6 In order to determine the effect of OA treatment in the production of inflammatory mediators,
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8 we next examined in sera the levels of Th1 cytokines, TNF α and IFN γ , as well as the
9
10 chemokines MCP-1 and MIP-1 α at day 21-24 after disease induction. As shown in Fig. 5A,
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12 any OA treatment (before or after the clinical onset) diminished the presence of the screened
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14 cytokines and chemokines compared with untreated mice. Accordingly, the high levels of
15
16 TNF α detected in spinal cord tissue of EAE-mice decreased by 56 % in both OA1- or OA2-
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18 treated EAE-animals as compared to untreated ones, whereas Th2 cytokine IL-10 expression
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20 increased significantly (Fig. 5B).
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27 **3.3. Effect of OA treatment on the humoral immune phase on EAE mice.**

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29 We next evaluated whether OA treatment also targeted the antigen-specific immune response
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31 in EAE animals. We determined the anti-MOG total IgG, IgG1, and IgG2a titers by ELISA in
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33 serum samples obtained on day 21 after immunization. As shown in Fig. 5C anti-MOG total
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35 IgG could only be measured in sera from EAE mice, whereas healthy animals showed an
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37 almost complete absence of anti-MOG antibody titers. In addition, EAE mice treated with
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39 OA, either OA1 or OA2 protocol, showed a significant reduction in this marker of humoral
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41 immune reaction. At a serum dilution of 1:60, the mean optical density (OD) measured at
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43 450 nm was 0.709 ± 0.100 and 0.703 ± 0.095 in OA1- and OA2-treated EAE mice,
44
45 respectively. In vehicle-treated EAE mice the mean OD values observed were 1.744 ± 0.116
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47 ($n = 7$ for each group), and in control animals OD values were 0.064 ± 0.004 ($n = 7$).
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49 Curiously, in untreated EAE mice the expression levels of the IgG1 isotype were higher than
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51 those of IgG2a, but while anti-MOG IgG1 titers diminished after OA treatments, anti-MOG
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53 IgG2a titers remained unchanged.
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3.4. Oleanolic Acid reduces infiltration in EAE mice.

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3 Paralleling the immune-inflammatory parameters studied above, CNS tissue sections were
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5 also incubated with anti-Mac-1/CD11b, a marker for macrophages/microglia, in order to
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7 detect the effect of OA on infiltrating/intrinsic inflammatory cells.
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11 Inflammatory cells are found within the CNS in acute EAE and are associated with clinical
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13 signs. As shown in Figure 6, an abundance of inflammatory cells was present either in spinal
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15 cord or in the granular layer of cerebellum of EAE mice, as expected (qualitative evaluation),
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17 while positive staining was not observed in CNS tissue sections of control mice. These cells
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19 showed morphology of macrophage-like or/and of ramified microglia (microglial-like cells).
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21 However, in OA treated EAE mice a substantially reduced infiltration was observed, as
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23 judged by a minimal CD11b/Mac-1 immunoreactivity on the different tissue sections, these
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25 results being linked to an improvement of the clinical symptoms of EAE.
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3.5. Effect of OA treatment on leukocyte recruitment in the CNS microvasculature of EAE mice.

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37 Previous studies have shown that cellular recruitment from the circulation to the CNS can be
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39 visualized by intravital microscopy techniques. These experiments have demonstrated that the
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41 mononuclear cells infiltrate around and within the pial venules of EAE mice [19]. Therefore,
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43 in this study we used intravital microscopy to directly observe leukocyte-endothelial
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45 interactions in the cerebral-microvasculature of EAE mice and investigate whether OA
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47 treatment, after or before the onset of the disease, modulate this recruitment. Leukocyte-
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49 endothelial cell interactions were evaluated at day 21-24 post-immunization, when the group
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51 of untreated mice showed hind limb paralysis.
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Very little adhesion (Fig. 7A) and no leukocyte rolling (Fig. 7B) were observed in the brain microcirculation of healthy control mice. In contrast, an increase for both rolling and adhesion was observed in brain of EAE mice. Around 38 rolling cells/min and 15 adherent cells/area/min were observed in postcapillary venules of symptomatic mice. However, in EAE animals that were treated with OA after or before the onset of physical symptoms (both OA1 and OA2), the adhesive events diminished significantly when compared to untreated animal, and a similar reduction was noted in the rolling events. Interestingly, OA treatment in presymptomatic mice (OA2) led to a remarkably more effective leukocyte recruitment reduction than OA treatment in symptomatic mice (OA1), 55% vs 35% respectively. Representative photos of the different treatments (Fig. 7C) illustrate the reduction in adhesion capability in the presence of OA.

3.6. Effect of OA treatment on blood-brain barrier disruption of EAE mice.

Blood-brain barrier (BBB) breakdown is another underlying event in the pathogenesis of EAE, and because of the diminished cellular infiltration of the CNS in OA-treated EAE mice, we examined finally the BBB integrity.

Since Evans Blue dye binds to albumin in the blood, extravasation of the dye serves as a marker for BBB permeability and neurovascular damage. Therefore, CNS tissues were processed and analysed to detect Evans Blue dye extravasation in treated or untreated OA mice (both EAE and healthy) at day 21-24 postimmunization, when untreated EAE mice exhibited hind limb paralysis. As shown in Fig. 8, all three CNS tissues of EAE mice revealed a marked increase in the permeability of the BBB compared to healthy animals, especially in cerebellum and spinal cord. However, this enhanced permeability was significantly reduced in both OA1- and OA2- treated mice. The measurements of Evans Blue extravasation did not

1 significantly differ between both groups of OA-treated mice, EAE+OA1 or EAE+OA2.

2 Neither was any variance found between treated and untreated healthy mice.

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5 Thus, the changes in BBB function during EAE may also be modulated by OA treatment.
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4. Discussion

Multiple sclerosis (MS) is a multiphasic autoimmune CNS disease for which no cure is presently known. The adverse events associated with the widely used interferon- β , glatiramer acetate and mitoxantrone fully justify the search for alternative and less detrimental drugs. Much research has focused on new therapeutic approaches to inhibiting the immune-inflammatory processes that are believed to initiate CNS damage in MS. Oleanolic acid is an excellent candidate to be considered as an immunomodulatory agent for the treatment of MS patients, since oleanane triterpenoids are multifunctional drugs that exhibit both anti-inflammatory and neuroprotective activities, targeting multiple cytokines and key signaling molecules important in neurodegenerative disorders [13,16,22].

We used a model of nonrelapsing, chronic sustained form of disease and demonstrated for the first time that OA, at a dose previously proved safe and therapeutically active on rodents [20,21], undoubtedly inhibited the development of EAE in treated mice using two different trial protocols: after (OA1, therapeutic) or before (OA2, semiprophylactic) the appearance of early clinical signs. In both, OA1- and OA2-treated mice, the pathological hallmarks of MS - Th1 response, blood brain barrier disruption, infiltration of immune cells through the endothelium of the BBB, and their subsequent entry into the CNS, as well as microglial activation - were significantly diminished and accompanied by a remarkable attenuation of the severity of clinical symptoms and a delay in their onset as compared with vehicle-treated animals.

At first sight, the broad effectiveness of OA on multiple signs of EAE indicates that it may affect an early phase in the immuno-inflammatory response leading to EAE, which was confirmed by the finding that triterpene attenuated the generation of certain Th1 cytokines and chemokines which are critical for the progression of the disease. We observed that OA promoted a dramatic decrease in OPN and TNF α levels expressed in CNS tissues of EAE mice, and diminished the presence in sera of soluble TNF α , IFN- γ , MIP-1 α or MCP-1.

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Conversely, secretion of Th2 cytokines such as IL-10 was induced, as increased levels of these were found in the spinal cord of EAE-treated mice. This increase in IL-10 concurrent with decreases in TNF α and IFN- γ could potentially be considered as a favorable shift in Th1/Th2 bias, since EAE induction is associated with Th1 cytokines but Th2 cytokines cause recovery from the disease [23]. In addition, because of the potential therapeutic effect of IL-10 on autoimmune diseases, it is worth noting the significant ($P<0.001$) increase in IL-10 in healthy OA-treated mice compared with vehicle-treated animals, which opens a new consideration for the profilactic administration of OA (under evaluation).

However, not only Th2 cells can secrete IL-10, but it can also be produced by other cell types, such as macrophages, dendritic cells or B cells, being therefore considered by some investigators, as an immunoregulatory cytokine [24]. In fact, the protective changes in TNF- α and IL-10 frequently observed in MS and EAE during pregnancy or with estriol treatment represents an immunoregulatory, rather than a Th2, environment [25].

There is then a clear concordance between the mildness of clinical symptoms in OA-treated animals and the cytokine profile found, although we will explore in further studies whether these changes represent an immunoregulatory or a Th2 phenotype.

OPN, in particular, is considered a potent modulator of autoimmune demyelinating diseases, and studies on OPN^{-/-} knockout mice have confirmed its pivotal role in controlling Th1/Th2 polarization. Although OPN^{-/-} knockout mice develop EAE, these mice display an evolution and severity of the disease distinct from their wild-type littermates. Such mice exhibit decreased inflammatory infiltrates and diminished production of IFN- γ , TNF α and IL-12, but increased IL-10 levels [26]; this evidence is consistent with the general cytokine-profile found here.

1 On the other hand, the occurrence of high levels of TNF α are mainly associated with changes
2 in BBB permeability, as well as with the presence of inflammatory mediators and cells
3 infiltrated in CNS tissues both in MS patients [27] and in EAE models [28]. As previously
4 reported, we have found high levels of TNF α both in serum and spinal cord of EAE mice,
5 which are associated with increased neurovascular permeability (under the assumption that its
6 presence in spinal cord may be due to activated CNS-resident cells or to TNF α -positive cells
7 from the circulation).

8 Consistent with high TNF α levels, we also found elevated expression of the adhesion
9 molecule ICAM-1, which has been demonstrated to be necessary for inflammatory cell
10 trafficking through post-capillary venules into the brain parenchyma and whose upregulation
11 is dependent upon TNF α production triggered by the loss of BBB integrity [29]. Interestingly,
12 we found that OA-induced suppression of inflammatory mediators correlates with the profile
13 of its actions on BBB permeability and on cell infiltration into the CNS.

14 In addition, CNS tissues of EAE mice treated with OA, both therapeutically or
15 semiprophylactically, exhibited diminished levels of ICAM-1 compared with untreated EAE
16 mice. In this study, intravital fluorescence microscopy of cerebral microvasculature revealed
17 that OA-treated EAE mice present a diminished leukocyte rolling and adhesion when
18 compared with that observed in untreated EAE mice. Therefore, it is possible that OA blocks
19 leukocyte recruitment to brain by reducing ICAM-1 expression.

20 The inflammatory response found in EAE mice is closely related to BBB damage [5].
21 Irrespective of the clinical forms of EAE, BBB breakdown is a hallmark in the development
22 of EAE, as well as in human MS which exacerbates the inflammatory response by providing
23 circulating cells and factors the access to privileged CNS tissues, and soluble CNS products
24 access to the circulation. The precise mechanisms mediating the onset of BBB dysfunction are
25 still controversial. Besides inflammatory cytokines including TNF α and IL-1 [30], or enzymes
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1 such as metalloproteinases [31], disruption of CNS homeostasis can occur in the absence of
2 important inflammatory mediators through the action of factors such as arachidonic acid
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4 metabolites [32] and free radicals such as peroxynitrite [33] or NMDA receptor agonists [34].
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6 In the present study, we observed that the loss of BBB integrity in C57BL/6J EAE-mice
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8 presents tissue differences, and similar to what has been previously found in other EAE-mice
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10 models, BBB permeability was predominantly disrupted in the cerebellum and spinal cord
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12 [35]. In addition, these regional differences in BBB permeability closely correlated with the
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14 expression pattern of parameters that contributes to inflammation and cell accumulation: OPN
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16 and ICAM-1 upregulation was also prominent in cerebellum and spinal cord. Therefore, it can
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18 be hypothesized that the expression of these molecules may either contribute to, or be induced
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20 in conjunction with BBB permeability changes, although such is not the goal of this study.
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22 Daily OA treatment, starting before or after the onset of the disease, significantly inhibited
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24 BBB disruption in all CNS tissues of EAE mice. It remains to be further investigated whether
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26 OA actions in neurovascular functions during EAE are exclusively related to its anti-
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28 immune/inflammatory properties or represent pleiotropic effects. Although some natural
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30 triterpenes have shown neuroprotective effects [36], inhibitory properties in arachidonic-acid-
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32 metabolizing enzymes [37], anti-oxidative properties and membrane-stabilizing actions [20],
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34 and inhibitory activity in the induction of ICAM-1 [38], no study to our knowledge had in fact
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36 been performed linking these actions to BBB integrity.
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38 Finally, although major attention is usually given to these Th-cell-mediated events in EAE,
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40 specific autoantibodies and B cell responses are also thought to contribute to the exacerbation
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42 of experimental and human MS [39]. Consistent with what has previously been reported, we
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44 found in this study that MOG-induced EAE showed higher concentrations of anti-MOG IgG
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46 antibodies of the IgG1 isotype than of the IgG2a isotype [40,41]. The continuous
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48 administration of OA (OA1 and OA2) significantly reduced IgG MOG antibody production,
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1 particularly the IgG1 isotype. Thus, inhibition of the pathogenic antibodies production against
2 MOG may also be a contributing mechanism by which OA exerts its beneficial actions on this
3 complex autoimmune disorder. A similar inhibition of the humoral response has been
4 previously described for IFN- τ treatment [42]. However, while IFN- τ actions suggest
5 inhibition of B cell clonal expansion by induction of terminal differentiation, it remains
6 unclear as to how OA diminishes humoral autoimmunity.

7 Together these findings provide a new insight into the neuroprotective actions of this natural
8 triterpenoid. However, the precise mechanism(s) responsible for such actions as well as the
9 major signaling molecules involved, are currently unclear and will need to be determined. In
10 this regard, it is interesting to note that the immunomodulatory actions found for OA, such as
11 inhibition of production of inflammatory mediators or diminished cell infiltration of the spinal
12 cord, correlates with those described for several cannabinoid ligands, particularly agonists for
13 CB2 cannabinoid receptors [43,44,45]. CB2 are highly expressed in inflammatory cells, such
14 as microglia, cell population also sensitive to triterpenoids [16], and which respond to both
15 kinds of molecules by reducing microglial activation and cytokine production. Thus, an
16 attractive hypothesis to investigate, regarding underlying OA mechanism of action, is whether
17 oleanolic acid modulates the synthesis of endogenous CB ligands or its receptors, as well as
18 the existence of a possible common mechanism and sites of action.

19 In summary, our findings demonstrate for the first time that the ability of oleanolic acid to
20 ameliorate neurological signs of EAE mice is probably due to inhibitory actions in both the
21 inflammatory arm and humoral component of the disease. Therefore, both a semiprofilactic
22 and therapeutic administration of OA can be considered as an effective therapeutical agent for
23 ongoing EAE and, as such, shows a promising potential for the treatment of MS in humans, as
24 well as other Th1 inflammatory diseases.

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6 encephalomyelitis and induces encephalitogenic T cell apoptosis: partial involvement
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Figures

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5 Figure 1.- Molecular structure of Oleanolic Acid.
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10 Figure 2.- Oleanolic acid influence on EAE disease progression. (A,B) Effect on clinical
11 signs, and (C,D) on body weight. C57BL/J6 mice were immunized with MOG₃₅₋₅₅ and given
12 oleanolic acid daily i.p. from day 12 (OA1) or 7 (OA2) post-immunization until the end of the
13 experiment (21-24 post-immunization). Arrows indicate when treatment was initiated. Values
14 are means \pm SD. ■, EAE + vehicle (n=18); ■, EAE + OA 50 mg/kg (n=10); □, control +
15 vehicle (n=10). For some points, error bars are not visible because the deviations are smaller
16 than the symbol sizes. Statistically significant differences were indicated for clinical
17 symptoms by * P <0.01 and * P <0.001 versus untreated EAE-mice; and for body weight by
18 * P <0.001 versus control and ** P <0.001 versus untreated EAE-mice..
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34 Figure 3.- Effect of Oleanolic acid treatments on maximum clinical scores. (A) Effect on
35 clinical sign evolution, and (B) on disease severity. C57BL/6 mice were immunized with
36 MOG₃₅₋₅₅ and given oleanolic acid daily i.p. from day 12 (OA1) or 7 (OA2) post-
37 immunization until severe clinical signs were reached (score of 4), at this time mice were
38 euthanized. Values are means \pm SD, n=7 in all groups. ■, EAE + vehicle; ■, EAE + OA1 50
39 mg/kg; □, EAE + OA2. The difference between EAE-untreated and EAE-OA groups was
40 highly significant (P < 0.001).
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54 Figure 4.- OPN and ICAM-1 protein expression in CNS tissues of untreated and treated EAE
55 mice. On day 21 after immunization with MOG₃₅₋₅₅, protein concentrations of OPN and
56 ICAM-1 were measured by commercial ELISA in spinal cord, cerebral cortex and cerebellum
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1 extracts of control, control OA, EAE, EAE+OA1, and EAE+OA2 mice. Results were
2 expressed as the mean \pm SD from seven animals per group. Statistically significant
3 differences were indicated by $*P<0.001$ versus control and $**P<0.001$ versus untreated
4 EAE-mice.
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11 Figure 5.- Effect of OA treatments on cytokine, chemokine and anti-MOG_{35–55} antibody
12 levels in EAE mice. On day 21 after immunization with MOG_{35–55}, TNF α , IFN γ , MCP-1,
13 MIP-1 α and IL-10 protein concentrations were measured, as explained in Materials and
14 Methods, in serum samples (A) or in spinal cord extracts (B) of control, control+OA, EAE,
15 EAE+OA1, and EAE+OA2 mice. (C) Sera were also evaluated by ELISA for titers of
16 pMOG_{35–55}-specific immunoglobulins at 1/60 dilution. Results were expressed as the mean \pm
17 SD from seven animals per group. Statistically significant differences were indicated by
18 $*P<0.001$ versus control, $**P<0.01$ versus untreated EAE-mice and $***P<0.001$ versus
19 untreated EAE-mice.
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37 Figure 6.- OA treatment improves immunohistological outcomes in spinal cords and
38 cerebellum of EAE mice. Typical sections of cellular infiltration on spinal cord and
39 cerebellum, at day 21 post-immunization, in control, EAE, EAE+OA1 and EAE+OA2 mice,
40 monitored by anti-Mac-1/CD11b, a marker for macrophages/microglia, are shown. Spinal
41 cord panels show three different histological sections visualized with a 20X lens. Cerebellum
42 left and right panels represent a same section visualized with a 10X and a 20X lens,
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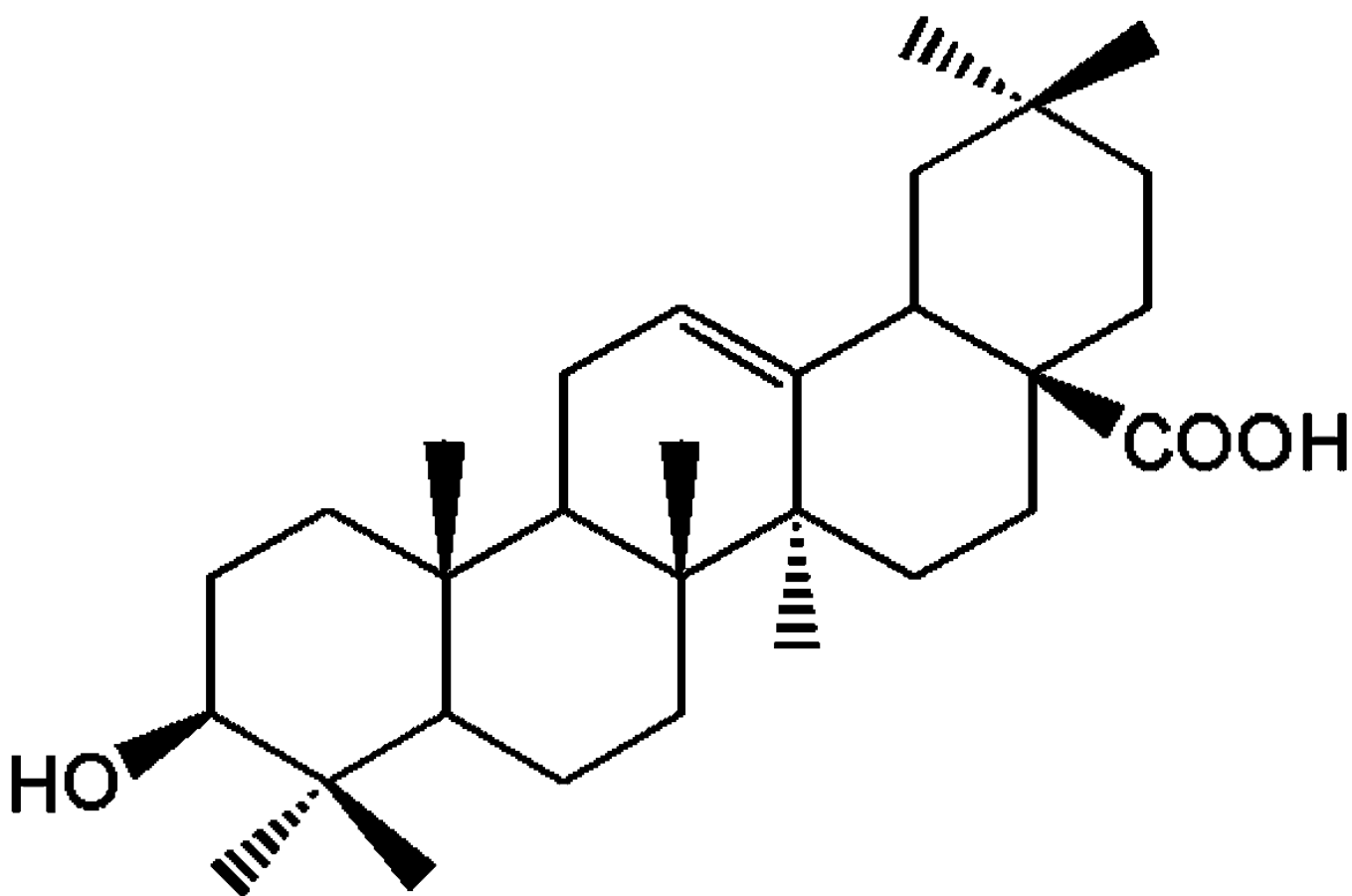
56 Figure 7. Leukocyte recruitment to the brain over the course of EAE. Intravital microscopy
57 was used to assess the firm arrest (A) and rolling flux (B) of leukocytes on brain
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1 microvasculature, at day 21 post-immunization. The protocol included control, EAE,
2 EAE+OA1 and EAE+OA2 groups visualized on day 21 post-immunization. Results are
3 shown as mean \pm SD of cells per minute. *Significant ($P < 0.001$) difference compared to
4 control, **significant ($P < 0.001$) difference compared to untreated EAE. C, Representative
5 images of the pial microvasculature in healthy, untreated-EAE mice and OA1- or OA2-treated
6 EAE-mice are shown. Note that the marked increase in the number of fluorescently labelled
7 leukocytes interacting with the cerebral endothelium in EAE mice declined in EAE+OA1 and
8 EAE+OA2 mice. In all groups, $n=10$.

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22 Figure 8.- Changes in blood-brain barrier permeability of OA treated or untreated EAE-mice.
23 Evans blue dye was used as a measurement of plasma protein extravasation in (A) spinal cord,
24 (B) cerebral cortex and (C) cerebellum 21-24 days post-immunization. Bars represent means
25 \pm SD, for $n = 6$ mice. * Significant ($P < 0.001$) difference compared to control, ** significant
26 ($P < 0.001$) difference compared to untreated EAE.
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Figure 1

Oleanolic Acid

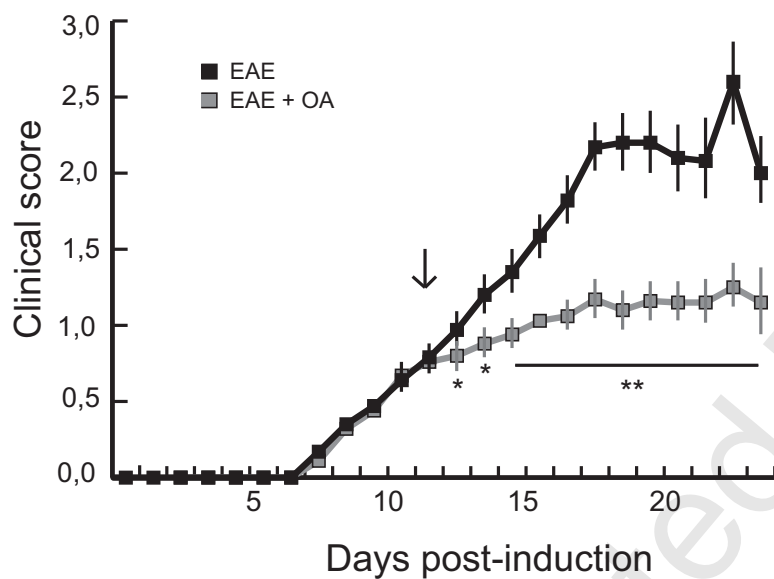


3 β -hydroxyolean-12-en-28-oic acid

Figure 2

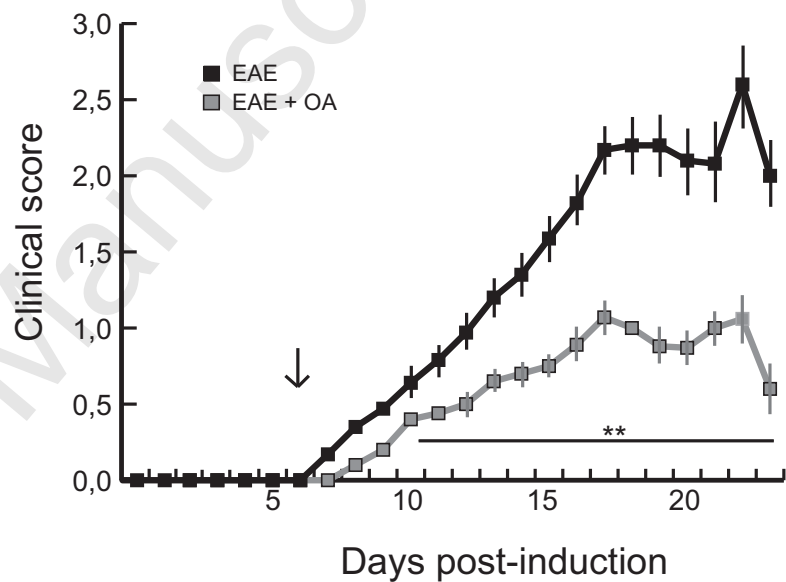
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OA1



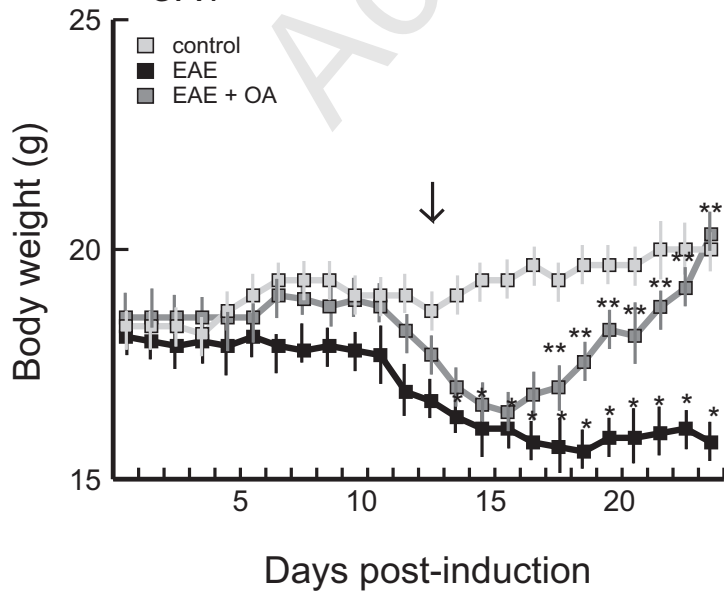
B

OA2



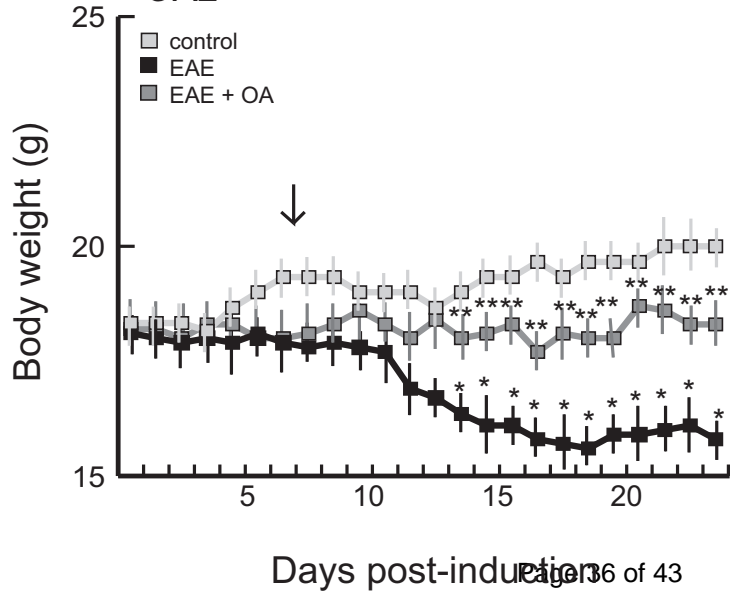
C

OA1

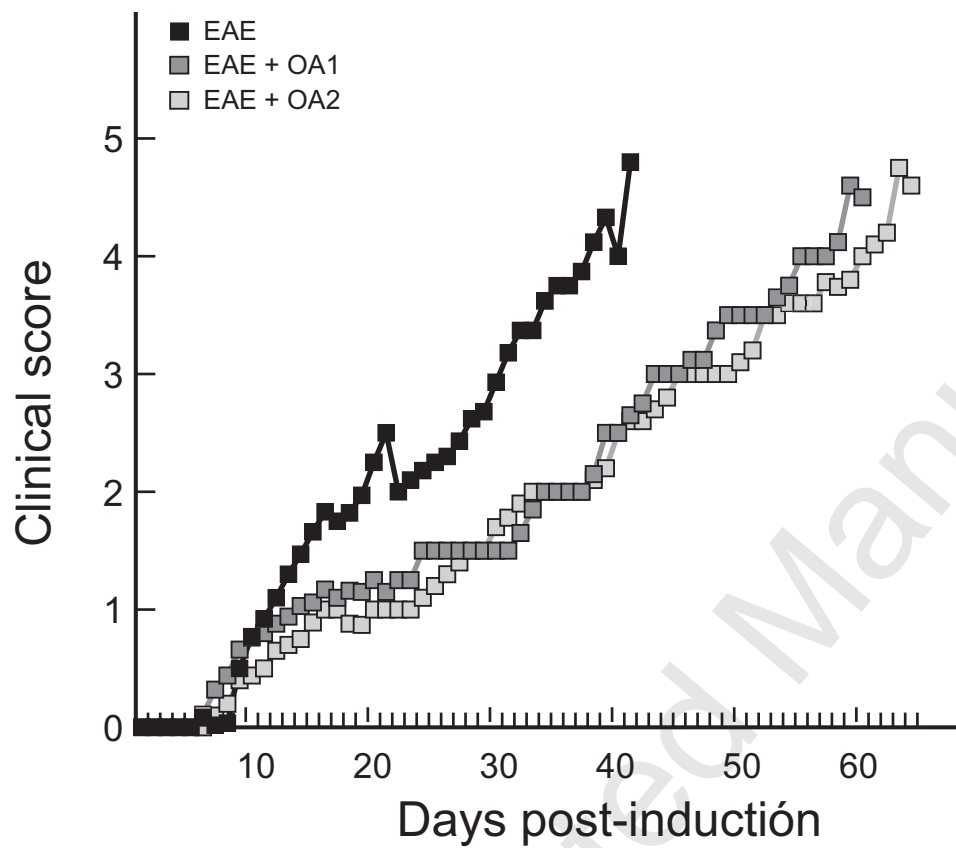


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OA2



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B

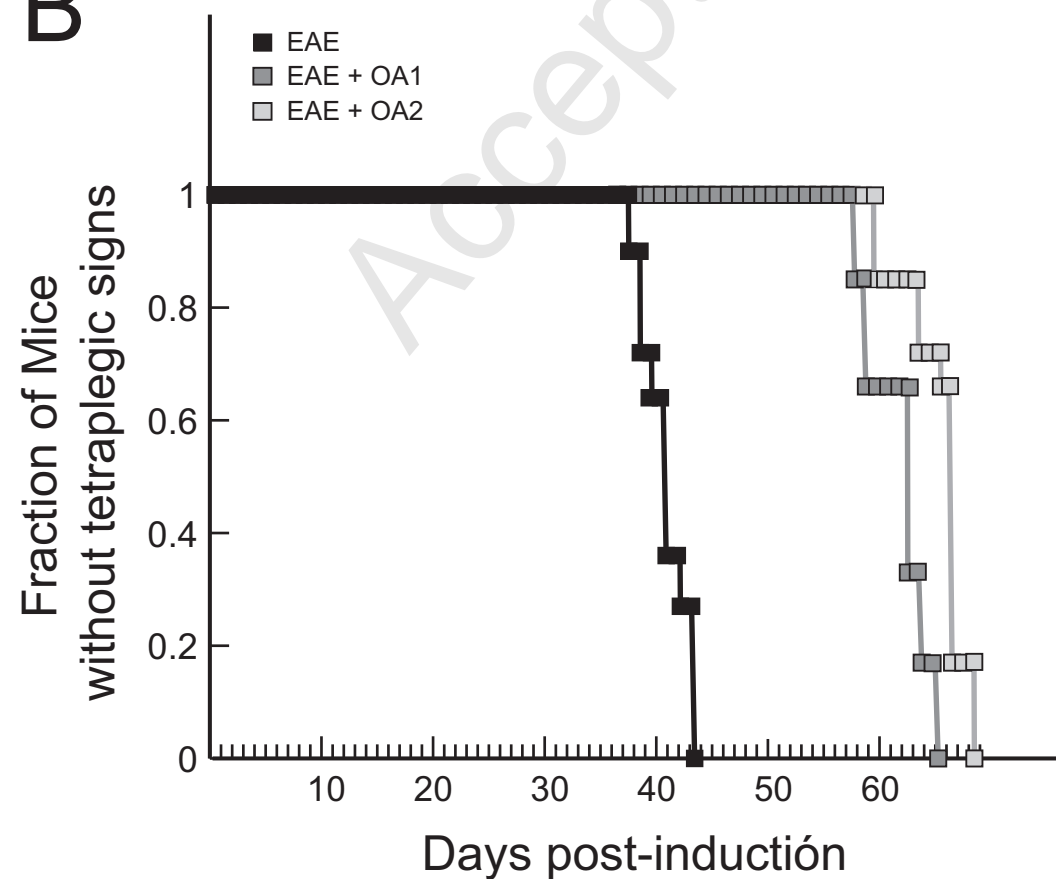
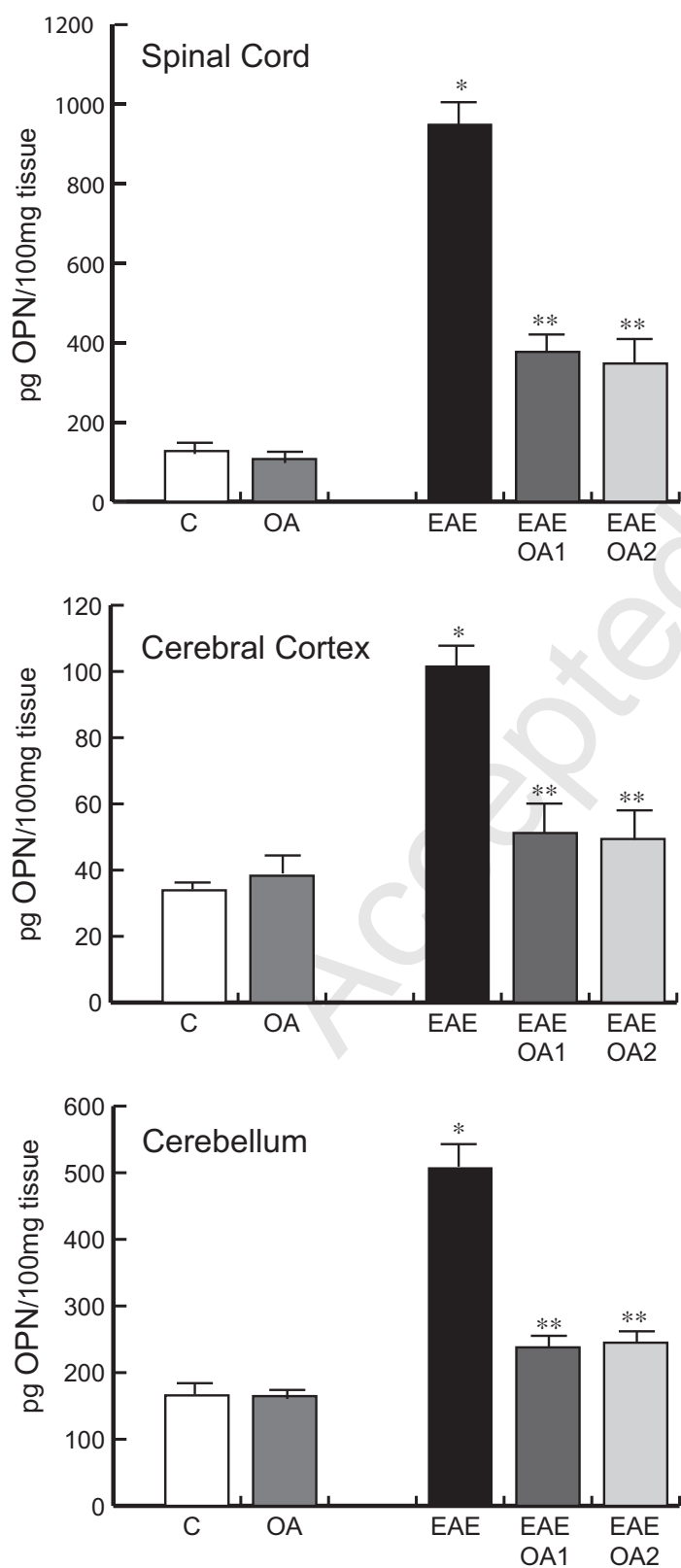
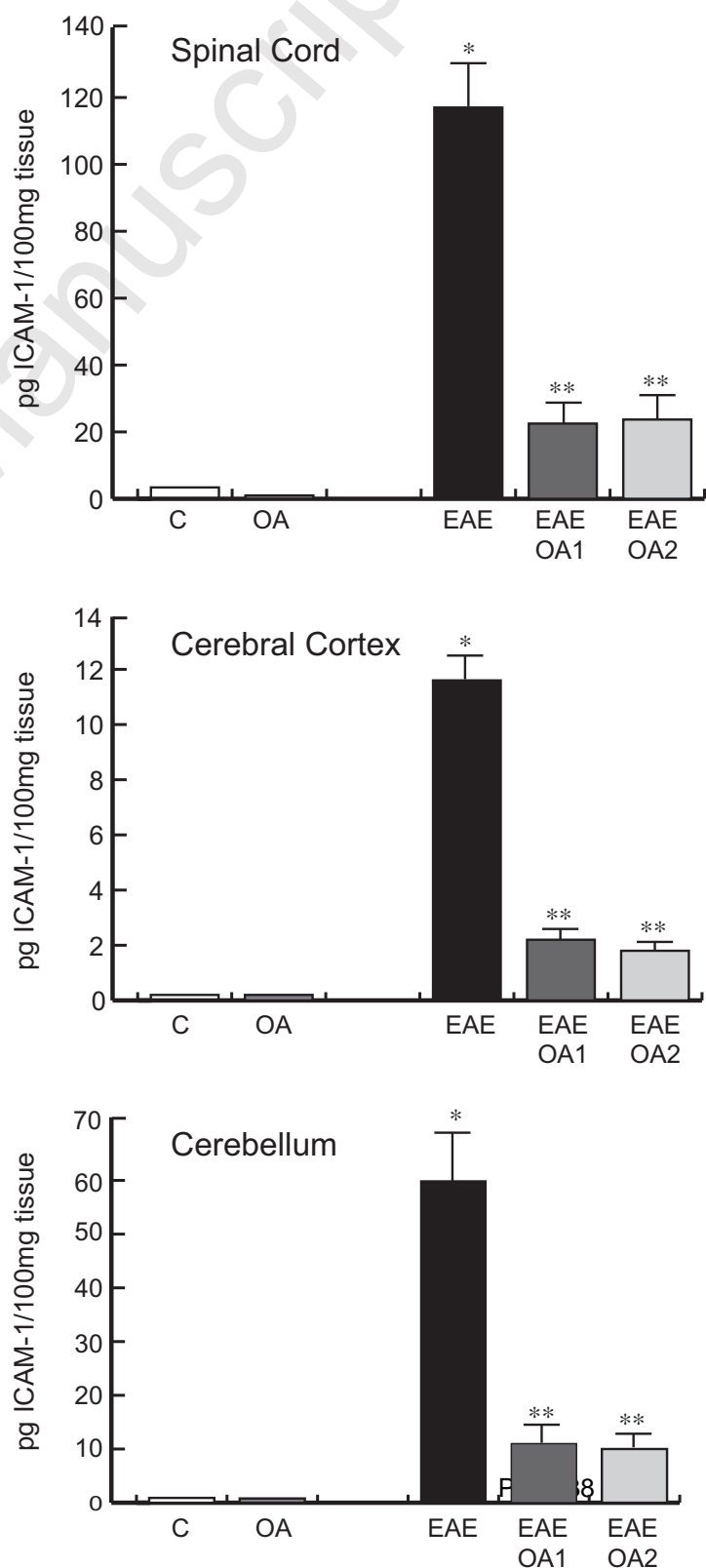


Figure 4

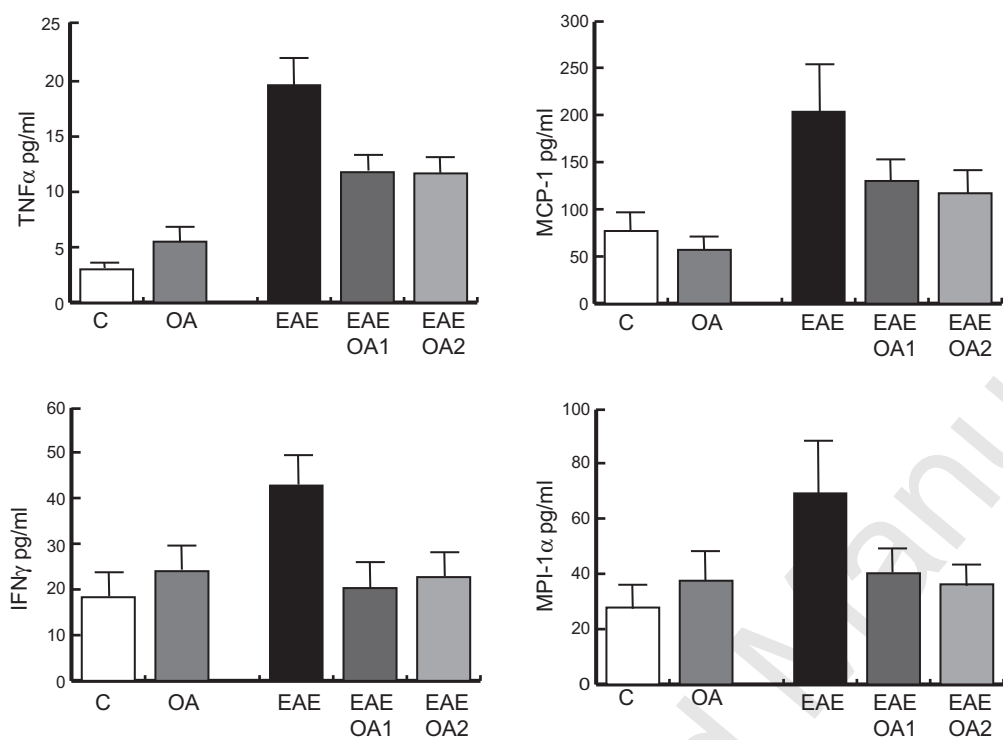
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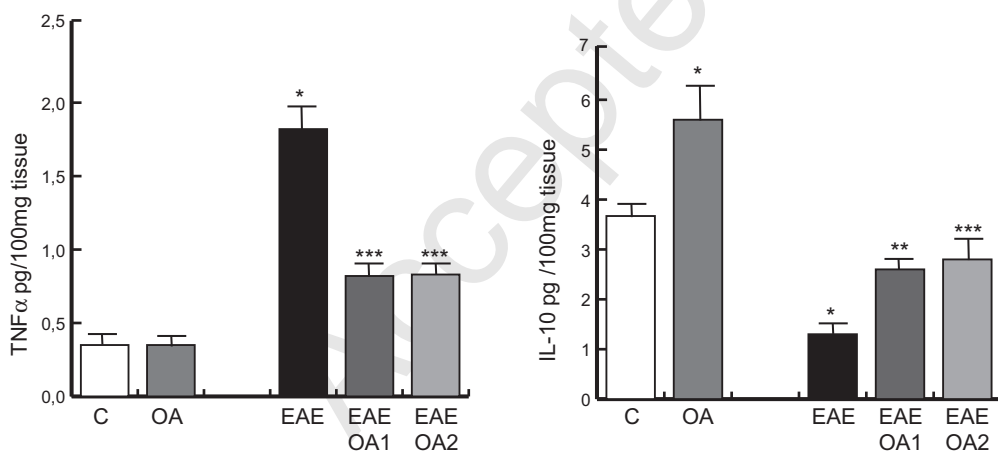
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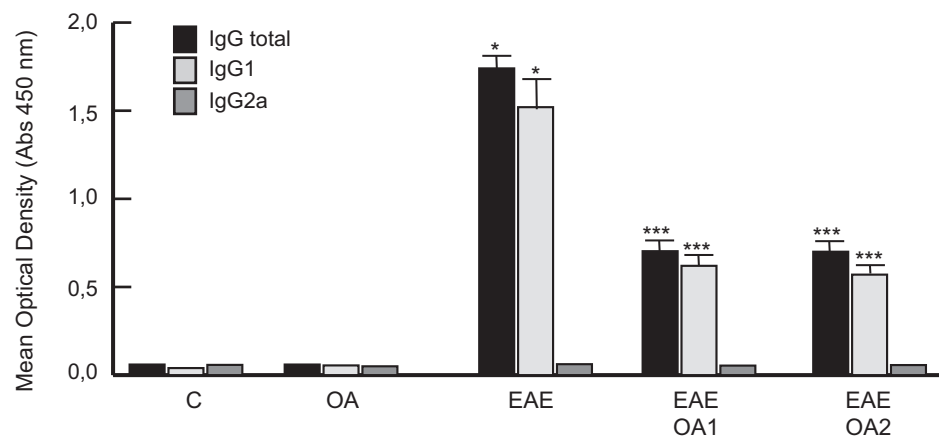


Figure 6

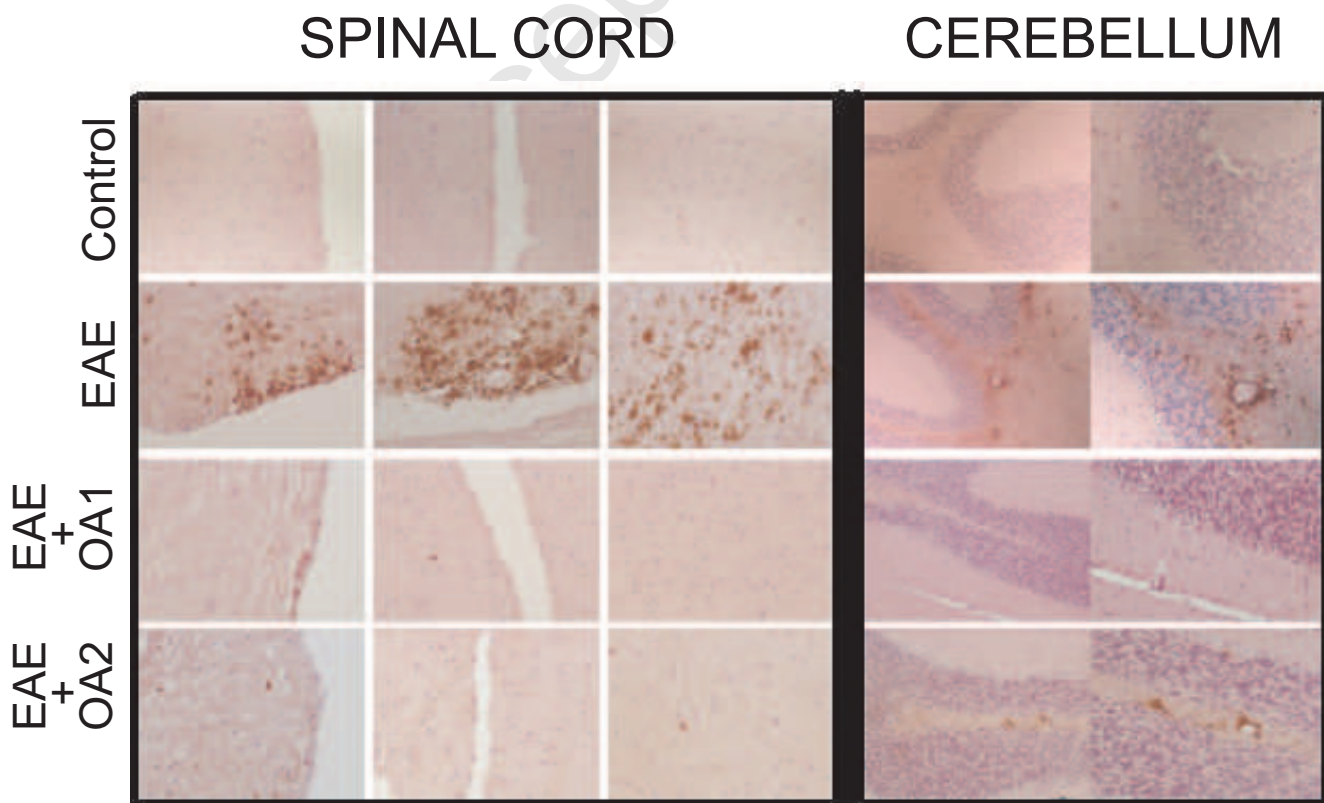


Figure 7

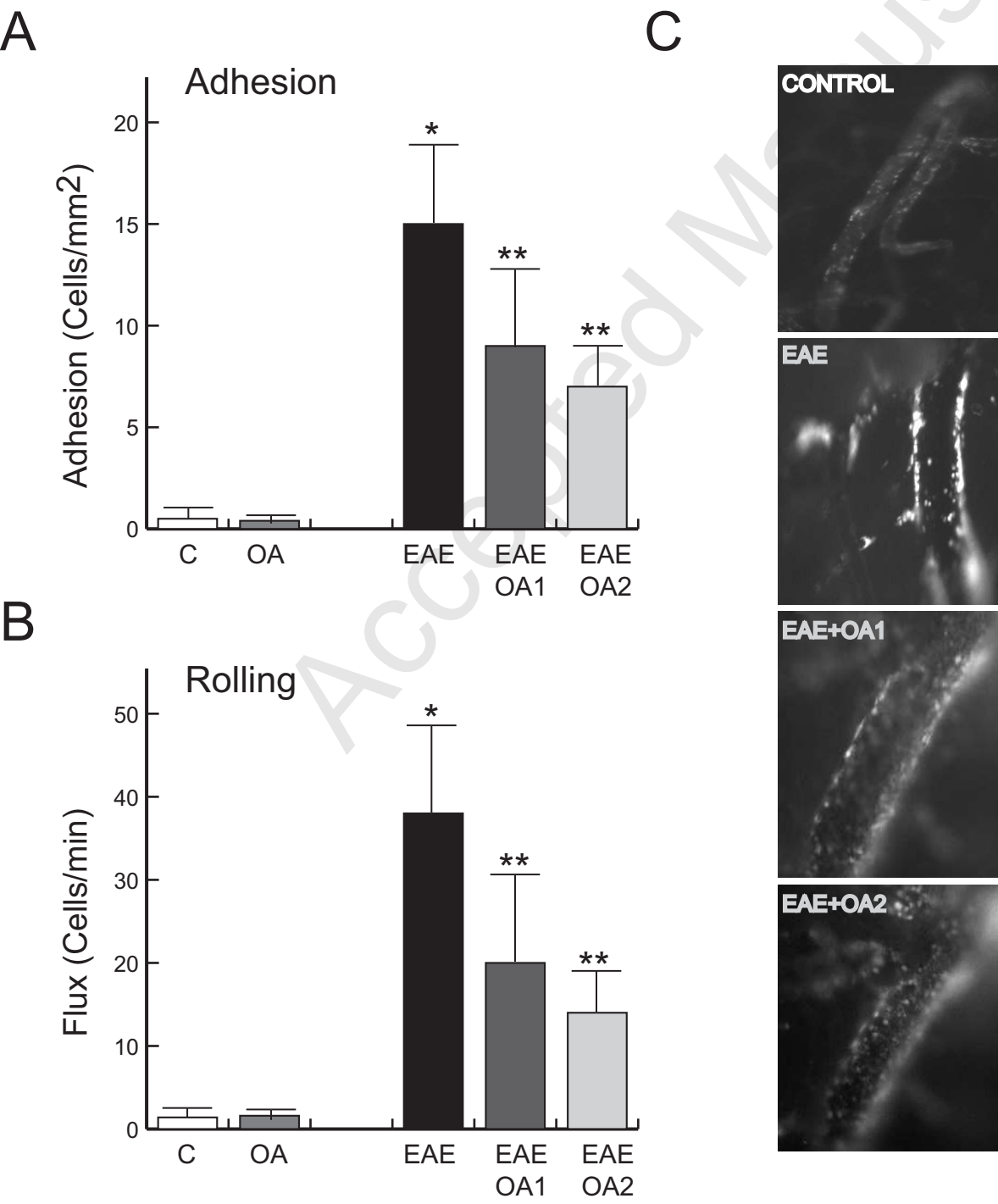
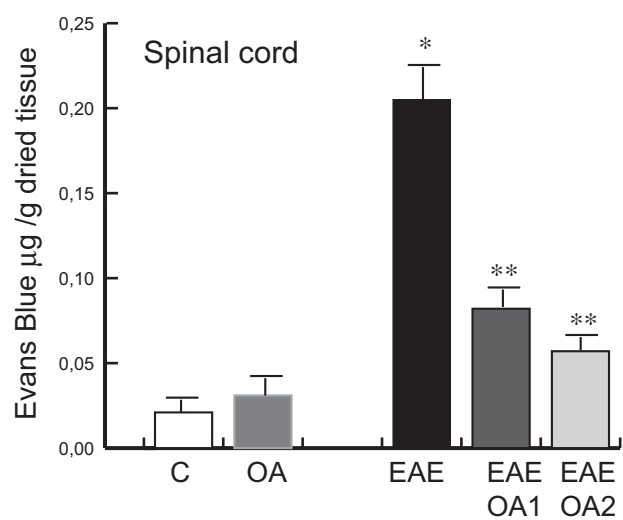
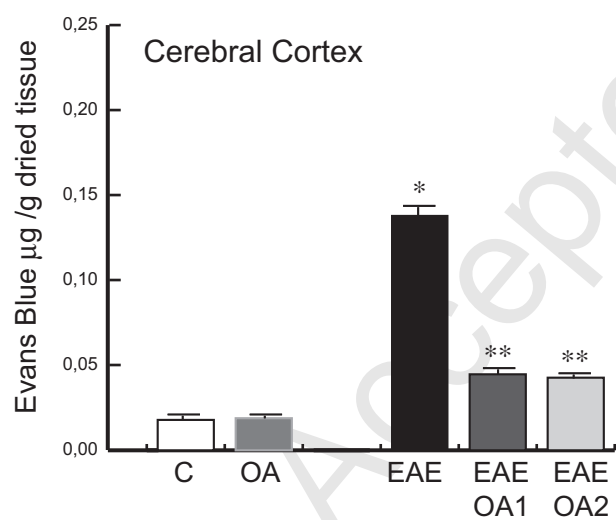


Figure 8

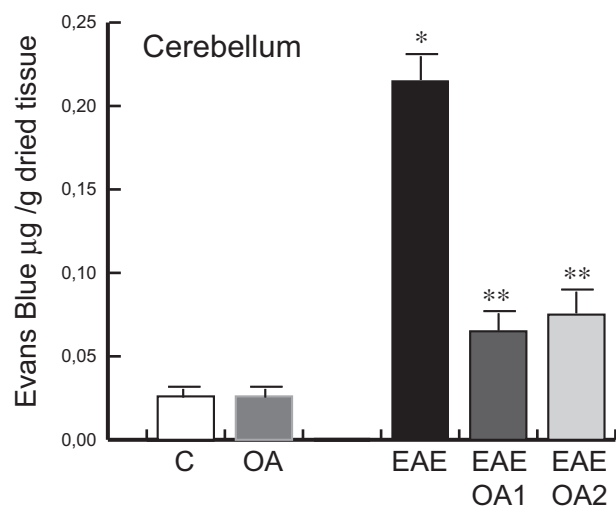
A



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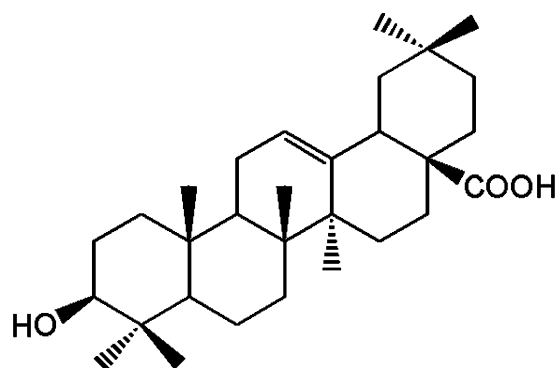


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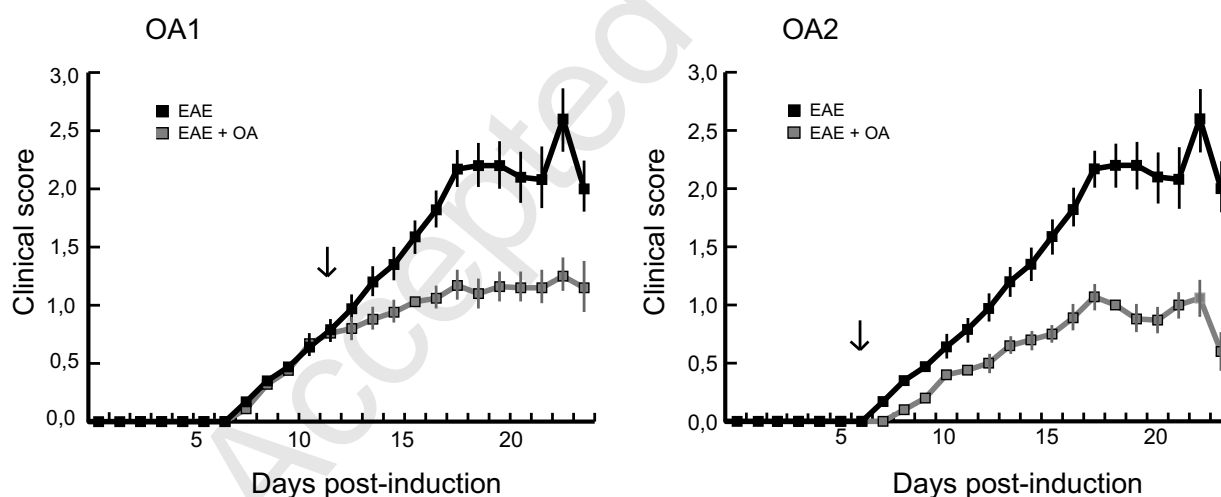
Semiprofilactic and therapeutic administration of the natural triterpene, oleanolic acid, can ameliorate the clinical signs of the experimental autoimmune encephalomyelitis.

A



Molecular structure of Oleanolic Acid

B



C

