



Beneficial actions of oleanolic acid in an experimental model of multiple sclerosis: A potential therapeutic role

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ABSTRACT

Multiple sclerosis (MS) is a chronic autoimmune inflammatory disease for which there exist no therapies without undesired side effects. Thus, the establishment of less toxic treatments is an ongoing challenge. Nowadays, research on medicinal plants has been attracting much attention, since screening of its active principles could prove useful in identification of safe and innovative pharmaceutical molecules. In this study we investigated the therapeutic effect of oleanolic acid (OA) a plant-derived triterpene with potent anti-inflammatory and immunomodulatory activities, whose actions on CNS diseases remain far from completely characterized. We focussed on the potential therapeutic effect of oleanolic acid (OA) on an accepted experimental model of MS, the experimental autoimmune encephalomyelitis (EAE). We have found that OA treatment, before or at the early onset of EAE, ameliorates neurological signs of EAE-mice. These beneficial effects of OA seem to be associated with a reduction of blood–brain barrier leakage and lower infiltration of inflammatory cells within the CNS, as well as with its modulatory role in Th1/Th2 polarization: inhibition of proinflammatory cytokines and chemokines, and stimulation of anti-inflammatory ones. Moreover, EAE-animals that were treated with OA had lower levels of anti-MOG antibodies than untreated EAE-mice.

Our findings show that the administration of the natural triterpenoid OA reduces and limits the severity and development of EAE. Therefore, OA therapy might be of clinical interest for human MS and other Th1 cell-mediated inflammatory diseases.

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1. Introduction

Multiple sclerosis (MS) is an immuno-inflammatory, degenerative and often disabling disease of the central nervous system (CNS). The mechanisms by which this disease appears and progresses are heterogeneous in nature and still unclear, but studies of patients with MS suggest that the observed demyelination in the CNS is a result of a T-cell-mediated autoimmune response [1]. Unfortunately, MS is a frustrating disease because there is no definitive therapy. Conventional therapeutic strategies, including immunosuppressive drugs and steroids, present undesirable side effects, particularly in long-term treatment. It thus becomes necessary to establish less toxic therapeutic options.

Experimental allergic encephalomyelitis (EAE) is an experimental model widely used for the study of MS. The development

and progression of clinical signs in EAE are associated with loss of blood–brain barrier (BBB) integrity and upregulation of adhesion molecules expression in CNS tissues, resulting in the migration of vascular inflammatory cells into the CNS compartment [2,3]. This inflammatory cascade triggers upregulation of various proinflammatory mediators produced by infiltrating leukocytes and resident glial cells in the CNS, which are determining factors in the tissue damage [4,5].

In recent years, numerous studies describing the therapeutic properties of extracts from plants used in traditional medicine have been developed. Indeed, the use of such extracts as complementary and alternative medicine has lately increased, and also serves as an interesting source of drug candidates for the pharmaceutical industrial research [6]. Oleanolic acid (OA), a natural pentacyclic triterpene widely found in a variety of plants, has been shown to display numerous biological properties with therapeutic potential [7,8]. It is present in the leaves and fruits of *Olea europaea*, among other vegetables. In fact, it is found in pomace olive oil, the principal source of fat in the Mediterranean

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diet [9] and supports the hypothesis that some components of olive oil may contribute to the beneficial properties of this diet for human health. It has been reported that OA has anti-inflammatory, anti-tumorigenic, anti-diabetic and anti-viral properties, as well as cardioprotective, hepatoprotective and immunomodulatory effects [10–12]. Recently, a number of synthetic oleanane triterpenoid derivatives based on oleanolic acid has been synthesized, in an attempt to increase its potency; some of these are currently undergoing clinical trials for the treatment of solid tumors [13].

In addition, OA is clinically used in China for the treatment of hepatitis B, and as an adjunct therapy for the prevention of hepatotoxicity [14]. In recent years, OA or herbal extracts containing OA have been demonstrated to present immunomodulatory actions [12] and promote improvement in brain function [15]. Synthetic analogs have also been revealed as novel neuroprotective agents [16]. However, no information regarding its *in vivo* effect on neuroinflammatory diseases is available. In this study, we demonstrate that OA significantly reduces the clinical signs of EAE when administered both before and after onset of the disease. We outline its effects on inflammatory parameters such as cellular infiltration and BBB dysfunction, as well as on the expression levels of cytokines, chemokines and adhesion molecules.

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-Gutiérrez from the Instituto de la Grasa, Sevilla, Spain. The molecular structure of OA is shown in Fig. 1.

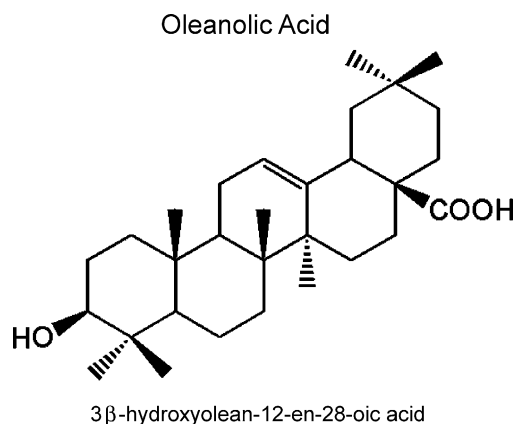


Fig. 1. Molecular structure of oleanolic acid.

2.2. Active induction of EAE

C57BL/6 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.

Chronic progressive disease was induced in adult 8–10-week-old female C57BL/6 mice with myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide. Briefly, mice were injected in the tail base bilaterally with 100 μ l of an innoculum containing 100 μ g 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 2 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: semiprophylactic, 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 0–5, 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 2.5, partial hind limb paralysis; grade 3, complete hind limb paralysis; grade 3.5, complete hind limb paralysis and fore limb weakness; grade 4, tetraplegic; grade 5, moribund state or death. Scores of the two blinded investigators were average. Data were plotted as daily mean clinical score for all animals in a particular treatment group. Scores of asymptomatic mice (score = 0) were included in the calculation of the daily mean clinical score for each group.

2.5. Histological studies

CNS tissues from five representative animals from the experimental groups were dissected between 21 and 24 days after immunization. The brain, cerebellum and spinal cord were fixed and embedded in paraffin. Paraffin-embedded tissues were cut on a microtome (5 μ m thickness) and incubated sequentially with anti-Mac-1/CD11b (macrophages/microglia), horseradish peroxidase-conjugated anti-goat antibody, and diaminobenzidine chromogen. Histological examination was performed with a Nikon Eclipse 90i (Nikon Instruments Inc., Amstelveen, The Netherlands) connected to a DXM1200C digital camera (Nikon Instruments Inc., Amstelveen, The Netherlands). Hematoxylin was used for background staining. Sections from 4 to 10 segments per mouse were examined blindly by one investigator.

2.6. Intravital microscopy in mouse brain

Intravital microscopy of the mouse cerebrovasculature was performed as previously described [18]. Briefly, the mice were anesthetized by i.p. injection of a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine and the tail vein was cannulated for administration of fluorescent dyes. A craniotomy was performed using a high-speed drill (Dremel, Madrid, Spain) and the dura matter was removed to expose the underlying pial vasculature. The mouse was maintained at 37 °C throughout the experiment and the exposed brain was continuously superfused with artificial cerebrospinal fluid buffer (132 mM NaCl, 2.95 mM KCl, 1.71 mM CaCl₂, 0.64 mM MgCl₂, 24.6 mM NaHCO₃, 3.71 mM dextrose and 6.7 mM urea, pH 7.4) at 37 °C.

In order to observe leukocyte/endothelium interactions, leukocytes were labelled *in vivo* immediately before each recording, using rhodamine-6G (5 mg/kg body weight). Mice were injected via the tail vein with 200 µl of a 0.05% rhodamine-6G solution: 5 mg of rhodamine-6G were dissolved in 10 ml of 0.9% saline solution. Rhodamine-6G selectively stains mitochondria and achieves 99.99% staining of leukocytes for up to 5 min after injection of the concentration used in this study [18]. Then, leukocytes were examined using a Zeiss Axioplan 2 (Thornwood, NY, USA) imaging microscope connected to an AxioCam MR digital camera using the AxioVision AC imaging software and an Acroplan 20×/0.50W Ph2 lens. Eight different postcapillary venules of diameter between 30 and 70 µm were chosen for observation. All experiments were recorded for later analysis. Rolling leukocytes were defined as white cells moving at a velocity less than that of erythrocytes. Leukocytes were considered adherent to the venular endothelium if they remained stationary for 30 s or longer. Leukocyte adhesion was expressed as cells/mm² of venular surface area, as shown previously [19].

2.7. Experimental protocol for intravital microscopy

Leukocyte rolling and adhesion were observed by intravital microscopy at day 21–24 post-immunization with MOG_{35–55}. Animals were subjected to different protocols corresponding to different treatment with OA as mention above: mice were treated daily with 50 mg/kg/d OA from either day 7 (OA2) or 12 (OA1) post-immunization.

2.8. BBB permeability measurement

In order to evaluate BBB disruption induced by EAE induction, we measured the extravasation of Evans Blue dye (EB) as a marker of albumin extravasation. At 21–24 days following EAE induction, mice were injected i.p. with 1 ml of 4% w/v Evans Blue. After 4 h, mice were sacrificed, perfused, and brain and spinal cord were removed. Dye was extracted from the CNS tissue for 2–3 days in formamide (4 ml/g of wet tissue) at room temperature. Extracted dye concentration was determined by measuring the absorbance at 650 nm. CNS tissue was dried 24 h at 60 °C and weighed.

Calculations were based on external standard readings, and extravasated dye was expressed as µg of Evans Blue/mg dried weight of brain tissue.

2.9. Assays for cytokines, chemokines and adhesion molecules levels detection

Mice ($n = 7$, per group) were treated i.p. with 50 mg/kg/d OA as mentioned above. Animals were sacrificed at day 20–24 post-immunization and CNS tissue samples (cerebral cortex, cerebellum and spinal cord) were prepared by homogenization in ice-cold PBS supplemented with protease inhibitor cocktail (Sigma–Aldrich, St

Louis, MO, USA) using a tissue-tearor homogenizer (Cole-Parmer Instrument, IL, USA). The samples were then centrifuged for 15 min at 4000 rpm in order to separate extracellular supernatant from the cell pellet. Aliquots of supernatant were prepared and stored at –80 °C until cytokine assays were performed. OPN, ICAM-1, TNFα and IL-10 levels were examined by commercial ELISA kits following manufacturer's instructions. The concentration of the cytokines was determined by extrapolation from the appropriate standard curve, and was expressed as pg/100 mg tissue.

Th1 cytokines and chemokines (TNFα, IFNγ, MCP-1 and MIP-1α) were assayed from plasma using a multiplex assay kit (Lincoplex, Linco Research, St. Charles, MO, USA) according to the instructions of the manufacturer. Assay standard curves were created with the stock provided by the kit, and the assay buffer served as the background (0 concentration). The concentrations of the cytokines were calculated by matching the optical density of the samples against a regression equation established with the standard curve. The assay was performed in duplicate for the background, standards, controls, and samples.

2.10. Detection of autoantibodies

Serum was collected from animals on day 21 after immunization. Level of antibodies directed against MOG was determined using the enzyme-linked immunoassay (ELISA) technique. 96-well polystyrene microtiter plates were coated with 0.5 µg/well of MOG_{35–55} peptide by overnight incubation in PBS at 4 °C. After blocking with 5% BSA, the wells were incubated in duplicate with the serum samples diluted 1:60 in PBS plus 10% FCS for 2 h at 20 °C. After washing, HRP-conjugated goat anti-mouse IgG, IgG1 or IgG2a polyclonal antibodies were subsequently added for 90 min. After another washing, adding the substrate, and arresting the reaction with 0.1N HCl, absorbance was read at 450 nm. The results were expressed as mean optical density at 450 nm.

2.11. Statistical analyses

Statistical analysis was performed with the GraphPad Prism Version 4 software (San Diego, CA, USA) by analysis of variance (ANOVA). Analyses were performed using two-way RM-ANOVA for comparison clinical parameters, and one-way ANOVA for comparison of parameters (cytokines, extravasation, leukocytes and MOG antibodies) at day 21 post-infection. A *post hoc* analysis was made by the Bonferroni's multiple comparison test. Results are expressed as mean ± SD; p values < 0.05 were considered statistically significant.

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 MOG_{35–55} 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 and 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 and B). In

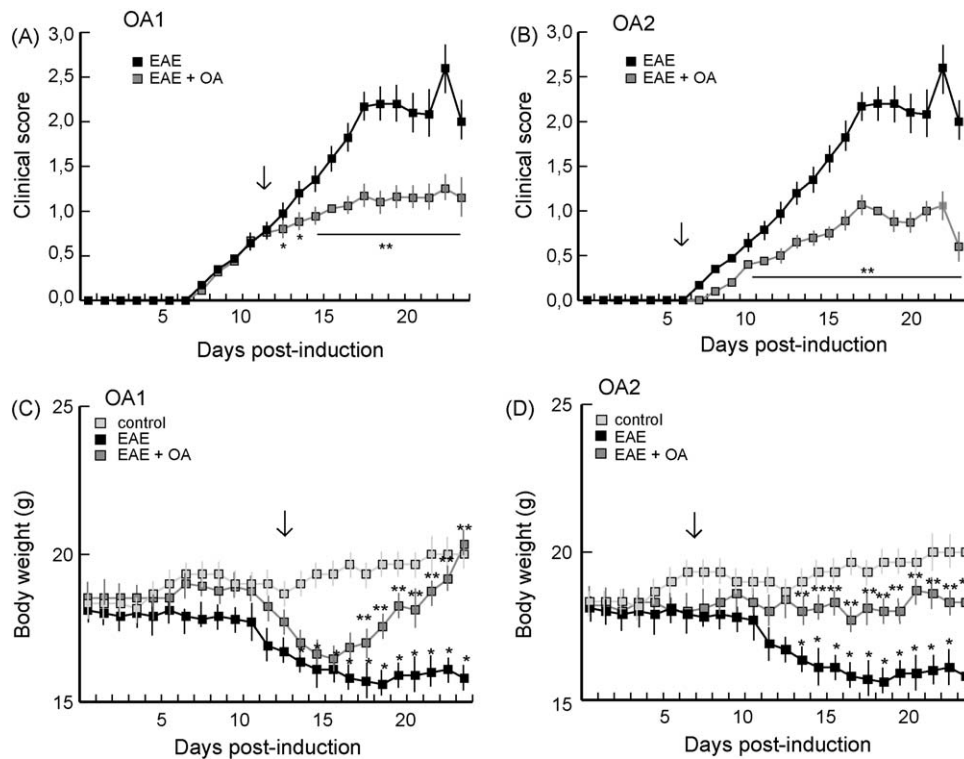


Fig. 2. Oleanolic acid influence on EAE disease progression. (A and B) Effect on clinical signs, and (C and D) on body weight. C57BL/6 mice were immunized with MOG_{35–55} and given oleanolic acid daily i.p. from day 12 (OA1) or 7 (OA2) post-immunization until the end of the experiment (21–24 post-immunization). Arrows indicate when treatment was initiated. Values are means \pm SD. (■) EAE + vehicle ($n = 18$); (◼) EAE + OA 50 mg/kg ($n = 10$); (□) control + vehicle ($n = 10$). For some points, error bars are not visible because the deviations are smaller than the symbol sizes. Statistically significant differences were indicated for clinical symptoms by * $p < 0.01$ and ** $p < 0.001$ versus untreated EAE-mice; and for body weight by * $p < 0.001$ versus control and ** $p < 0.001$ versus untreated EAE-mice.

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 and 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 (Fig. 2A), while untreated animals progressively deteriorated. This OA1 group also showed a remarkable improvement in body weight (Fig. 2C). The effect observed on the weight parameter is not the result of a direct effect of the compound on weight gain, because the molecule itself does not affect the weight of healthy animals (data not shown).

We next performed experiments to assess the impact of oleanolic acid treatment on the long-term evolution of clinical symptoms (endpoint: tetraplegic stage). In Fig. 3 we show both evolution of clinical symptoms (Fig. 3A), and time to maximum score using Kaplan–Meier survival analysis (Fig. 3B). Although in all groups, mice continued to deteriorate over the time, OA1- and OA2-treatments delayed the clinical disease. The average clinical score on both OA–EAE groups was 2.5 (partial hind limb paralysis) at the time of maximum score of untreated EAE-mice (score of 4) was achieved, and body weight loss by day 40 was approximately 20% for OA1 and 16% for OA2, opposed to 50% for untreated EAE-animals (data not shown), indicating a marked amelioration of symptoms by OA. Mice that developed severe EAE (score of 4) were euthanized at this time. Differences in clinical evolution for the

treated EAE-mice were not found significant between OA1 and OA2 groups.

3.2. Expression of cytokines and chemokines in the CNS and serum of OA-treated EAE-mice

To investigate whether the diminished EAE severity resulting from OA treatment could be associated with a reduced immune-inflammatory reaction, we studied both cellular and humoral responses. Thus, the modulation of some inflammatory events, such as accumulation of cytokines or adhesion molecules, in the sera or CNS tissue of OA-treated EAE-mice was first examined.

We studied the levels of osteopontin (OPN), a proinflammatory cytokine, and ICAM-1, an adhesion molecule, in CNS tissue extracts at days 21–24 after disease induction. In agreement with clinical signs, the concentration of OPN and ICAM-1 (Fig. 4A and B) in cerebral cortex, cerebellum and spinal cord of EAE-mice was significantly increased compared with control animals. In contrast, both OA1 and OA2 treatment notably diminished the expression of both proteins. As shown in Fig. 4A, OPN decreased up to 60% in the spinal cords of OA1-treated EAE-mice with a mean of (377 ± 48) pg/100 mg tissue, and 63% in the spinal cords of OA2-treated EAE-mice with a mean of (347 ± 65) pg/100 mg tissue compared with (949 ± 68) pg/100 mg tissue in untreated EAE-mice ($p < 0.001$). In cerebellum the levels of OPN in EAE-mice and controls were (507 ± 45) pg/100 mg tissue and (165 ± 17) pg/100 mg tissue, respectively, and a decrease of 53.2% in OA1-treated EAE-mice with a mean of (237 ± 36) pg/100 mg tissue, and 51.8% in OA2-treated EAE-mice with a mean of (244 ± 18) pg/100 mg tissue was observed, compared with untreated EAE-mice ($p < 0.001$). In cerebral cortex the levels of OPN in EAE-mice and healthy controls were of (101.6 ± 6.2) pg/100 mg tissue and

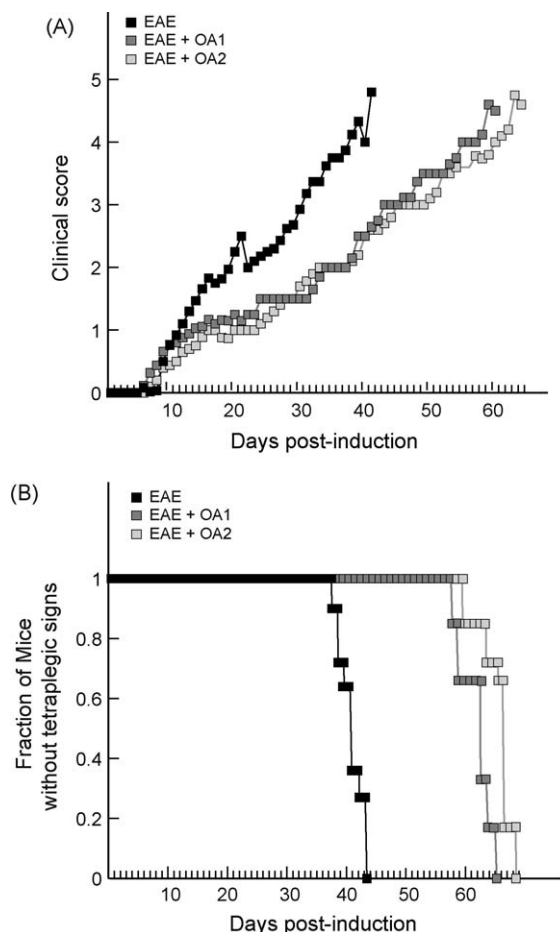


Fig. 3. Effect of oleanolic acid treatments on maximum clinical scores. (A) Effect on clinical sign evolution, and (B) on disease severity. C57BL/6 mice were immunized with MOG_{35–55} and given oleanolic acid daily i.p. from day 12 (OA1) or 7 (OA2) post-immunization until severe clinical signs were reached (score of 4), at this time mice were euthanized. Values are means \pm SD, $n = 7$ in all groups. (■) EAE + vehicle; (▒) EAE + OA1 50 mg/kg; (□) EAE + OA2. The difference between EAE-untreated and EAE-OA groups was highly significant ($p < 0.001$).

(33.9 ± 2.6) pg/100 mg tissue, respectively, and a decrease of 49.3% in OA1-treated EAE-mice with a mean of (51.2 ± 10) pg/mg tissue and 51% in OA2-treated EAE-mice with a mean of (49.4 ± 11) pg/mg tissue was observed as compared with untreated EAE-mice ($p < 0.001$).

In addition, as shown in Fig. 4B, ICAM-1, an important endothelial adhesion molecule involved in recruiting cells into the brain, was highly expressed on the CNS tissues obtained from EAE-mice: (117 ± 9.8) pg/100 mg spinal cord, (59.9 ± 9.1) pg/100 mg cerebellum and (11.6 ± 1.2) pg/100 mg cerebral cortex. In contrast, detected levels of ICAM-1 significantly decreased in tissues obtained from OA-treated EAE-mice, as expected. In OA1- or OA2-treated EAE-mice, ICAM-1 expression diminished in spinal cord by 80.2% and 79.7% respectively, in cerebellum by 81% and 82% respectively, and in cerebral cortex by 81% and 84.5%, respectively.

In order to determine the effect of OA treatment in the production of inflammatory mediators, we next examined in sera the levels of Th1 cytokines, TNF α and IFN γ , as well as the chemokines MCP-1 and MIP-1 α at days 21–24 after disease induction. As shown in Fig. 5A, any OA treatment (before or after the clinical onset) diminished the presence of the screened cytokines and chemokines compared with untreated mice. Accordingly, the high levels of TNF α detected in spinal cord tissue

of EAE-mice decreased by 56% in both OA1- or OA2-treated EAE-animals as compared to untreated ones, whereas Th2 cytokine IL-10 expression increased significantly (Fig. 5B).

3.3. Effect of OA treatment on the humoral immune phase on EAE-mice

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

3.4. Oleanolic acid reduces infiltration in EAE-mice

Paralleling the immune-inflammatory parameters studied above, CNS tissue sections were also incubated with anti-Mac-1/CD11b, a marker for macrophages/microglia, in order to detect the effect of OA on infiltrating/intrinsic inflammatory cells.

Inflammatory cells are found within the CNS in acute EAE and are associated with clinical signs. As shown in Fig. 6, an abundance of inflammatory cells was present either in spinal cord or in the granular layer of cerebellum of EAE-mice, as expected (qualitative evaluation), while positive staining was not observed in CNS tissue sections of control mice. These cells showed morphology of macrophage-like or/and of ramified microglia (microglial-like cells). However, in OA-treated EAE-mice a substantially reduced infiltration was observed, as judged by a minimal CD11b/Mac-1 immunoreactivity on the different tissue sections, these results being linked to an improvement of the clinical symptoms of EAE.

3.5. Effect of OA treatment on leukocyte recruitment in the CNS microvasculature of EAE-mice

Previous studies have shown that cellular recruitment from the circulation to the CNS can be visualized by intravital microscopy techniques. These experiments have demonstrated that the mononuclear cells infiltrate around and within the pial venules of EAE-mice [19]. Therefore, in this study we used intravital microscopy to directly observe leukocyte–endothelial interactions in the cerebral-microvasculature of EAE-mice and investigate whether OA treatment, after or before the onset of the disease, modulate this recruitment. Leukocyte–endothelial cell interactions were evaluated at days 21–24 post-immunization, when the group of untreated mice showed hind limb paralysis.

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

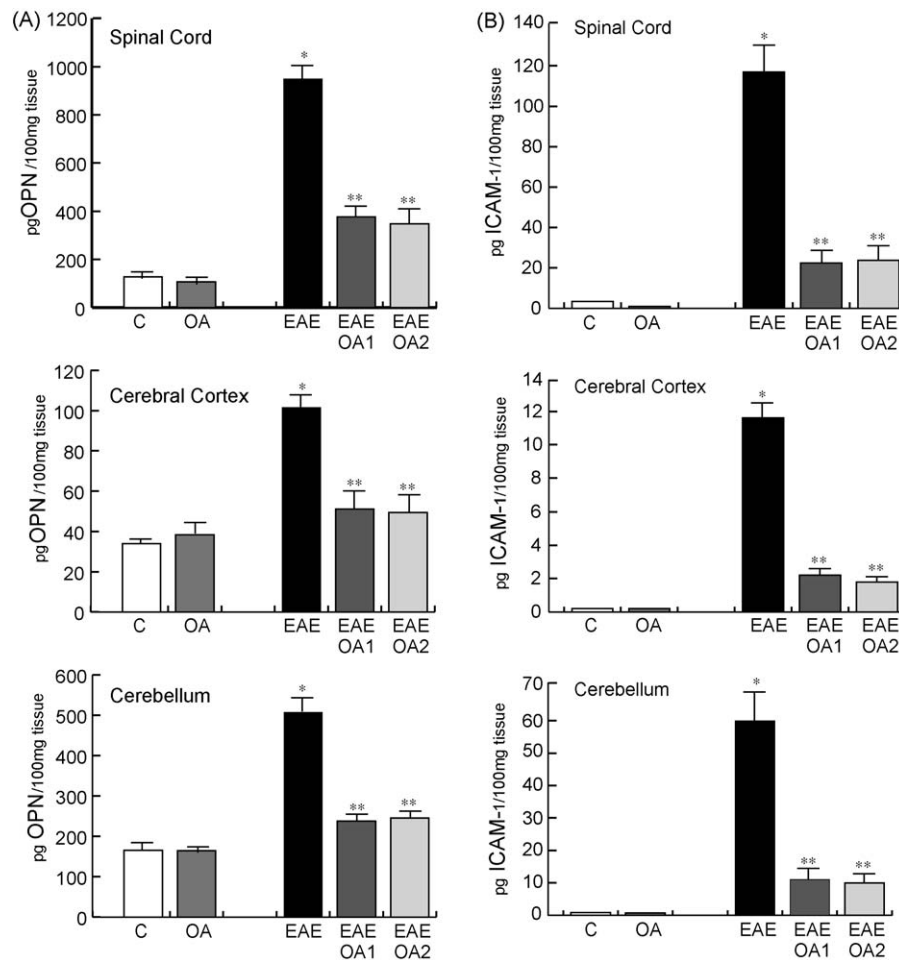


Fig. 4. OPN and ICAM-1 protein expression in CNS tissues of untreated and treated EAE-mice. On day 21 after immunization with MOG_{35–55}, protein concentrations of OPN and ICAM-1 were measured by commercial ELISA in spinal cord, cerebral cortex and cerebellum extracts of control, control OA, EAE, EAE + OA1, and EAE + OA2 mice. Results were expressed as the mean \pm SD from seven animals per group. Statistically significant differences were indicated by * $p < 0.001$ versus control and ** $p < 0.001$ versus untreated EAE-mice.

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% versus 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 days 21–24 post-immunization, 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 significantly differ between both groups of OA-treated mice, EAE + OA1 or EAE + OA2. Neither was any variance found between treated and untreated healthy mice.

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

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

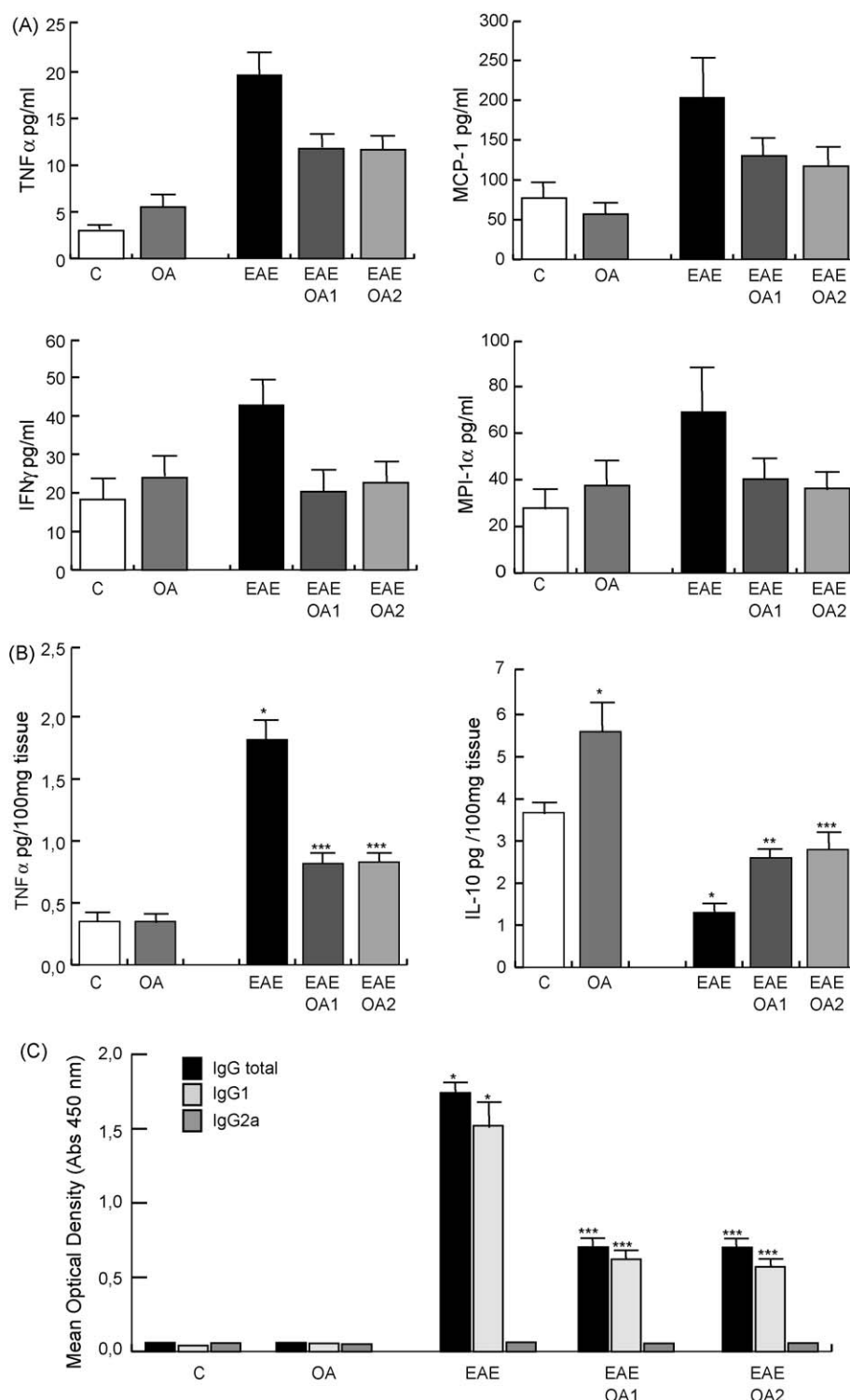


Fig. 5. Effect of OA treatments on cytokine, chemokine and anti-MOG_{35–55} antibody levels in EAE-mice. On day 21 after immunization with MOG_{35–55}, TNF α , IFN γ , MCP-1, MIP-1 α and IL-10 protein concentrations were measured, as explained in Section 2, in serum samples (A) or in spinal cord extracts (B) of control, control + OA, EAE, EAE + OA1, and EAE + OA2 mice. (C) Sera were also evaluated by ELISA for titers of pMOG_{35–55}-specific immunoglobulins at 1/60 dilution. Results were expressed as the mean \pm SD from seven animals per group. Statistically significant differences were indicated by * $p < 0.001$ versus control, ** $p < 0.01$ versus untreated EAE-mice and *** $p < 0.001$ versus untreated EAE-mice.

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

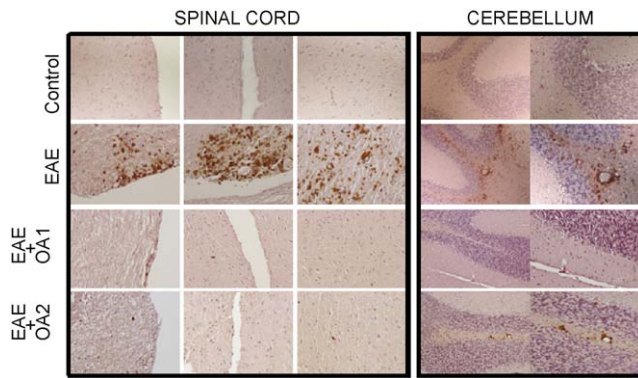


Fig. 6. OA treatment improves immunohistological outcomes in spinal cords and cerebellum of EAE-mice. Typical sections of cellular infiltration on spinal cord and cerebellum, at day 21 post-immunization, in control, EAE, EAE + OA1 and EAE + OA2 mice, monitored by anti-Mac-1/CD11b, a marker for macrophages/microglia, are shown. Spinal cord panels show three different histological sections visualized with a 20 \times lens. Cerebellum left and right panels represent a same section visualized with a 10 \times and a 20 \times lens, respectively.

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.

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

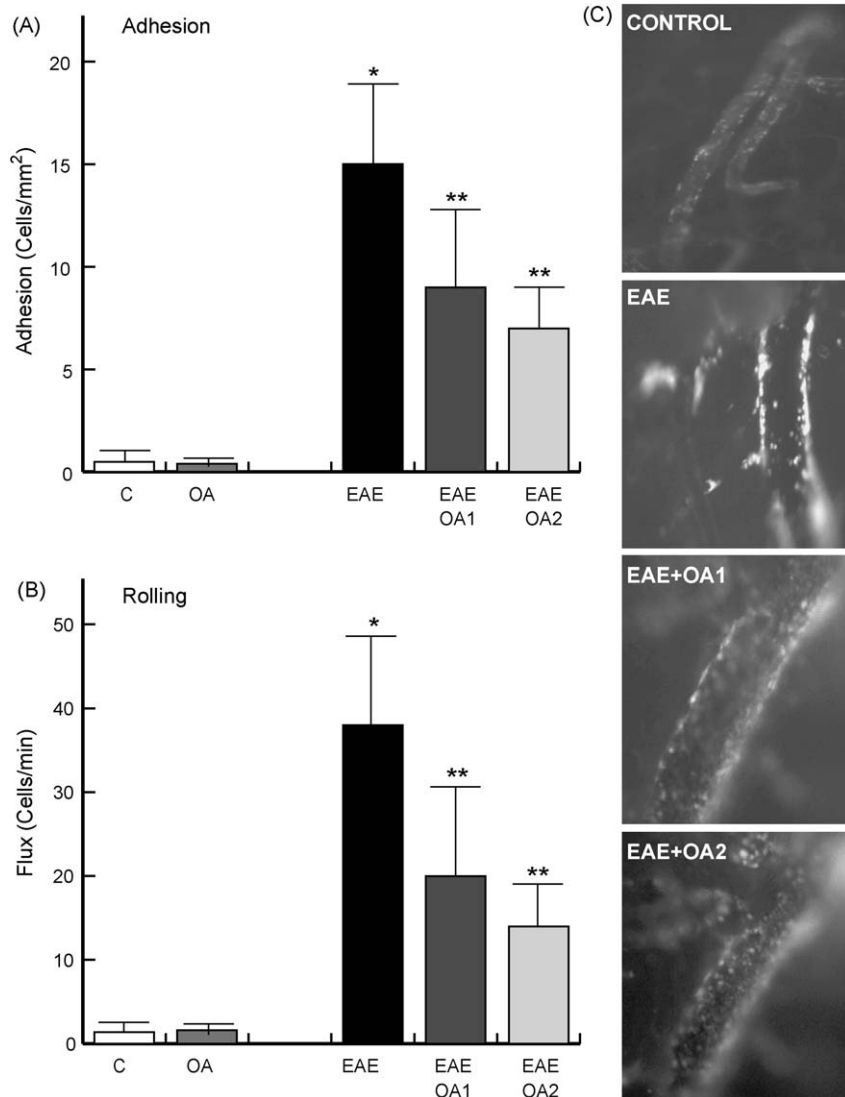


Fig. 7. Leukocyte recruitment to the brain over the course of EAE. Intravital microscopy was used to assess the firm arrest (A) and rolling flux (B) of leukocytes on brain microvasculature, at day 21 post-immunization. The protocol included control, EAE, EAE + OA1 and EAE + OA2 groups visualized on day 21 post-immunization. Results are shown as mean \pm SD of cells per minute. *Significant ($p < 0.001$) difference compared to control, **significant ($p < 0.001$) difference compared to untreated EAE. (C) Representative images of the pial microvasculature in healthy, untreated EAE-mice and OA1- or OA2-treated EAE-mice are shown. Note that the marked increase in the number of fluorescently labelled leukocytes interacting with the cerebral endothelium in EAE-mice declined in EAE + OA1 and EAE + OA2 mice. In all groups, $n = 10$.

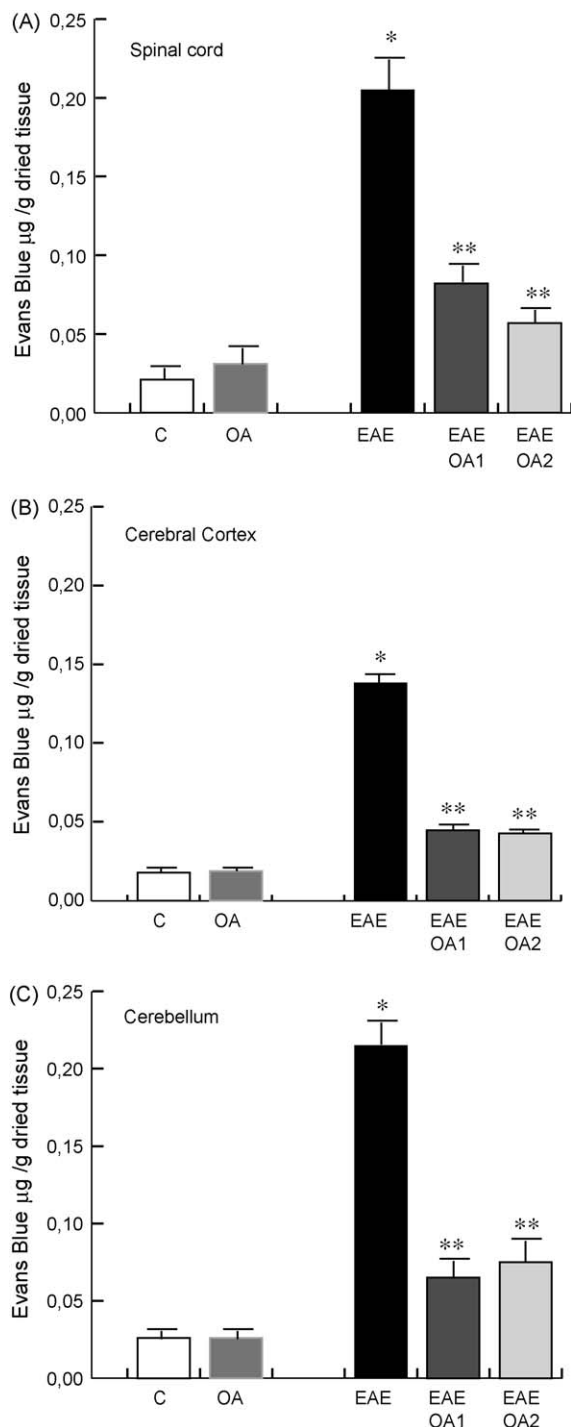


Fig. 8. Changes in blood–brain barrier permeability of OA-treated or untreated EAE-mice. Evans Blue dye was used as a measurement of plasma protein extravasation in (A) spinal cord, (B) cerebral cortex and (C) cerebellum 21–24 days post-immunization. Bars represent means \pm SD, for $n = 6$ mice. *Significant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

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.

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

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

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

The inflammatory response found in EAE-mice is closely related to BBB damage [5]. Irrespective of the clinical forms of EAE, BBB breakdown is a hallmark in the development of EAE, as well as in human MS which exacerbates the inflammatory response by providing circulating cells and factors the access to privileged CNS tissues, and soluble CNS products access to the circulation. The precise mechanisms mediating the onset of BBB dysfunction are still controversial. Besides inflammatory cytokines including TNF α and IL-1 [30], or enzymes such as metalloproteinases [31], disruption of CNS homeostasis can occur in the absence of important inflammatory mediators through the action of factors such as arachidonic acid metabolites [32] and free radicals such as peroxynitrite [33] or NMDA receptor agonists [34]. In the present study, we observed that the loss of BBB integrity in C57BL/6J EAE-mice presents tissue differences, and similar to what has been previously found in other EAE-mice models, BBB permeability was predominantly disrupted in the cerebellum and spinal cord [35]. In addition, these regional differences in BBB permeability closely correlated with the expression pattern of parameters that contributes to inflammation and cell accumulation: OPN and ICAM-1 upregulation was also prominent in cerebellum and spinal cord. Therefore, it can be hypothesized that the expression of these molecules may either contribute to, or be induced in conjunction with BBB permeability changes, although such is not the goal of this study. Daily OA treatment, starting before or after the onset of the disease, significantly inhibited BBB disruption in all CNS tissues of EAE-mice. It remains to be further investigated whether OA actions in neurovascular functions during EAE are exclusively related to its anti-immune/inflammatory properties or represent pleiotropic effects. Although some natural triterpenes have shown

neuroprotective effects [36], inhibitory properties in arachidonic-acid-metabolizing enzymes [37], anti-oxidative properties and membrane-stabilizing actions [20], and inhibitory activity in the induction of ICAM-1 [38], no study to our knowledge had in fact been performed linking these actions to BBB integrity.

Finally, although major attention is usually given to these Th-cell-mediated events in EAE, specific autoantibodies and B cell responses are also thought to contribute to the exacerbation of experimental and human MS [39]. Consistent with what has previously been reported, we found in this study that MOG-induced EAE showed higher concentrations of anti-MOG IgG antibodies of the IgG1 isotype than of the IgG2a isotype [40,41]. The continuous administration of OA (OA1 and OA2) significantly reduced IgG MOG antibody production, particularly the IgG1 isotype. Thus, inhibition of the pathogenic antibodies production against MOG may also be a contributing mechanism by which OA exerts its beneficial actions on this complex autoimmune disorder. A similar inhibition of the humoral response has been previously described for IFN- γ treatment [42]. However, while IFN- γ actions suggest inhibition of B cell clonal expansion by induction of terminal differentiation, it remains unclear as to how OA diminishes humoral autoimmunity.

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

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

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