Natural Triterpenes Modulate Immune-Inflammatory Hallmarks to Protect against Experimental Autoimmune Encephalomyelitis. Therapeutic Implications for Multiple Sclerosis.

R Martín¹, M Hernández, Cl. Cordova, M L Nieto*

Instituto de Biología y Genética Molecular, CSIC-Universidad de Valladolid, Spain.

Running title: Natural triterpenes actions in autoimmunity

*Corresponding Autor:
Mª Luisa Nieto, Instituto de Biología y Genética Molecular, C/Sanz y Forés 3, 47003 Valladolid, Phone number: 34-983184836, Fax number: 34-983184800. E-mail: mlnieto@ibgm.uva.es

Footnotes:
¹ Present address: Instituto de Ciencias del Corazón (ICICOR), Hospital Clínico Universitario, Valladolid
Summary

Background and purpose: Multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), are inflammatory demyelinating diseases that develop as a result of deregulated immune responses causing glia activation and CNS tissues destruction. Oleanolic acid and erythrodiol are natural triterpenes that display strong antiinflammatory and immunomodulatory activities. We recently described that oleanolic acid beneficially influences the course of established EAE. We now extend our previous observations to erythrodiol, and also address the efficacy of both molecules, in protecting against neuroinflammatory diseases, when administrated under different regimens.

Experimental approach: The utility of both triterpenes in disease prevention was evaluated at a clinical and molecular level: in vivo through their prophylactic administration to myelin oligodendrocyte protein-immunized C57BL/6 mice, and in vitro through their addition to stimulated-BV2 microglial cells.

Results: Triterpenes protected against EAE by restricting the infiltration of inflammatory cells into the CNS and by preventing blood-brain barrier disruption. Triterpenes-pretreated EAE-mice exhibited less leptin secretion, and switched cytokine production towards a Th2/regulatory profile: levels of Th1 and Th17 cytokines decreased, while expression of Th2 cytokines was up-regulated in both serum and spinal cord. Triterpenes also affected the humoral response causing auto-antibody production inhibition. In addition, in vitro triterpenes abrogated ERK and rS6 phosphorylation, reduced the proliferative response, phagocytic properties, and synthesis of
proinflammatory mediators induced by the addition of inflammatory stimuli to microglia.

**Conclusions and implications:** Both triterpenes restricted the development of the characteristic hallmarks of EAE. We envision these natural products as novel helpful tools for intervention in autoimmune and neurodegenerative diseases including MS.

**Keywords:** Encephalomyelitis; Neuroimmunology; Inflammation; Microglia; Pharmacology; Triterpenes

**Abbreviations**

ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; ERY, erythrodiol; EAE, experimental autoimmune encephalomyelitis; BBB, evaluate blood-brain barrier; EB, Evans Blue; CFA, complete Freund’s adjuvant; CNS, central nervous system; COX-2, cyclooxygenase-2; IL-4, interleukin-4; IFNγ, interferon-γ; IFNτ, interferon-τ; IL-6, interleukin-6; IL-10, interleukin-10; IL-17, interleukin-17; iNOS, inducible nitric oxide synthase; i.p., intraperitoneal; MOG, myelin oligodendrocyte glycoprotein; rS6, ribosomal protein S6; TNFα, tumor necrosis factor α; MS, multiple sclerosis; OA, oleanolic acid.
Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disease directed against myelin proteins of the brain and spinal cord, which is considered as one of the major neurological illness of young adults (Noseworthy et al., 2000). The precise cause of MS is unknown, but one theory is that it might be triggered by exposure to a viral infection or environmental influences. The disease takes dissimilar courses in different people and can go into four main pathological subtypes, even leading to death in the very progressive form (Lassmann et al., 2001).

Experimental autoimmune encephalomyelitis (EAE) induced in susceptible strains of animals provides the best available model for understanding events in MS and to test new drugs that could lead to novel therapies (Steinman, 1999). MS/EAE pathogenesis is driven mostly by a Th1-mediated autoimmune response. The development of the disease includes breakdown of the blood brain barrier, infiltration of the CNS - brain and spinal cord - by myelin-reactive T cells and macrophages, activation of resident CNS cells (microglia and astrocytes), demyelination and axonal loss (Merrill and Benveniste 1996; Benveniste, 1997; Engelhardt, 2006).

Microglial cells are active participants throughout the MS disease process. “Activated” microglia produces inflammatory cytokines, free radicals and attracts immune cells into the CNS. A diffuse activation of microglia throughout the brain serves as a source of inflammation from inside the CNS in chronic MS/EAE, while at latter stages of the disease a chronically activated microglia is associated with impaired neural function (Rasmussen, 2007).

Other components of the immune system that play crucial roles in MS/EAE pathogenesis include dendritic and B cells, antibodies, as well as inflammation-related enzymes, cytokines, and chemokines. Thus, cyclooxigenase-2 (COX-2) and inducible
nitric oxide synthase (iNOS) enzymes and pro-inflammatory cytokines such as interferon-γ (IFNγ), tumor necrosis factor-α (TNFα) or interleukin-17 (IL-17) are considered to be pathogenic, while the Th2 cell-related cytokines interleukin-4 (IL-4) and interleukin-10 (IL-10) have been shown to down-regulate the immune response in acute EAE (Sospedra and Martin, 2005; Hafler 2004; Imitola et al 2005). Much progress has been made over the past decade in elucidating the causes and molecular basis of MS, but in spite of the extensive research performed to develop new pharmacotherapeutic approaches to slow down the disease progression, there are still no optimal therapies available, due to both unwanted side effects of the drugs and the clinical and immunopathologic heterogeneity of this disease (Hemmer, 2006). Oleanolic acid (OA) and erythrodiol (ERY) are two natural triterpenes of the oleanane group present in many vegetables, including the leaves and fruits of Olea Europea. They have been recognized to have hepatoprotective, antiinflammatory, and antihyperlipidemic properties. Indeed, oleanolic acid has been promoted in China as an oral drug for human liver disorders. Data that correlated well with the traditional use of Olea Europea in African and European Mediterranean countries, where this plant has been utilized widely in folk medicine as a diuretic, hypotensive, hypoglucomic, emollient, febrifuge and tonic, for urinary and bladder infections, for headaches, as well as a therapy for inflammatory pain (Dold and Cocks, 1999). Recently, a number of synthetic oleanane triterpenoid derivatives have been synthesized based on oleanolic acid with more potent activities, some of which are currently being developed for the treatment of chronic kidney diseases (Pergola et al, 2011) or as an attractive new therapeutic option for cancer patients by enhancing the effect of immunotherapy (Nagaraj et al, 2010). In the last years, a variety of novel pharmacological properties of triterpenoids have been reported: i) beneficial effects on cardiovascular system due to
antioxidant and vasorelaxant activities (Rodriguez-Rodriguez et al., 2006), ii) interaction with cytochrome P450s, iii) antiproliferative activities on tumoral cells by activating apoptotic programs (Martin et al., 2007; Martin et al, 2009), iv) effects on intracellular redox balance and protective effects against lipid peroxidation, as well as v) immunomodulatory effects (Marquez-Martin et al., 2006). Besides, we have shown that OA has a therapeutic effect on an experimental model of MS (Martin et al., 2010), demonstrating that intraperitoneal (i.p.) administration of OA, in mice with established EAE, is capable of reducing important hallmarks related to EAE disease. However, the potential of these biologically active molecules on maintenance of health has not been addressed in depth, although disease prevention is a major goal on public health, particularly because of the shifting of the concept from “disease care” to “heath care”. Therefore, it has been of interest in the present study to verify the possible influence of early administration of OA and ERY, an intermediate from which oleanolic acid is formed and on which no previous data exist, on health promotion in our EAE model. Our findings confirmed that both ERY and OA strongly slowed down the clinical manifestation of the disease, and we correlated the magnitude of improvement for EAE with the decrease of the immuno-inflammatory response.
Materials and Methods

Disease Induction and Treatment

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) and provided food and water ad lib. All experimental protocols reviewed and approved by the Animal Ethics Committee of the UVa, were performed in compliance with the European Communities directive 86/609/ECC and Spanish legislation (BOE 252/34367-91, 2005) regulating animal research.

Immunization

EAE was induced in 8-10-week-old female C57BL/J6 mice by subcutaneous immunization with 100 µg of myelin oligodendrocyte glycoprotein (MOG) _35-55_ peptide (MEVGWYRSPFSRVVHLGYRNGK; from Dr F. Barahona, CBM, Madrid) emulsified in complete Freund’s adjuvant (CFA) containing 0.4 mg Mycobacterium tuberculosis (H37Ra; Difco, Detroit, MI, USA) on day 0. Additionally, mice received 300 ng of Pertussis toxin intraperitoneal (i.p.) on days 0 and 2. Clinical signs of EAE were assessed daily in a double-blind manner 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 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. After mice scored level 4 for 2 days were given disease severity grade of 5 and euthanized.

*Triterpene Treatment Procedure*

A) MOG-Immunized mice were treated daily with 50 mg/kg/d of OA or ERY by i.p. injection beginning at different times.

* Groups OA<sub>0</sub> and ERY<sub>0</sub>: triterpenes treatment started at the immunization day.
* Groups OA<sub>-7</sub> and ERY<sub>-7</sub>: triterpenes treatment began on day -7, before EAE induction.
* Groups OA<sub>12</sub> and ERY<sub>12</sub>: triterpenes treatment began on day 12 after EAE induction.

B) Control groups (without EAE induction):

* Group control, C: treated daily with 0.2% w/v DMSO
* Groups OA and ERY: healthy mice treated with the triterpenes for an equivalent period of time than the corresponding EAE mice.

Animals were studied in two different periods of time:

i) 30 days post-immunization, when EAE mice showed hind limb paralysis, or

ii) at the day when severe symptoms (score 5) on each animal group were apparent: at day 40 (on untreated EAE mice) or at day 110 (on triterpenes treated EAE mice) after immunization.

Control mice (without EAE induction) were also injected daily with OA or ERY for an equivalent period of time.

OA and ERY (Extrashynthese, Genay Cedex, France) were 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).
Histological Studies

Spinal cord tissue was obtained from five representative animals of the different experimental groups on day 30 after immunization. Tissues were fixed and embedded in paraffin, cut on a microtome (5 µm thicknesses), stained with Eosin-Hematoxylin. Histological examination was performed with a Nikon Eclipse 90i (Nikon Instruments Inc) connected to a DXM1200C digital camera (Nikon Instruments Inc). Sections from 4–10 segments per mouse were examined blindly by one investigator.

Intravital Microscopy in Mouse Brain

Intravital microscopy of the mouse cerebromicrovasculature was performed as previously described (Martin et al., 2010). Briefly, mice were anesthetized at day 30 post immunization 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, USA) 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 at 37 °C.

Leukocytes were fluorescently labeled by i.v. administration of rhodamine 6G (5 mg/kg body weight) and visualized by a Zeiss Axioplan 2 imaging microscope connected to an AxioCam MR digital camera using the AxioVision AC imaging software and an Acroplan 20x/0.50W Ph2 lens. Eight different postcapillary venules of diameter between 30 and 70 µm were chosen for observation. Rolling leukocytes were defined as white cells moving at a velocity less than that of erythrocytes. Leukocytes remaining stationary for 30 s or longer were considered adherent to the venular endothelium.
Leukocyte adhesion was expressed as cells/mm$^2$ of venular surface area, as shown previously (Martin et al, 2010).

**Evaluation of Cytokines and MOG-specific Antibodies by an Enzyme-Linked Immunosorbent Assay (ELISA)**

Anti-MOG-specific IgM and IgG isotypes were detected in serum samples collected from animals on day 30 after immunization, using ELISA. In brief, 96-well polystyrene microtiter plates were coated with 0.5 mg/well of MOG$_{35-55}$ peptide diluted in PBS overnight in a humidified chamber followed by PBS washing and blocking for 1 hour with 5% BSA in PBS. Wells were incubated in duplicate with serum samples diluted 1:60 in PBS for 2 hours at room temperature. After washing, HRP-labeled rat anti-mouse IgM, anti-mouse IgG, anti-mouse IgG1 and anti-mouse IgG2a (1:2000) from Serotec (Sigma-Aldrich, St Louis, MO, USA) 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. Data are expressed as mean optical density at 450 nm.

Leptin levels in serum samples and spinal cord tissue were determined by ELISA (RayBiotech, Norcross, GA, USA). For cytokine quantification (IL-4, IL-6, IL-10, IL-17, TNF$\alpha$, and IFN$\gamma$), cell culture medium, serum and spinal cord tissue were analyzed by ELISA according to the manufacturer’s protocols (eBioscience, San Diego, CA, USA). Spinal cords were removed on day 30 after immunization or at the severe stage of the disease (score 5), weighed and then frozen at -80°C. Spinal cord (SC) tissue was homogenized by using a tissue homogenizer, (Cole-Parmer Instrument, IL, USA) in an ice bath in 0.5 ml ice-cold PBS supplemented with 0.4 M NaCl, 0.05% Tween 20, 0.5% BSA and a protease inhibitor cocktail: 20 $\mu$g/ml of leupeptin, 20 KI units of aprotinin, 0.1 mM phenylmethylsulphonyl fluoride (Sigma–Aldrich, St Louis, MO, USA), and
centrifuged at 10,000 rpm for 10 minutes at 4°C. Supernatant were stored at -80 °C until cytokine assays were performed. Total protein was assayed using the Bradford method. A 50- to 100-µl sample of each supernatant was used for tests.

Data were processed and expressed as pg of cytokine/mg of SC wet weight, or pg of cytokine/ml for serum samples, and plotted.

**Blood-Brain Barrier permeability measurement**

To evaluate blood-brain barrier (BBB) disruption, we measured the extravasation of Evans Blue dye (EB) as a marker of albumin extravasation. At 30–31 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 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 mg of Evans Blue/mg dried weight of tissue.

**Cell Culture**

Murine BV-2 cells, an immortalized murine microglia cell line, exhibit phenotypic and functional properties comparable to those of primary microglia and hippocampal neurons (Bocchini et al., 1992). BV-2 cells (a gift from Prof. J. Bethea, Miller School of Medicine, Miami, USA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) high sucrose, supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin, and kept at 37 °C in 5% CO₂. Cells were seeded in 96-well plates (5x10⁴ cells/well) or 60 mm culture dishes (3x10⁶ cells/well.).
**Proliferation assay**

Cell proliferation was quantified by using the Promega kit, Cell Titer 96\(^{®}\)Aqueous One Solution Cell Proliferation Assay, according to the manufacturer's recommendations. Briefly, cells were seeded in 96-well plates and serum starved for 24 h. Then, cells were treated in triplicate with IFN\(_\gamma\), leptin or LPS, in the presence or absence of the triterpenes. After 24 h of incubation, formazan product formation was assayed by recording the absorbance at 490 nm in a 96-well plate reader (OD value). Formazan is measured as an assessment of the number of metabolically active cells and expressed in percentages relative to FBS stimulated cells. Cell viability was assessed by trypan blue exclusion.

**Western blot analysis**

Cells were washed with PBS and harvested in Laemmli SDS sample buffer. Protein extracts were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% BSA-TBST at room temperature and then incubated for 18 h at 4\(^\circ\)C with the indicated antibodies including ERK 1/2 (Zymed Laboratories), rabbit p-ERK1/2, p-rS6 (Cell Signaling Technology), COX-2 (sc-1745, Santa Cruz Biotech, CA), actin (sc-8432, Santa Cruz Biotech, CA) and iNOS (BD Biosciences, Lexington, KY). After washing with TBST buffer, a 1:2.000 (v/v) dilution of horseradish peroxidase-labelled IgG was added at room temperature for 1 h. The blots were developed using enhanced chemiluminescence.

**Phagocytosis assays**

Cells were stimulated in serum-free media with or without 100UI/ml of IFN\(_\gamma\), 1\(\mu\)g/ml of LPS or 0.5 \(\mu\)M of leptin for 24 h, in the presence or absence of different doses of OA
or ERY and then exposed to 0.1 mg/ml of FITC-labelled dextran (MW 40,000) for 2 h. Non-internalized particles were removed by vigorous washing with cold PBS (pH 7.4) prior to measuring fluorescence at 480 nm excitation and 520 nm emission on either a Flow Cytometer (Gallios™, Beckman Coulter, USA) or a Fluoroskan multiwell plate reader (TECAN Genios Pro; Tecan Group Ltd, Switzerland). Cultures without fluospheres were used (blank wells) as background. Each culture condition was done in triplicate, and three independent experiments were performed. To confirm that the fluospheres were accumulated intracellularly, a Leica TCS SP5X confocal microscope was used with the Leica LAS AF acquisition software and a x60 oil objective.

**Statistical Analyses**

Statistical analysis was performed with the GraphPad Prism Version 4 software (San Diego, CA, U.S.A.) by analysis of variance (ANOVA). Analyses were performed using repeated measures-ANOVA (two-way ANOVA) for comparison of clinical parameters, and one-way ANOVA for comparison of parameters such as cytokines, extravasation, leukocytes and MOG antibodies. A post hoc analysis was made by the Bonferroni's multiple comparison test. P < 0.05 was considered statistically significant.
**Results**

**Effects of Preventive Treatment with Oleanolic Acid or Erythrodiol on Clinical EAE.**

Female C57BL/6 mice exhibit active EAE after immunization with the MOG_{35-55} peptide. In this experimental model we compared the effects of two pentacyclic triterpenes, oleanolic acid (OA) and erythrodiol (ERY) administrated at a dose (50 mg/kg) previously proved to be both safe and therapeutically relevant in rodents (Jeong, 1999; Senthil et al., 2007; Martin et al., 2010), in two regimens: 7 days before immunization (day -7; OA_{-7}, ERY_{-7}) or at the day of induction (day 0; OA_{0}, ERY_{0}). The clinical analysis of the different groups of animals is shown in Figure 1. The placebo-treated animals developed after 12 to 31 days neurological symptoms of active EAE, consisting of tail limpness and a mild-to-moderate paraparesis, as well as progressive weight loss. Interestingly, when OA or ERY were administered from the induction day, clinical disease was markedly less severe and mice had a later onset of the clinical signs compared to sick untreated animals (Fig. 1A). First neurological symptoms (score 1) were observed at day 11 with mean day of onset 13.49±2.12 in untreated EAE mice, while OA_{0} or ERY_{0} animals showed no clinical signs at that time and reached a similar score (tail atony) on day 27 (mean 33.1±1.8 and 33.83±1.8, respectively). When triterpenes were administered 1 week before EAE induction, clinical disease remained mostly suppressed for the duration of the experiment (until day 30^{th} post-induction). No motor problems were appreciated in ERY_{-7} treated EAE-mice and only minimal pathological abnormalities were developed in the OA_{-7} group: 1/10 mice showed inability to curl the distal end of the tail (score 0.5, p < 0.001) (data not shown).
Figures 1B and C show differences between EAE mice treated with placebo or triterpenes from immunization day in the long-term progression and severity of the disease. The mean clinical score on both triterpene-EAE groups was 1.5 (clumsy gait and/or impaired righting ability) while maximum score on untreated EAE-mice was achieved. Mice were euthanized when they developed severe EAE (ethical end point). There was a dramatic difference in the time-course of mice reaching severe EAE when comparing placebo with OA₀- or ERY₀-treated EAE mice. Analyzing the days spent at each neuro-severity score level, we found that the group of drug-treated EAE mice spent significantly more time (about 2.4 folds) at score 0, no symptoms, as well as at scores 1 and 2, mild disability, than the vehicle treated group. This slowing down of the disease development was more notorious in the progression towards complete hind limb paralysis and fore limb weakness, as the ratio between the time spent on score 3 in triterpenes-treated versus placebo-treated EAE mice was 4.9 (data not shown).

We also compared the prophylactic and therapeutic efficacy of both molecules on the progression of disease severity. As shown in figure 1D, a notable difference was observed between the groups that received either triterpenes or vehicle. Likewise, differential effectiveness was also observed according to the administration time. Thus, on mice that received the triterpenes 12 days after immunization (onset of symptomatic disease; OA₁₂, ERY₁₂) -therapeutic treatment- the time interval to serious motor impairment was clearly delayed when compared with the placebo group, from 40±1 to 61±2 days. Mice given triterpenes from immunization day - prophylactic treatment - reached the higher disability score three times later than those receiving placebo, about 115±2 days. No major differences were observed between ERY- and OA-treated EAE mice.
The EAE disease is also associated with a progressive body weight reduction. Mice began to lose weight just before the onset of the clinical signs, showing a significant mean body weight decrease of 18-20% ($p < 0.001$ versus healthy group) at day 30-31 post-induction, reaching a maximum loss of a 43% on day 40 (data not shown). On the contrary, body weight of EAE animals treated with ERY or OA from the induction day showed only a slight decrease, 3-4% ($p > 0.05$ versus healthy group), and those EAE-mice treated starting on day -7 did not show any significant variation ($p > 0.05$ versus healthy group) (Fig. 2A). No differences were found in body weight between treated and untreated healthy mice (data not shown).

Next, because leptin, a cytokine-like hormone, regulates body weight through inhibition of food intake and stimulation of energy expenditure, we wondered whether triterpenes-mediated EAE protection was also associated with a modulation of the leptin levels. As shown in Figure 2B and C, 30 days after EAE induction leptin levels were significantly increased in serum and spinal cord tissue of EAE mice compared to healthy control mice, in contrast OA and ERY treatment from immunization day markedly diminished the enhanced leptin production of EAE mice. In addition, leptin levels were also quantified on spinal cord tissue from untreated- or triterpene-treated EAE mice at day 40 and 110 after immunization, respectively, when the strong/severe disease was developed (score 5). As shown in Figure 2D, leptin levels in all EAE groups (treated or untreated) was slightly higher compared to those found at day 30, but interestingly they showed an identical pattern.

**Prophylactic administration of Oleanolic acid or Erythrodiol protect from inflammatory cells recruitment into the CNS**
To investigate whether the marked changes observed in the clinical scores, corresponded to differences in CNS tissue inflammation, histological analysis was performed on spinal cord tissues collected on day 30 from all experimental groups. Qualitative microscopic examination of longitudinal spinal cord sections from EAE mice (Figure 3A) showed a strong leukocyte infiltration compared with samples from unimmunized mice. By contrast, fewer inflammatory cells were detected in triterpenes-treated mice starting from days -7 or 0 after immunization. Cellular influx was absent in control healthy mice.

The migration of leukocytes through post-capillary venules and into the brain parenchyma occurs in a multi-step manner (Carvalho-Tavares et al., 2000). These leukocyte/endothelium interactions in the pial microcirculation of mice were evaluated using intravital microscopy at day 30 after induction. In the brain of unimmunized healthy mice scarce leukocyte recruitment was observed, while EAE induced an increase in rolling cells and adherent leukocytes on pial vessel walls (Fig 3B) compared to healthy mice. Prophylactic treatment with OA or ERY revealed a significant lower number of these events when compared to placebo-treated EAE mice.

**Triterpene treatment abrogates MOG-specific Ab production.**

The effect of prophylactic treatment on serum antibody responses was also assessed on day 30 after MOG immunizations, since MOG-specific antibodies can enhance CNS inflammation increasing EAE severity (Linnington et al., 1988). As shown in Fig. 4A, EAE mice produced a remarkable MOG-specific IgG and IgM antibody responses, compared with unimmunized mice. Both OA- and ERY-treatment, starting at day 0 of EAE induction, promoted a significant reduction in the levels of MOG-specific IgM (67.7% and 64.4%, respectively), and IgG (78.9% and 77.8%, respectively) compared to
untreated EAE mice. Similarly, the high levels of MOG-specific IgG1 subclass found on EAE mice were significantly attenuated in the triterpene-treated EAE groups (80% for OA, and 78.2% for ERY). No significant changes on anti-MOG IgG2a subclass levels were observed among any experimental group. Healthy animals treated with either placebo, OA or ERY showed an almost complete absence of anti-MOG antibody titers.

Moreover, in sera from triterpene-treated mice from day -7, the levels of specific-antibodies observed were also significantly smaller when compared with untreated sick mice and even lower than those found on OA0- and ERY0-treated EAE mice.

**Preventive Treatment with Oleanolic Acid or Erythrodiol reduces Blood-Brain-Barrier permeabilization**

One of the early and central events in MS pathogenesis is the breakdown of the blood-brain barrier (BBB). To investigate whether prophylactic administration of AO or ERY to EAE mice resulted in reduced BBB disruption, Evans blue (EB) dye leakage was measured in brains and spinal cord from mice at day 30-31 post-immunization. As shown in Figure 4B, EB extravasation was increased in spinal cord, cerebellum and cerebral cortex from placebo-treated EAE mice, compared to healthy animals. This effect was significantly reduced in CNS tissues from EAE mice treated with the triterpenes from immunization day. The data from the EB extravasation assay revealed that triterpenes administration one week before EAE induction triggered a protection 10% ($p < 0.05$) higher than when treatment begun at the immunization day (data not shown). No differences between treated versus untreated healthy mice, or between OA- versus ERY-treated EAE mice were found.
In addition, since it has been recently demonstrated that Th17 cytokines impairs BBB integrity by disrupting tight junctions (Kebir et al., 2007), we examined whether triterpenes-treatment affected the expression levels of the major Th17 cytokine, IL-17A. As shown in Figure 5A, the production of IL-17A in serum and spinal cords of animals with EAE was up-regulated, and OA or ERY treatment suppressed this production.

**Preventive Treatment with Oleanolic Acid or Erythrodiol Switches the Cytokine Profile on EAE mice.**

After demonstrating that OA and ERY protect from BBB breakdown on EAE mice, we wondered whether prophylactic triterpenes-treatment would also prevent the altered Th1/Th2 balance that contributes to the pathogenesis of EAE, triggering a cytokine bias mainly associated with protection or recovery from disease. Therefore, spinal cord tissue and serum from mice treated with either vehicle, OA or ERY were assessed for inflammatory markers. We found that both triterpernes significantly reduced the levels of cytokines (TNFα, IFNγ, and IL-6) known to be proinflammatory (Fig. 5 B) and up-regulated in EAE, whereas they increased the expression of the anti-inflammatory cytokines IL-4, IL-10 (Fig. 5 C), compared to sham-treated EAE mice. Interestingly, in healthy mice, IL-4 and IL-10 up-regulation was observed in both sera and spinal cord tissue in the triterpenes-treated group, compared to placebo-treated animals. No effects were observed related to proinflammatory cytokines between the different groups of unimmunized mice (treated control versus non-treated control). No apparent difference was observed related to inflammatory cytokines between the groups of healthy mice.

To ascertain the inflammatory status of the triterpenes-treated EAE mice when strong EAE was developed, we also analyzed the expression levels of the inflammatory TNFα and the anti-inflammatory IL-10 in spinal cord tissues from triterpenes-treated or
untreated EAE mice, when disease reach maximal score in each group. Surprisingly, the expression pattern of the cytokines TNFα and IL-10 were very similar to what we obtained from spinal cord tissues harvested at day 30 post-immunization (Figure 5D).

Treatment with Oleanolic Acid or Erythrodiol Reduces the Inflammatory Response in Microglial Cells.

We next investigated whether the anti-inflammatory effect found in vivo, upon OA and ERY treatment in EAE mice, comprises also attenuation of some of the hallmarks of “activated” microglia such as the phagocytic properties, the high proliferative capacity, and the ability to release cytokines. We used immortalized mouse BV-2 cells to mimic the microglial activation observed in neurodegenerative disorders.

Proliferation and survival. We stimulated BV-2 microglia cells with specific inflammatory stimuli. As shown in Figure 6A, at 24 h of incubation 100 UI/ml of IFNγ, 1 μg/ml of LPS and 0.5 μM of leptin stimulated cell proliferation without significant differences among them. Pre-treatment of BV-2 cells with different doses of OA or ERY reduced the mitogenic response of the cells to the inflammatory stimuli in a dose-dependent manner. The presence of the triterpenes had no significant influence on the viability of either resting or activated BV-2 cells. In addition, this growth-inhibitory effect was paralleled by impaired activation/phosphorylation of ERK 1/2 and of the ribosomal protein S6 (rS6), key constituents of, respectively, the MAPK and mTOR signal transduction pathways, which play a central role in the regulation of cell growth and proliferation (Figure 6B)

Inflammatory mediators. We next investigated the ability of these triterpenes to regulate expression of inflammatory mediators in the BV2 microglia cell line. As shown in Figure 6C, stimulation of BV-2 cells with IFNγ, LPS or leptin led to a strong increase
in the production of COX-2 and iNOS, whereas the presence of 15 μM of OA or of ERY fully inhibited the up-regulation of these enzymes. Both triterpenes also significantly attenuated stimuli-induced protein expression of TNFα, which is known to promote autocrine signaling in microglia (Fig. 6D)

*Phagocytosis.* We assessed the effect of triterpenes on the phagocytic capacity of BV-2 cells by incubating activated microglial cells for 2 h with FITC-labeled dextran beads, followed both flow cytometry analysis and fluorescence quantification. The ability of BV-2 cells to ingest latex beads has previously been carefully documented (Bocchini et al., 1992). As shown in Figure 7A, BV-2 cells, after IFNγ, LPS and leptin treatment for 2 h, significantly enhanced its phagocytic capacity, compared to resting cells. However, in the presence of OA or ERY the fluorescence recorded, as an ingestion index, was dramatically reduced. In Figure 7B, data from flow cytometry analysis also points in that way. In a separate experiment, the cells were also stained with DAPI and studied using a confocal microscope to visually confirm the ingestion of dextran beads (Fig. 7C).
Discussion

In this study we have demonstrated that prophylactic administration of the natural oleanane-type triterpenes OA and ERY confer significant protection against development of EAE, an accepted experimental model to study MS. The protective role was manifested at clinical, histological, and molecular levels. Triterpenes delayed the onset and decreased the severity of the disease, by preventing up-regulation of specific antibodies and inflammatory cytokines, and stabilizing the BBB integrity, thus hampering the migration of leukocytes in the CNS. This suggests that the beneficial and protective effects of triterpenes are mediated, at least in part, through restraining immune-inflammatory responses at a systemic level, as well as within the CNS. Accordingly, data from the in vitro model also revealed that the presence of AO or ERY abrogated the inflammatory parameters of activated microgial cells, pointing to a likely regulatory effect of triterpenes on key CNS-resident innate immune cells.

Since pretreatment with OA or ERY dramatically influence the outcome of the disease affecting neurological symptoms and body weight, among other events, we hypothesized that one of the potential mechanisms for the triterpenes-mediated beneficial effect on EAE might be the suppression of endogenous leptin production. Leptin is a hormone with metabolic functions that influences food intake, immunity and inflammation. Leptin serum concentration has been found augmented in both EAE and MS patients (Matarese et al., 2008). It has been reported that a significant surge in serum leptin precedes disease onset and persists until clinical scores peaks. Circulating leptin is able to enter the brain accounting probably for the observed food-intake inhibition and body-weight loss of EAE mice, and also playing a crucial role in the regulation of inflammatory processes by acting directly on brain-resident immune cells.
In keeping with this statement, our findings show that BV2 microglia cells rapidly respond to leptin by synthesizing proinflammatory mediators and increasing its proliferative and phagocytic activities, events implicated in neuroinflammation and neurodegeneration. Besides, leptin-deficient mice are resistant to experimentally induced autoimmune disorders including EAE (Matarese et al., 2001), and leptin neutralization improves the course of the disease (De Rosa et al., 2006). Here we show that the systemic and local levels of leptin on triterpenes-treated mice at day 31 after immunization were significantly lower than those of untreated-EAE mice. Then, assuming that leptin is a factor that bridges metabolism, nutritional status and immune response, our findings support that lowering leptin levels may be one mechanism by which triterpenes might prevent EAE disease. However, our data from treated-EAE mice at the severe score of the disease reveal leptin levels in spinal cord tissue very similar to those obtained from spinal cord tissues harvested at day 30 after-immunization, thus suggesting that leptin lowering by triterpenes in EAE affects the onset and evolution of the disease, rather than its prevention. Subsequent studies should focus on the exact role of leptin, and the mechanisms through which OA and ERY exert this inhibiting effect on leptin levels.

In addition, we also found, that the clinical symptoms correlated with the degree of inflammation of the CNS in both triterpene- and placebo-treated EAE mice. Therefore, a second mechanism of action by which triterpenes protect from EAE might be by acting on a phase the lymphocyte entry into the CNS and its subsequent cascade of inflammatory events. In keeping with previously established, after mice immunization motor weakness developed and inflammatory cells accumulated in CNS tissues. In contrast, early administration of OA or ERY conferred protection against an increase in
cell adhesion and rolling flux within the CNS microvasculature, reducing the number of infiltrating cells into the CNS, and significantly delaying the disorders of motor function.

It is a general statement that drugs affecting the different stages of leukocyte recruitment may have broad application in the modulation of chronic inflammatory diseases in which leukocyte accumulation is a hallmark of disease pathology. Accordingly, several laboratories have reported the ability of triterpenes to affect leukocyte recruitments by modulating the expression of surface molecules. The pentacyclic triterpenoid acids oleanolic and ursolic decreased TNFα-induced E-selectin expression on endothelial cells (Takada et al., 2010), and similarly tripterine inhibited the expression of adhesion molecules in activated endothelial cells (Zhang et al., 2006). OA and some oleanane-type triterpenoids isolated from fabaceous plants reduced ICAM-1 expression in monocytic cells to the same extent dexamethasone did (Ahn et al., 2002). In addition, we have recently shown that OA reduced VCAM expression on CNS tissues of mice with established EAE, as well as extravasation of lymphocytes into the perivascular space (Martin et al., 2010).

On top of this, leukocyte trafficking into the CNS and their subsequent infiltration in the brain or spinal cord parenchyma may also be controlled by the functional integrity of the BBB (and blood spinal cord barrier), whose properties, are in turn, modulated by molecules such as chemokines or cytokines (Merrill and Benveniste, 1996; Minagar and Alexander, 2003; Engelhardt, 2006). A recent study has demonstrated that in EAE/MS, BBB disruption precedes perivascular cell infiltration, as well as clinical development of the disease (Wuerfel et al., 2007). Several studies on EAE have shown that keeping
the BBB intact is crucial to protect from the disease, thus, the naturally-occurring products berberine (Ma et al., 2010), oleanolic acid (Martin et al., 2010), and lipoic acid (Schreibelt et al., 2006), or the synthetic FTY720 (Foster et al., 2009), when administered at the clinical onset of EAE, reduced its severity by reducing BBB permeabilization, which correlated with a decreased leukocyte infiltration and inflammation into the CNS. Here, we have found that prophylactic treatment with OA or ERY resulted not only in a significant protection against BBB disruption, but also against the presence of cytokines that promote a strong inflammatory response and that may affect BBB function.

We have particularly focused on IL-17 because of its distinctive role in permeabilizing human BBB to soluble molecules and circulating CD4\(^+\) lymphocytes (Kebir et al., 2007). In addition, adoptive transfer of myelin-specific CD4\(^+\) Th17 cells has been shown to induce the selective upregulation of potent chemoattractants for leukocytes within the spinal cord of recipient mice (Carlson et al., 2008), while treatment with IL-17A-blocking antibodies revealed a beneficial effect on EAE (Uyttenhove and van Snick, 2006). Moreover, treatments including the synthetic polypeptide glatiramer (Begum-Haque et al., 2008) or the glucocorticoid methylprednisolone (Miljković et al., 2009) have shown to be effective altering the progression of multiple sclerosis and its animal model, EAE, by reducing the secretion of IL-17 as well as IFN\(_{\gamma}\), and protecting the integrity of the BBB. In our study, mice that were immunized following OA or ERY treatment did not express IL-17 and IFN\(_{\gamma}\) in spinal cord tissue, and its serum concentration was significantly lower than that of sham-treated EAE mice, paralleling triterpenes protective action on the BBB.
In the same direction, we have also found that triterpenes pretreatment, markedly attenuated the expression of other inflammatory cytokines, such as IL-6 and TNFα - whose high levels observed in EAE/MS correlate with the dysregulation of the BBB too (Sharief et al., 1992; Quintana et al., 2009) -, while increased the presence of the protective anti-inflammatory cytokines IL-4 and IL-10. Interestingly, this Th1/Th2 bias promoted by triterpenes treatment is maintained even once the disease was developed and reached severe levels. Therefore, limitation/restriction of Th1 and Th17 cytokine production in the CNS and systemic circulation, and promotion of Th2-type immune response, could be determining and account for the beneficial effects of triterpenes modulating the development (onset and progression) of clinical EAE.

Given the down-modulation of the proinflammatory cytokines in CNS tissues of triterpenes pre-treated EAE mice, we thought that the immuno-regulatory activities of OA and ERY might include actions on both circulating and CNS-resident immune cells. Therefore assuming that both triterpenes, being lipophylic molecules, may penetrate the BBB, as already demonstrated for some of them, we hypothesized that their effects on preventing inflammation of CNS tissues might be, in part, mediated through restraining microglia activation.

Although microglial activation has important reparative functions in the CNS, in infection, inflammation, or injury it may go beyond control and eventually produce detrimental effects that override the beneficial effects. Several ischemic and neurodegenerative disorders are associated with proliferation and over activation of microglia (Lull and Block, 2010). In fact, it has been proposed that innate immunity cells, including microglia, play an important role in EAE, by providing a permissive cytokine microenvironment that potentiates the immune response within the CNS.
(Heppner et al., 2005; Rasmussen et al., 2007). Our in vitro data have shown that functions of activated microglia, such as proliferation, phagocytosis and expression of proinflammatory mediators including TNFα, COX-2 and iNOS, are suppressed in the presence of OA and ERY. These evidences are in line with several in vitro studies describing triterpenes actions on multiple cellular targets from the innate immune system. Natural triterpenes have shown to abrogate production of the proinflammatory cytokines on human mononuclear cells (Marquez-Martin et al., 2006), synthetic derivatives decrease the expression on COX-2 e iNOS in murine macrophages (Suh et al., 1998), and restrain the neurotoxic activities of microglia cells though inhibition of ROS and TNFα secretion (Tran et al., 2008).

Along with Th cell-mediated events in EAE, another potential mechanism involved in the protective action of triterpenes, includes inhibition of the autoreactive humoral response. Studies on B cell-deficient mice show that B cells and antibodies are not necessary in MOG-induced EAE, but antibodies do influence the disease course and/or severity of the lesions by exacerbating CNS inflammation and demyelination (Linnington et al., 1988). Moreover, high serum antibodies or enhanced numbers of B cells secreting antibodies against myelin antigens have been observed in MS patients. Herein, we showed that anti-MOG antibody production was significantly lessened in mice treated either the triterpenes. This might be due to triterpenes induced inhibition of B cell activation and/or proliferation, as previously described in EAE mice treated with an inhibitor peptide mimicking SOCS-1 (Mujtaba et al., 2005), or with IFNτ (Mujtaba et al., 1998). Further studies would be interesting to investigate both options.
Besides immune-mediated inflammation, oxidative stress is another important mechanism involved in EAE/MS (Haider et al. 2011; Gilgun-Sherki et al., 2004). We have focussed on the immunomodulatory effect of OA and ERY in EAE, but we have not characterized, and neither has it been documented in murine EAE, their antioxidants capabilities. Further studies to address their ability to restrain lipid, proteins and DNA oxidation, which underlie axonal damage and oligodendrocytes death should be developed. Interestingly, other olive-related components, such as the polyphenols (efficient scavengers of free radicals), have been reported to effectively protect against multiple sclerosis (Greeling et al., 2003). It has been reported that a diet supplemented with dry olive leaf extracts (which are rich in polyphenols, flavonoids and tannins) has a beneficial effect in EAE in rats (Miljković et al., 2009), and the olive oil extract Oliplus, containing 45.5% polyphenols, 4.2% hydroxytyrosol, 2.2% tyrosol and 9.2% oleuropeine is effective on inhibiting gelatinases involved in the pathogenesis of multiple sclerosis (Luzzi et al. 2011). These data suggest a potential valuable synergism between triterpenes and polyphenols that deserves deeper studies, which could support their administration (alone or in combination) as a food supplementation for the patients suffering from CNS autoimmunity.

In summary, we conclude that prophylactic treatment with OA or ERY can protect mice from EAE by modulating both the cellular and humoral arm of the immune response. In addition the in vitro results also suggest that the triterpenes activity might involve regulatory mechanisms related to effector functions of microglial cells. Therefore, we propose OA and ERY as compounds with promising multi-level immunomodulating characteristics, useful for therapeutic intervention in multiple sclerosis and other autoimmune and/or neuroinflammatory diseases.
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References


synthase (iNOS) and inducible cyclooxigenase (COX-2) in mouse macrophages. Cancer Res 58: 717-723.


**Figures**

**Figure 1.- Triterpenes effects on clinical symptoms in EAE mice.** (A) Effect on clinical signs evolution (n=15, in all groups). (B,C) Long term effects on disease progression and severity (n=10, in all groups). (D) Effect on survival (n=10, in all groups). C57BL/6 mice were immunized with MOG35-55 and given oleanolic acid or erythrodiol daily i.p. from day 0 (OA₀, ERY₀), or 12 (OA₁₂, ERY₁₂) after immunization until the end of the experiment. Long-term experiments are represented by Kaplan–Meier curves. Mice were sacrificed when severe neurological signs were apparent. Values are means ± SD. For some points, error bars are not visible because the deviations are smaller than the symbol sizes. The difference between EAE-untreated and EAE-triterpenes treated groups was highly significant (*p < 0.01, **p < 0.001).

**Figure 2.- Triterpenes effects on body weight-related parameters.** (A) Body weight, and leptin levels from (B) sera and (C,D) spinal cord tissue of untreated and treated EAE mice. C57BL/6 mice were immunized with MOG35-55 and given oleanolic acid or erythrodiol daily i.p. from the day of immunization, day 0 (OA₀, ERY₀) or 7 days before, day -7 (OA₋₇, ERY₋₇) until the end of the experiment (15 mice per group). Leptin protein levels were measured by commercial ELISA in serum samples (B) and in spinal cord extracts (C) from mice at day 30 post-immunization (*p < 0.01 versus control and **p < 0.05 versus untreated EAE-mice; seven mice per group), and in spinal cord extracts (D) from mice at the highest score of the disease: day 40 in untreated-EAE group, day 110 on triterpenes-treated group (*p < 0.001 versus control and **p < 0.001 versus untreated EAE-mice; seven mice per group). Results were expressed as the mean ± SD.
Figure 3.- Triterpene effects on leukocyte recruitment into CNS. (A) Spinal cord histological sections. Typical longitudinal sections of cellular infiltration on spinal cord, in mice from different groups, stained with eosin-hematoxilin and visualized with a 10X and a 20X lens. (B) Firm arrest and rolling flux of leukocytes on brain microvasculature studied by intravital microscopy. Results are shown as mean ± SD of cells per minute, n=10. Statistically significant differences were indicated by *p < 0.001 versus control, **p < 0.001 versus untreated EAE mice. C57BL/6 mice were immunized with MOG35–55 as explained in Materials and Methods and given oleanolic acid or erythrodiol daily i.p. from the day of immunization, day 0 (OA₀), or 7 days before, day -7 (OA₋₇) until day 31. In all groups n=10.

Figure 4.- Effect of triterpenes on Blood-brain barrier permeability and anti-MOG35–55 antibodies. On day 31 after immunization, (A) titers of pMOG35–55-specific immunoglobulins at 1/60 dilution were evaluated by ELISA, in sera samples from mice treated daily with OA or ERY from immunization day, or 7 days before immunization. Results were expressed as the mean ± SD; n=7 in all groups. (B) Blood-brain barrier permeability on mice of the indicated groups was evaluated by measuring the extravasation of Evans blue dye in spinal cord, cerebral cortex and cerebellum. Bars represent means ± SD, seven animals per group (*p < 0.001 versus control mice, **p < 0.001 versus untreated EAE mice).

Figure 5.- Effect of triterpenes treatments on cytokine expressions in EAE mice. IL-17, IFNγ, IL-6, TNFα, IL-4 and IL-10 protein concentrations were measured in spinal cord extracts or in serum samples from mice of the indicated groups at day 30 post-immunization (A,B,C) or at the day that animals developed strong disease: day 40
in untreated-EAE group and day 110 on triterpenes-treated group (D). Results were expressed as the mean ± SD from seven animals per group. Statistically significant differences were indicated by *p < 0.001 and ****p < 0.01 compared to control, and **p < 0.001 and ***p <0.01 compared to untreated EAE.

**Figure 6.- Triterpenes modulate BV-2 microglia cell activation.** BV-2 cells were pretreated for 30 min with the indicated doses of OA or ERY, and then were stimulated with 100 UI/IFNγ, 1 µg/ml of LPS or 0.5 µM of leptin. (A) After 24 h of incubation, cell proliferation was investigated, and expressed in percentages relative to FBS stimulated cells. (*p < 0.05 and ** p < 0.001 compared to stimuli without triterpene; n = 3). (B) After 15 min of incubation, ERK 1/2 and rS6 phosphorylation was identify in the cell lysates by western blot. (C) After 24 h of incubation, COX-2 and iNOS expression was identify in cell lysates by western blot, (D) and the presence of TNFα in the cell culture medium was quantified by commercial ELISA(** p < 0.001 compared to stimuli without triterpene; n = 3).

**Figure 7.- Triterpenes modulate phagocytic capabilities of BV-2 microglia cells.** After 24 h stimulation with 100 UI/IFNγ, 1 µg/ml of LPS or .5 µM of leptin, in the presence or absence of triterpenes, BV-2 cells were incubated for 2 h with 1 mg/mL FITC-labelled dextran, and phagocytosis was measured by fluorescence emission at 520 nm in a fluorimeter (A), in a flow cytometer (B) or in a confocal microscopy under a x60 oil objective (C). In A, values represents mean of cell fluorescence intensity ± SD, (*p < 0.001 compared to control and ** p < 0.001 compared to stimuli without triterpene; n=3). In the histograms, cells obtained after stimuli treatment in the absence of the triterpene (open black curves) are compared with cells treated in the presence of
the triterpene (open gray curves). Solid grey curves represent resting/control cells. Results are representative of three independent experiments.
Figure 1

A

B

C

D
Figure 3

A

B

Adhesion

Rolling

Graphs showing changes in adhesion and rolling with different treatments.
Figure 6

A

B

C

D

Cell proliferation (% of FBS)
Figure 7