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RESEARCH PAPER

Natural triterpenes modulate immune-inflammatory markers of experimental autoimmune encephalomyelitis: therapeutic implications for multiple sclerosis

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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 glial activation and destruction of CNS tissues. Oleanolic acid and erythrodiol are natural triterpenes that display strong anti-inflammatory and immunomodulatory activities. Oleanolic acid beneficially influences the course of established EAE. We now extend our previous observations to erythrodiol and address the efficacy of both compounds to protect against EAE, given 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.

KEY RESULTS

These triterpenes protected against EAE by restricting infiltration of inflammatory cells into the CNS and by preventing blood–brain barrier disruption. Triterpene-pretreated EAE-mice exhibited less leptin secretion, and switched cytokine production towards a Th2/regulatory profile, with lower levels of Th1 and Th17 cytokines and higher expression of Th2 cytokines in both serum and spinal cord. Triterpenes also affected the humoral response causing auto-antibody production inhibition. *In vitro*, triterpenes inhibited ERK and rS6 phosphorylation and 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 features of EAE. We envision these natural products as novel helpful tools for intervention in autoimmune and neurodegenerative diseases including MS.

Abbreviations

BBB, blood–brain barrier; CFA, complete Freund's adjuvant; EAE, experimental autoimmune encephalomyelitis; EB, Evans blue; iNOS, inducible nitric oxide synthase; MOG, myelin oligodendrocyte glycoprotein; rS6, ribosomal protein S6; MS, multiple sclerosis

Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disease directed against myelin proteins of the brain and spinal cord, and is considered as one of the major neurological diseases in 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 (BBB), 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 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 *et al.*, 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, COX-2 and inducible nitric oxide synthase (iNOS) enzymes and pro-inflammatory cytokines such as IFN-γ, TNF-α or IL-17 are considered to be pathogenic, while the Th2 cell-related cytokines IL-4 and IL-10 have been shown to down-regulate the immune response in acute EAE (Hafler, 2004; Imitola *et al.*, 2005; Sospedra and Martin, 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 immunopathological heterogeneity of this disease (Hemmer *et al.*, 2006).

Oleanolic acid and erythrodiol are two natural triterpenes of the oleanane group present in many vegetables, including the leaves and fruits of *Olea europea* (the olive tree). They have been recognized to have hepatoprotective, anti-inflammatory and antihyperlipidemic properties. Indeed, oleanolic acid has been promoted in China as an oral drug for human liver disorders. Data correlated well with the traditional use of *O. europea* in African and European Mediterranean countries, where this plant has been utilized widely in folk medicine as a diuretic, hypotensive, hypoglycaemic, 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 tumoural cells by activating apoptotic programmes (Martín *et al.*, 2007; 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 oleanolic acid has a therapeutic effect on an experimental model of MS (Martín *et al.*, 2010), demonstrating that i.p. administration of oleanolic acid, in mice with established EAE, is capable of reducing important biomarkers 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 'health care'. Therefore, it has been of interest in the present study to assess the influence of early administration of oleanolic acid and erythrodiol, 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 erythrodiol and oleanolic acid markedly slowed the clinical manifestations of the disease and we were able to correlate the magnitude of improvement for EAE with the decrease of the immuno-inflammatory responses.

Methods

Disease induction and treatment

All animal care and experimental protocols were reviewed and approved by the Animal Ethics Committee of the University of Valladolid and complied with the European Communities directive 86/609/ECC and Spanish legislation (BOE 252/34367-91, 2005) regulating animal research. 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 and provided food and water *ad libitum*.

Immunization. EAE was induced in 8 to 10-week-old female C57BL/J6 mice by subcutaneous immunization with 100 μg of myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK; from Dr F. Barahona, CBM, Madrid) emulsified in complete Freund's adjuvant containing 0.4 mg *Mycobacterium tuberculosis* (H37Ra; Difco, Detroit, MI, USA) on day 0. Additionally, mice received 300 ng of *Pertussis* toxin 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 abnor-

mality; 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 from two investigators, both unaware of the treatments, were averaged. 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. Mice scoring at level 4 for 2 days were automatically given a disease severity grade of 5 and killed.

Triterpene treatment procedure.

(A) MOG-Immunized mice were treated daily with 50 mg kg^{-1} day^{-1} of oleanolic acid or erythrodiol by i.p. injection beginning at different times.
- Groups OA_0 and ERY_0: triterpene treatment started at the immunization day.
- Groups OA_{-7} and ERY_{-7}: triterpene treatment started on day -7, before EAE induction.
- Groups OA_{12} and ERY_{12}: triterpene treatment started 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 the same time as the corresponding EAE mice.

Animals were studied at two different times:

(i) 30 days after immunization, when EAE mice showed hind limb paralysis, or
(ii) at the day when severe symptoms (score 5) in each animal group were apparent. This was at day 40 in untreated EAE mice and at day 110 for triterpene-treated EAE mice, after immunization.

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

Oleanolic acid and erythrodiol (Extrasynthese, Genay Cedex, France) were first dissolved in 2% w/v DMSO and then diluted with PBS for each experiment (the final concentration of DMSO was 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-haematoxylin. Histological examination was performed with a Nikon Eclipse 90i (Nikon Instruments, Inc., Amstelveen, the Netherlands) connected to a DXM1200C digital camera (Nikon Instruments Inc). Sections from 4–10 segments per mouse were examined by one investigator, without knowledge of the treatments.

Intravital microscopy in mouse brain

Intravital microscopy of the mouse cerebromicrovasculature was performed as previously described (Martín *et al.*, 2010). Briefly, mice were anaesthetized at day 30 post-immunization by i.p. injection of a mixture of 100 $mg \cdot kg^{-1}$ ketamine and 10 $mg \cdot kg^{-1}$ 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 CSF buffer at 37°C.

Leukocytes were fluorescently labelled by i.v. administration of rhodamine 6G (5 $mg \cdot kg^{-1}$ body weight) and visualized by a Zeiss Axioplan 2 imaging microscope (Hertfordshire, UK) connected to an AxioCam MR digital camera using the AxioVision AC imaging software and an Acroplan 20x/0.50W Ph2 lens. Eight different post-capillary 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 (Martín *et al.*, 2010).

Evaluation of cytokines and MOG-specific antibodies by 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 microtitre plates were coated with 0.5 mg per well of MOG_{35-55} peptide diluted in PBS overnight in a humidified chamber followed by PBS washing and blocking for 1 h with 5% BSA in PBS. Wells were incubated in duplicate with serum samples diluted 1:60 in PBS for 2 h at room temperature. After washing, HRP-labelled 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-α, and IFN-γ), cell culture medium, serum and spinal cord tissue were analysed 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. SC tissue was homogenized by using a tissue homogenizer (Cole-Parmer Instrument, Vernon Hills, 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 $\mu g \cdot mL^{-1}$ of leupeptin, 20 KI units of aprotinin, 0.1 mM phenylmethylsulphonyl fluoride (Sigma-Aldrich), and centrifuged at 3000× *g* for 10 min 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 per mg of spinal cord wet weight, or pg of cytokine per mL for serum samples.

BBB permeability measurement

To evaluate BBB disruption, we measured the extravasation of Evans blue (EB) dye 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 EB. After 4 h, mice were killed, perfused, and brain and spinal cords were removed. Dye was extracted for 2–3 days in formamide (4 mL·g^{-1} 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 EB per mg dried weight of tissue.

Cell culture

Murine BV-2 cells, an immortalized murine microglia cell line, exhibit phenotypic and functional properties comparable with 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, FL, USA) were cultured in Dulbecco's modified Eagle's medium high sucrose, supplemented with 10% fetal bovine serum (FBS), 100 U·mL^{-1} penicillin and 100 µg·mL^{-1} streptomycin, and kept at 37°C in 5% CO_2. Cells were seeded in 96-well plates (5×10^4 cells per well) or 60 mm culture dishes (3×10^6 cells per well.).

Proliferation assay

Cell proliferation was quantified by using the Promega kit (Madison, WI, USA), 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-γ, 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°C with the indicated antibodies including ERK 1/2 (Zymed Laboratories, South San Francisco, CA, USA), rabbit p-ERK1/2, p-rS6 (Cell Signaling Technology, Danvers, MA, USA), COX-2 (sc-1745, Santa Cruz Biotech, Santa Cruz, CA, USA), actin (sc-8432, Santa Cruz Biotech) and iNOS (BD Biosciences, Lexington, KY, USA). 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 100 UI·mL^{-1} of IFN-γ, 1 µg·mL^{-1} of LPS or 0.5 µM of leptin for 24 h, in the presence or absence of different doses of oleanolic acid or erythrodiol and then exposed to 0.1 mg·mL^{-1} 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, Fullerton, CA, USA) or a Fluoroskan multiwell plate reader (TECAN Genios Pro; Tecan Group Ltd, Zurich, 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 (Wetzlar, Germany) and a ×60 oil objective.

Statistical analyses

Statistical analysis was performed with the GraphPad Prism Version 4 software (San Diego, CA, USA) by ANOVA. Analyses were performed using repeated measures ANOVA (or 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 and erythrodiol given at a dose (50 mg·kg^{-1}) previously proven to be both safe and therapeutically relevant in rodents (Jeong, 1999; Senthil *et al.*, 2007; Martín *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 neurological symptoms of active EAE after 12 to 31 days, consisting of tail limpness and a mild-to-moderate paraparesis, as well as progressive weight loss. Interestingly, when oleanolic acid or erythrodiol were administered from the day of induction, clinical disease was markedly less severe and mice had a later onset of the clinical signs compared with untreated animals with EAE (Figure 1A). First neurological symptoms (score 1) were observed at day 11 with mean day of onset 13.5 ± 2 in untreated EAE mice, while OA_0 or ERY_0 animals showed no clinical signs at that time and a similar score (tail atony) was first reached on day 27 (mean values 33 ± 2 and 34 ± 2 days respectively). When the triterpenes were given as a pre-treatment, starting 1 week before EAE induction, clinical disease remained mostly suppressed for the duration of the experiment (until day 30 post-induction). No motor problems were observed in ERY_{-7} treated EAE-mice and only minimal pathological abnormali-

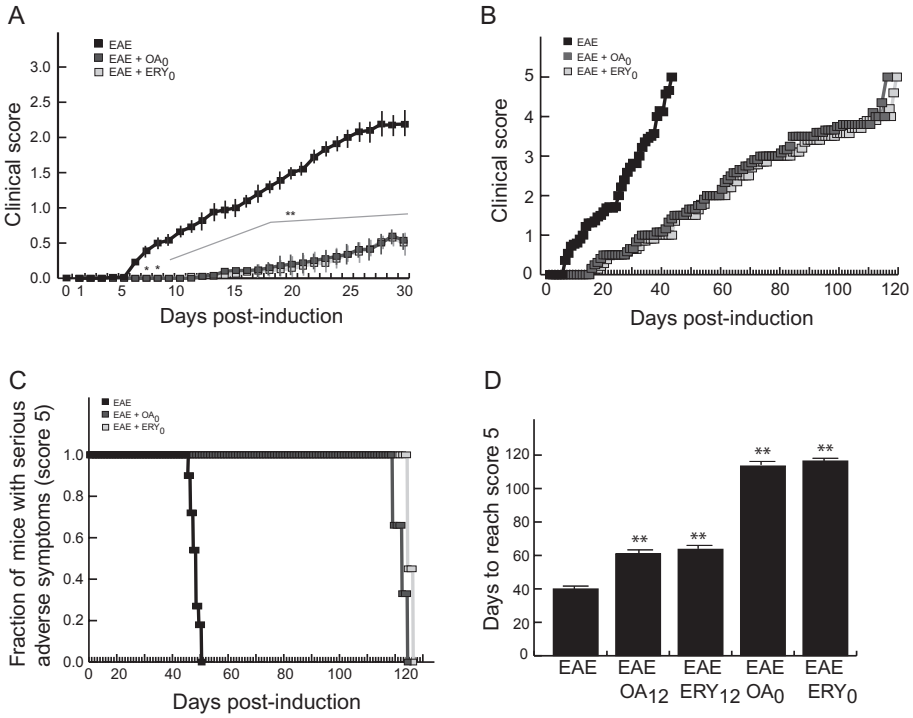

Figure 1

Effects of triterpenes on clinical symptoms in EAE mice. (A) Effect on the evolution of clinical signs ($n = 15$, 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 $MOG_{35–55}$ and given oleanolic acid or erythrodiol daily i.p. from day 0 (OA_0, ERY_0), or day 12 (OA_{12}, ERY_{12}) after immunization until the end of the experiment. Long-term experiments are represented by Kaplan–Meier curves. Mice were killed 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-triterpene treated groups was highly significant (*$P < 0.01$, **$P < 0.001$).

ties were developed in the OA_{-7} group. Only 1/10 mice showed inability to curl the distal end of the tail (score 0.5, $P < 0.001$) (data not shown).

Figure 1B,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 killed 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 OA_0- or ERY_0-treated EAE mice. Analysing the days spent at each neuro-severity score level, we found that the group of triterpene-treated EAE mice spent significantly more time (about 2.4-fold) 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 obvious in the progression towards complete hind limb paralysis and fore limb weakness, as the ratio between the time spent on score 3 in triterpene-treated versus placebo-treated EAE mice was 4.9 (data not shown).

We also compared the prophylactic and therapeutic efficacy of both compounds on the progression of disease severity. As shown in Figure 1D, a notable difference was observed between the groups that received either triterpene or vehicle. Likewise, differential effectiveness was also observed according to the administration time. Thus, in mice that received the triterpenes 12 days after immunization (onset of symptomatic disease; OA_{12}, ERY_{12}) – 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 the day of immunization – prophylactic treatment – reached the higher disability score three times later than those receiving placebo, about 115 ± 2 days. No major differences were observed between erythrodiol- and oleanolic acid-treated EAE mice.

The EAE model is also associated with a progressive body weight reduction. Mice started to lose weight just before the onset of the clinical signs, showing a significant mean body weight decrease of 18–20% ($P < 0.001$ vs. 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 erythrodiol or oleanolic acid from the induction day showed only a slight decrease, 3–4% ($P > 0.05$ vs. healthy group), and those EAE-mice treated starting on day -7 did not show any significant variation ($P > 0.05$ vs. healthy group) (Figure 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 stimu-

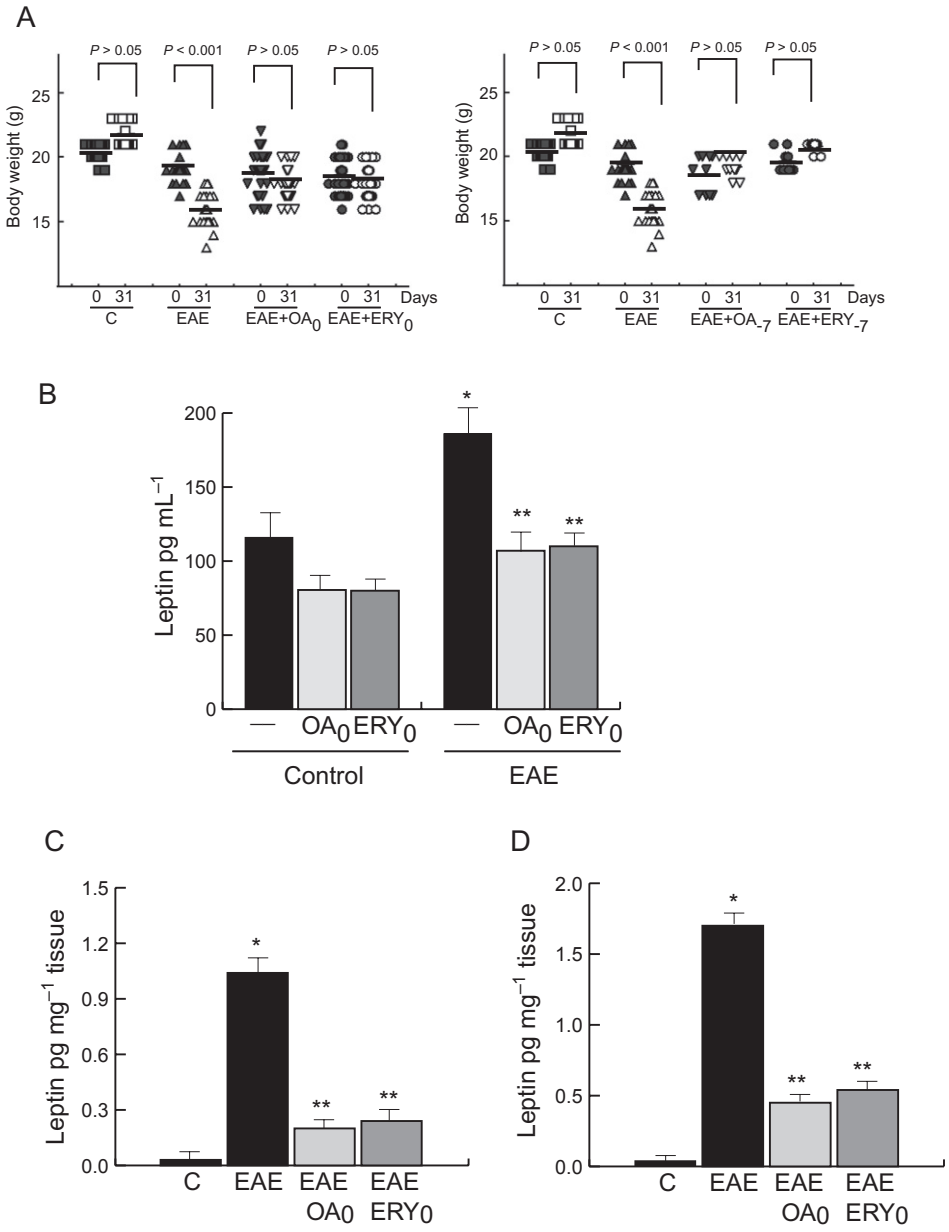

Figure 2

Effects of triterpenes 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 MOG_{35-55} and given oleanolic acid or erythrodiol daily i.p. from the day of immunization, day 0 (OA_0, ERY_0) 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 (*$P < 0.01$ vs. control and **$P < 0.05$ vs. 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 (*$P < 0.001$ vs. control and **$P < 0.001$ vs. untreated EAE-mice; seven mice per group). Results were expressed as the mean ± SD.

lation 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,C, 30 days after EAE induction leptin levels were significantly increased in serum and spinal cord tissue of EAE mice compared with healthy control mice. In contrast, oleanolic acid and erythrodiol treatment from immunization day markedly diminished the enhanced leptin production of EAE mice. In addition, leptin levels were also quantified in spinal cord tissue from untreated- or triterpene-treated EAE mice at days 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) were slightly higher compared with those found at day 30, but interestingly they showed an identical pattern.

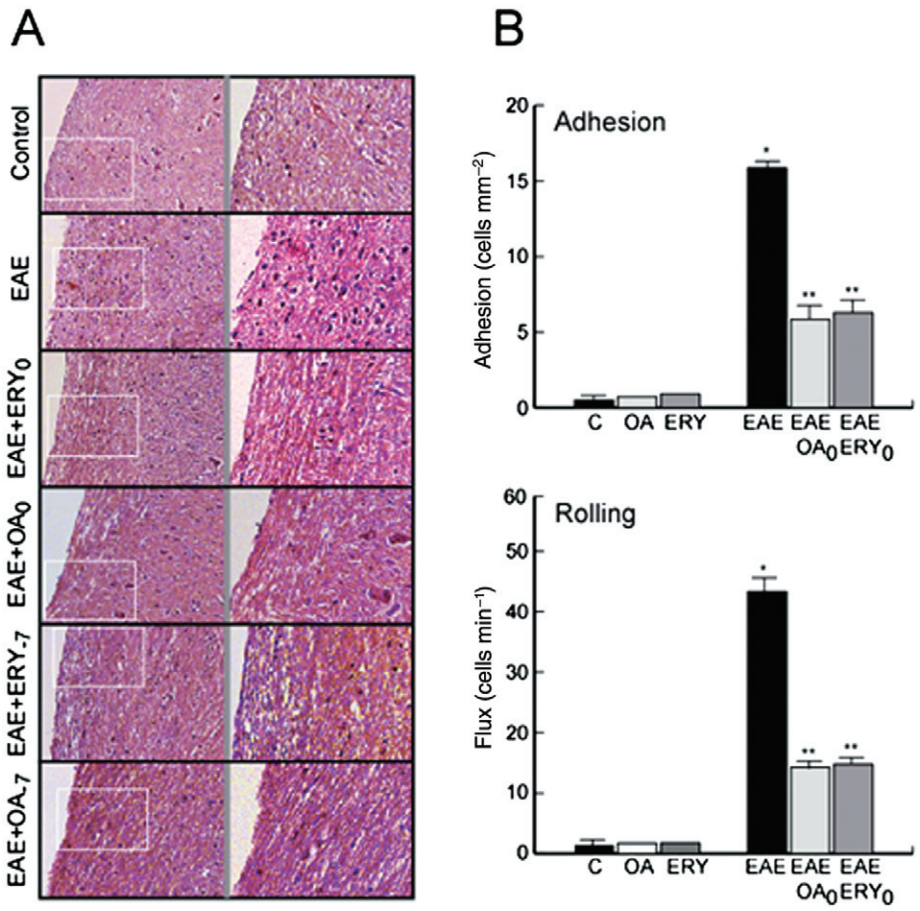

Figure 3

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-haematoxylin and visualized with a 10× and a 20× 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, $n = 10$. *$P <$ 0.001 versus control, **$P < 0.001$ versus untreated EAE mice. C57BL/6 mice were immunized with $MOG_{35–55}$ and given oleanolic acid or erythrodiol daily i.p. from the day of immunization, day 0 (OA_0), or 7 days before, day -7 (OA_{-7}) until day 31. In all groups, $n = 10$.

Prophylactic administration of oleanolic acid or erythrodiol protects against inflammatory cell 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, little leukocyte recruitment was observed, while EAE induced an increase in rolling cells and adherent leukocytes on pial vessel walls (Figure 3B), compared with healthy mice. Prophylactic treatment with oleanolic acid or erythrodiol revealed a significantly lower number of these events when compared with placebo-treated EAE mice.

Triterpene treatment decreases MOG-specific antibody production

The effect of prophylactic treatment on serum antibody responses was also assessed on day 30 after MOG immunizations, as MOG-specific antibodies can enhance CNS inflammation increasing EAE severity (Linington *et al.*, 1988). As shown in Figure 4A, EAE mice produced a remarkable MOG-specific IgG and IgM antibody responses, compared with unimmunized mice. Both oleanolic acid and erythrodiol treatment, starting at day 0 of EAE induction, significantly reduced the levels of MOG-specific IgM (67.7% and 64.4%, respectively), and IgG (78.9% and 77.8%, respectively) compared with untreated EAE mice. Similarly, the high levels of

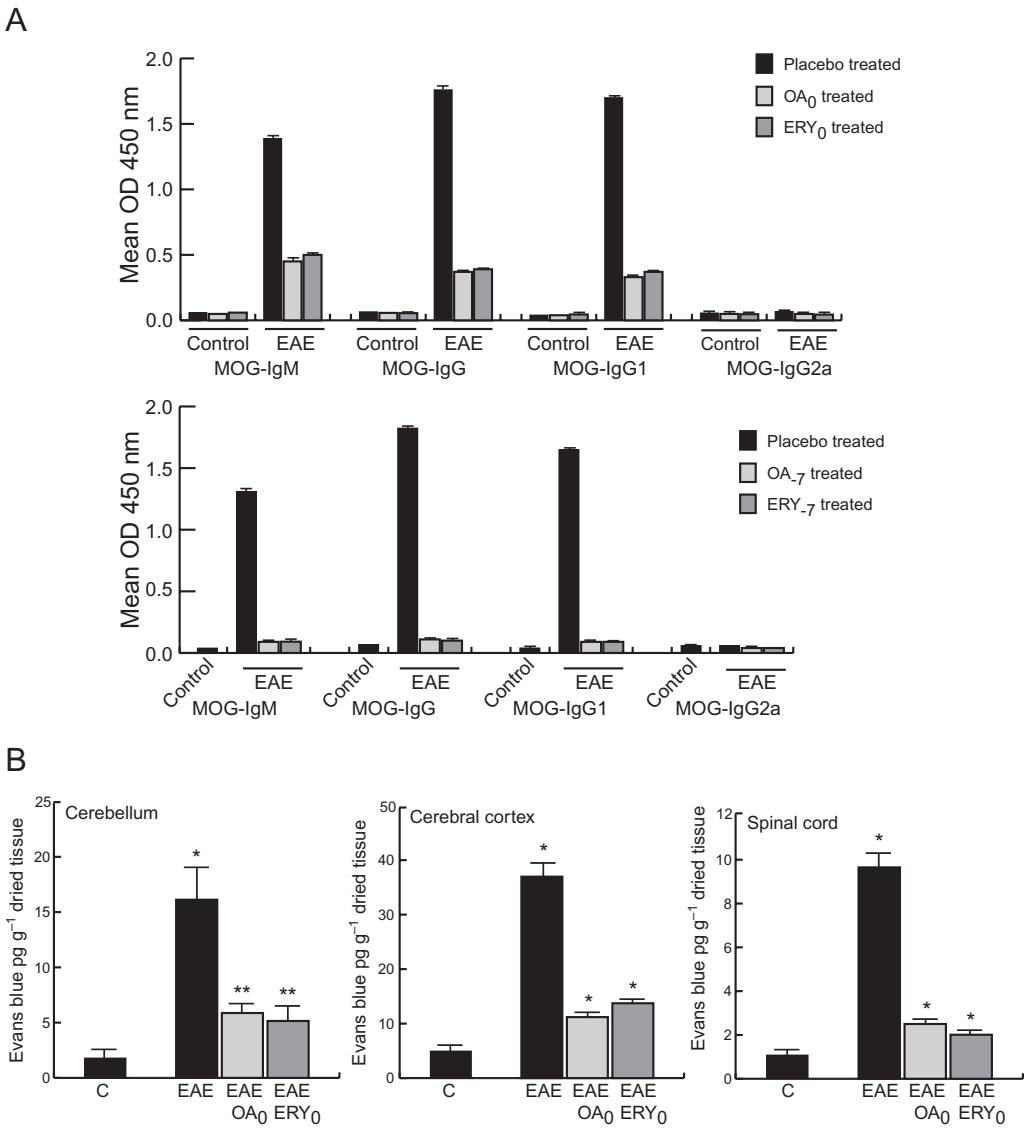

Figure 4

Effect of triterpenes on BBB permeability and anti-$MOG_{35–55}$ antibodies. On day 31 after immunization (A), titres of $pMOG_{35–55}$-specific immunoglobulins at 1/60 dilution were evaluated by ELISA, in serum samples from mice treated daily with oleanolic acid or erythrodiol from immunization day, or 7 days before immunization. Results were expressed as the mean ± SD; $n = 7$ in all groups. (B) BBB permeability on mice of the indicated groups was evaluated by measuring the extravasation of EB dye in spinal cord, cerebral cortex and cerebellum. Bars represent means ± SD, seven animals per group (*$P < 0.001$ vs. control mice, **$P < 0.001$ vs. untreated EAE mice).

MOG-specific IgG1 subclass found on EAE mice were significantly attenuated in the triterpene-treated EAE groups (80% for oleanolic acid, 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, oleanolic acid or erythrodiol showed an almost complete absence of anti-MOG antibody titres.

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 OA_0- and ERY_0-treated EAE mice.

Pretreatment with oleanolic acid or erythrodiol reduces BBB permeabilization

One of the early and central events in MS pathogenesis is the breakdown of the BBB. To investigate whether prophylactic administration of oleanolic acid or erythrodiol to EAE mice resulted in reduced BBB disruption, EB dye leakage was measured in brains and spinal cord from mice at days 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 with 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 1 week before EAE induction triggered a protection 10% ($P < 0.05$) higher than when treatment began at the immunization day (data not shown). No differences between treated and untreated healthy mice, or between oleanolic acid- and erythrodiol-treated EAE mice were found.

In addition, as it has been recently demonstrated that Th17 cytokines impair BBB integrity by disrupting tight junctions (Kebir *et al.*, 2007), we examined whether triterpene 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 oleanolic acid or erythrodiol treatment suppressed this production.

Pre treatment with oleanolic acid or erythrodiol switches the cytokine profile of EAE mice

After demonstrating that oleanolic acid and erythrodiol prevented BBB breakdown in EAE mice, we wondered whether prophylactic triterpene 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, oleanolic acid or erythrodiol were assessed for inflammatory markers. We found that both triterpenes significantly reduced the levels of cytokines (TNF-α, IFN-γ and IL-6) known to be pro-inflammatory (Figure 5B) and up-regulated in EAE, whereas they increased the expression of the anti-inflammatory cytokines IL-4, IL-10 (Figure 5C), compared with 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 triterpene-treated group, compared with placebo-treated animals. No effects were observed related to pro-inflammatory cytokines between the different groups of non-immunized mice (treated control vs. non-treated control). No differences related to inflammatory cytokines were observed between the groups of healthy mice.

To ascertain the inflammatory status of the triterpene-treated EAE mice when severe EAE had developed, we also analysed the expression levels of the inflammatory TNF-α and the anti-inflammatory IL-10 in spinal cord tissues from triterpene-treated or untreated EAE mice, when disease reaches maximal score in each group. Surprisingly, the expression pattern of the cytokines TNF-α and IL-10 was very similar to what we obtained from spinal cord tissues harvested at day 30 after 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*, after oleanolic acid or erythrodiol treatment in EAE mice, also included attenuation of some of the markers 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, after 24 h of incubation with either 100 UI·mL^{-1} of IFN-γ, 1 μg·mL^{-1} of LPS or 0.5 μM of leptin, cell proliferation was increased without significant differences among the stimuli. Pretreatment of BV-2 cells with different doses of oleanolic acid or erythrodiol 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 IFN-γ, LPS or leptin led to a strong increase in the production of COX-2 and iNOS, whereas the presence of 15 μM of oleanolic acid or of erythrodiol fully inhibited the up-regulation of these enzymes. Both triterpenes also significantly attenuated stimuli-induced protein expression of TNF-α, which is known to promote autocrine signalling in microglia (Figure 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-labelled dextran beads, followed by 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 IFN-γ, LPS and leptin treatment for 2 h, significantly enhanced its phagocytic capacity, compared with resting cells. However, in the presence of oleanolic acid or erythrodiol, the fluorescence recorded, as an ingestion index, was dramatically reduced. In Figure 7B, data from flow cytometry analysis also point 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 (Figure 7C).

Discussion

In this study we have demonstrated that prophylactic administration of the natural oleanane-type triterpenes, oleanolic acid and erythrodiol, confers 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

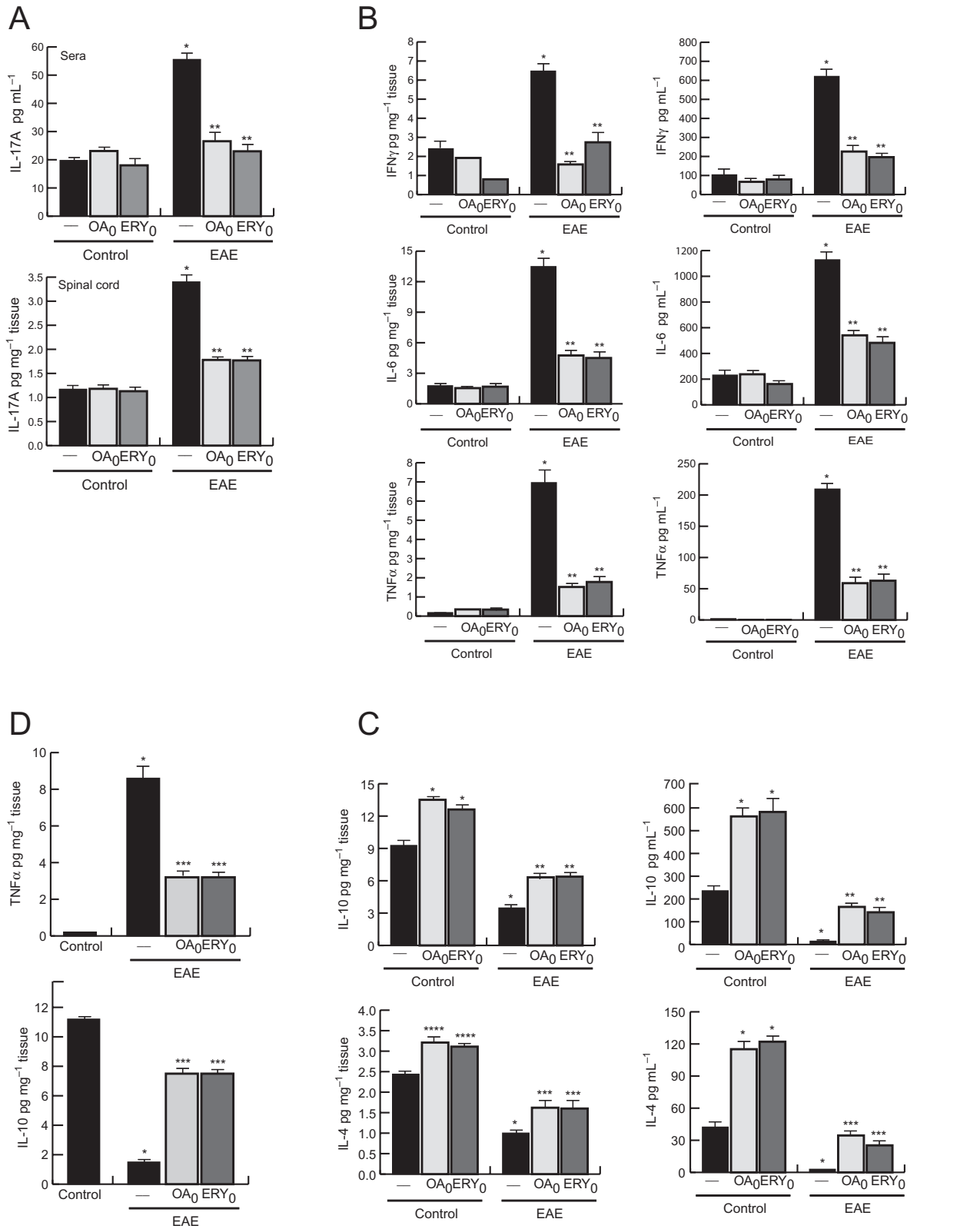

Figure 5

Effect of triterpene treatments on cytokine expressions in EAE mice. IL-17, IFN-γ, IL-6, TNF-α, 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 severe disease: day 40 in untreated-EAE group and day 110 in triterpene-treated groups (D). Results were expressed as the mean ± SD from seven animals per group. *$P < 0.001$ and ****$P < 0.01$ compared with control, and **$P < 0.001$ and ***$P < 0.01$ compared with untreated EAE.

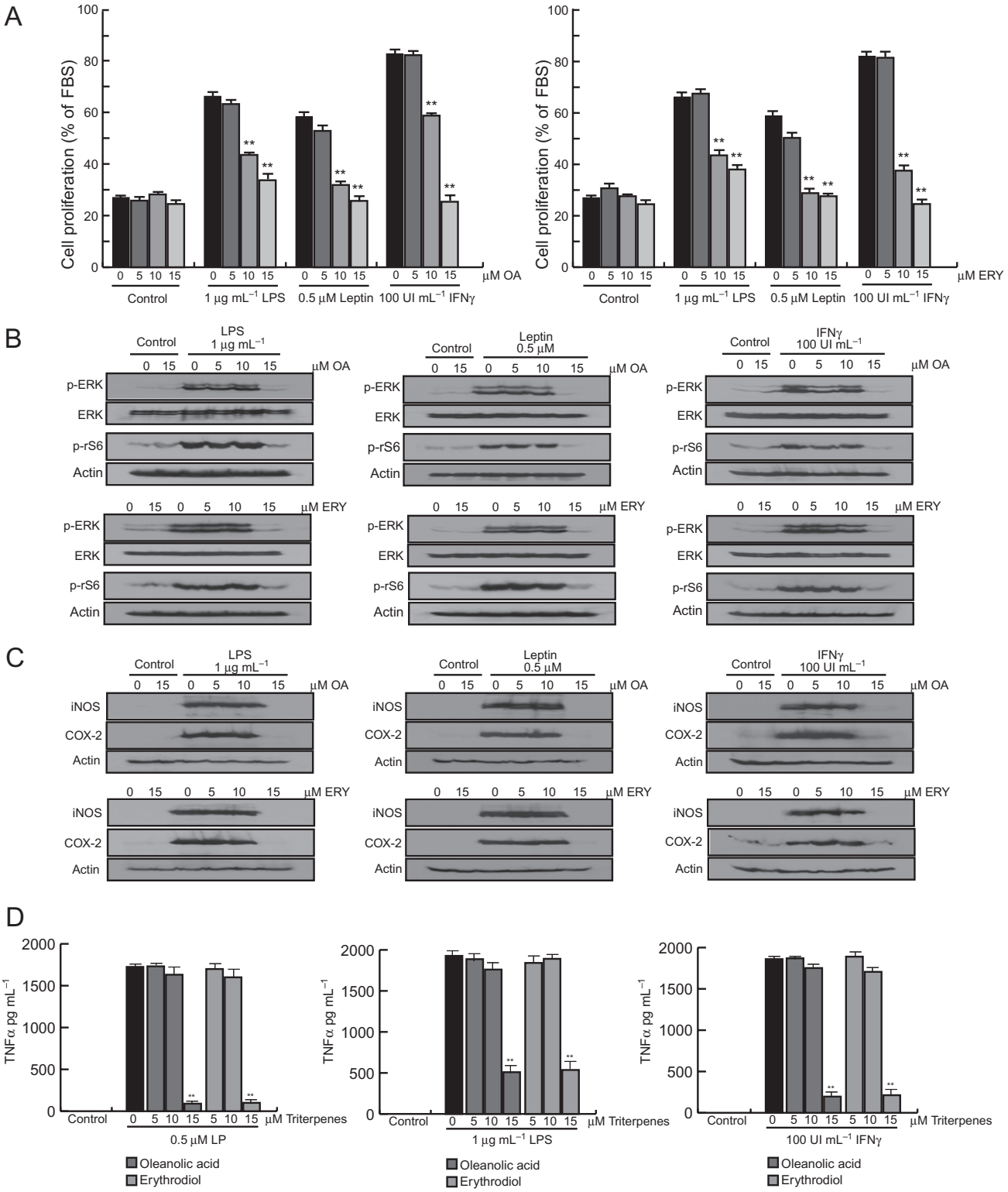

Figure 6

Triterpenes modulate BV-2 microglia cell activation. BV-2 cells were pretreated for 30 min with the indicated doses of oleanolic acid or erythrodiol, and then were stimulated with 100 UI mL^{-1} IFNγ, 1 μg·mL^{-1} 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 (**$P < 0.001$ compared with stimuli without triterpene; $n = 3$). (B) After 15 min of incubation, ERK 1/2 and rS6 phosphorylation was identified in the cell lysates by Western blot. (C) After 24 h of incubation, COX-2 and iNOS expression was identified 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 with stimuli without triterpene; $n = 3$).

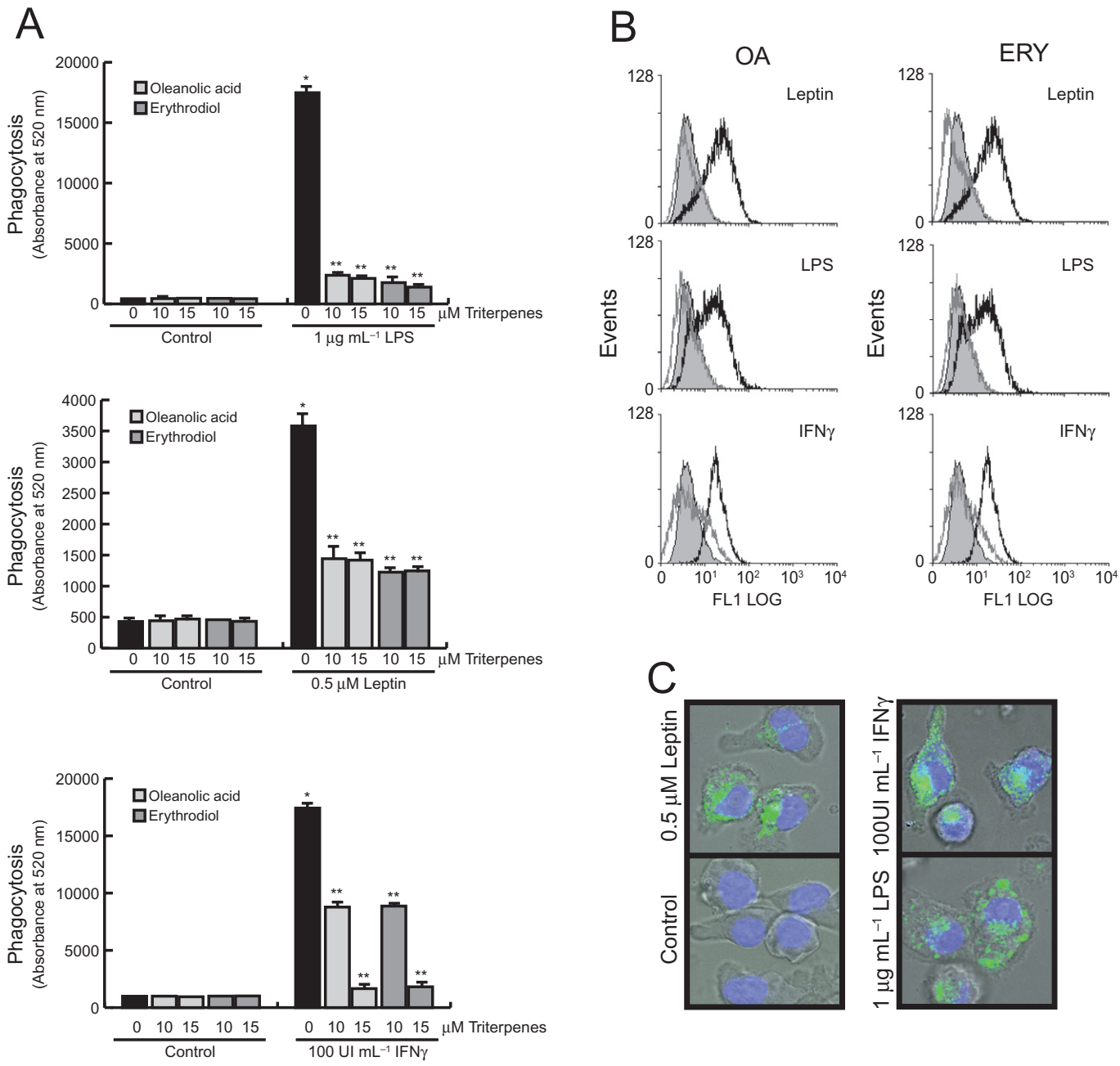

Figure 7

Triterpenes modulate phagocytic capabilities of BV-2 microglia cells. After 24 h stimulation with 100 UI mL^{-1} IFNγ, 1 µg·mL^{-1} of LPS or 0.5 µM of leptin, in the presence or absence of triterpenes, BV-2 cells were incubated for 2 h with 1 mg·mL^{-1} FITC-labelled dextran, and phagocytosis was measured by fluorescence emission at 520 nm in a fluorimeter (A), in a flow cytometer (B) or by confocal microscopy under a ×60 oil objective (C). In A, values represent mean of cell fluorescence intensity ± SD (*P < 0.001 compared with control and **P < 0.001 compared with 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 grey curves). Solid grey curves represent resting/control cells. Results are representative of three independent experiments.

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 oleanolic acid or erythrodiol markedly decreased the inflammatory parameters of activated microglial cells, pointing to a possible regulatory effect of triterpenes on key CNS-resident innate immune cells.

As pretreatment with oleanolic acid or erythrodiol dramatically influences 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 influence 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 the clinical scores peak. 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 pro-inflammatory 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 showed that the systemic and local levels of leptin in triterpene-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 oleanolic acid and erythrodiol 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 at a stage when lymphocytes enter the CNS and its subsequent cascade of inflammatory events. In keeping with earlier results, after immunization mice developed motor weakness and inflammatory cells accumulated in CNS tissues. In contrast, early administration of oleanolic acid or erythrodiol conferred protection against an increase in cell adhesion and rolling flux within the CNS microvasculature, reducing the number of cells infiltrating into the CNS, and significantly delaying the disorders of motor function.

It is a general statement that compounds affecting the different stages of leukocyte recruitment may have broad application in the modulation of chronic inflammatory diseases in which leukocyte accumulation is a marker of disease pathology. Accordingly, several laboratories have reported that triterpenes affect leukocyte recruitment by modulating the expression of surface molecules. The pentacyclic triterpenoid acids, oleanolic and ursolic, decreased TNF-α-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). Oleanolic acid and some oleanane-type triterpenoids isolated from *fabaceous* plants reduced ICAM-1 expression in monocytic cells as effectively as dexamethasone (Ahn *et al.*, 2002). In addition, we have recently shown that oleanolic acid reduced VCAM expression on CNS tissues of mice with established EAE, as well as the extravasation of lymphocytes into the perivascular space (Martín *et al.*, 2010).

Added to 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 the 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 in protecting from this disease. The naturally occurring products berberine (Ma *et al.*, 2010), OA (Martín *et al.*, 2010) and lipoic acid (Schreibelt *et al.*, 2006), or the synthetic compound 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 oleanolic acid or erythrodiol 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 up-regulation 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.*, 2009b) 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-γ, and protecting the integrity of the BBB. In our study, mice that were immunized following oleanolic acid or erythrodiol treatment did not express IL-17 and IFN-γ in spinal cord tissue, and its serum concentration was significantly lower than that of sham-treated EAE mice, paralleling the protective action of the triterpenes on the BBB.

In the same direction, we have also found that triterpene pretreatment markedly attenuated the expression of other inflammatory cytokines, such as IL-6 and TNF-α, whose high levels observed in EAE/MS correlated with dysregulation of the BBB (Sharief and Thompson, 1992; Quintana *et al.*, 2009). Triterpene pretreatment also increased levels of the protective anti-inflammatory cytokines IL-4 and IL-10. Interestingly, this Th1/Th2 bias promoted by triterpene treatment was maintained even after the disease developed and reached severe levels. Therefore, limitation or restriction of Th1 and Th17 cytokine production in the CNS and systemic circula-

tion, and promotion of Th2-type immune response, could be critical aspects of the beneficial effects of triterpenes in modulating the development (onset and progression) of clinical EAE.

Given the down-modulation of the pro-inflammatory cytokines in CNS tissues of EAE mice pretreated with triterpenes, we thought that the immuno-regulatory activities of oleanolic acid and erythrodiol might include actions on both circulating and CNS-resident immune cells. Therefore, assuming that both triterpenes, being lipophilic 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 ischaemic and neurodegenerative disorders are associated with proliferation and overactivation 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 pro-inflammatory mediators including TNF-α, COX-2 and iNOS, were suppressed in the presence of oleanolic acid and erythrodiol. These data are in line with several *in vitro* studies describing triterpene actions on multiple cellular targets from the innate immune system. Natural triterpenes inhibited production of the pro-inflammatory cytokines in human mononuclear cells (Marquez-Martin *et al.*, 2006) and synthetic derivatives decreased the expression of COX-2 and iNOS in murine macrophages (Suh *et al.*, 1998) and restrained the neurotoxic activities of microglia cells through inhibition of reactive oxygen species and TNF-α 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 (Linington *et al.*, 1988). Moreover, high serum antibodies or enhanced numbers of B cells secreting antibodies against myelin antigens have been observed in MS patients. Here, we have shown that anti-MOG antibody production was significantly decreased in mice treated with either of the triterpenes. This might be due to triterpene-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 IFN-τ (Mujtaba *et al.*, 1998). Further studies would be needed to investigate both options.

Besides immune-mediated inflammation, oxidative stress is another important mechanism involved in EAE/MS (Gilgun-Sherki *et al.*, 2004; Haider *et al.*, 2011). We have focussed on the immunomodulatory effect of oleanolic acid and erythrodiol in EAE, but we have not characterized, and neither has it been documented in murine EAE, their antioxidant capacities. Further studies to address their ability to restrain lipid, protein and DNA oxidation, which underlie axonal damage and oligodendrocyte 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 MS (Geerlings *et al.*, 2003). 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.*, 2009a), and the olive oil extract Oliplus, containing 45.5% polyphenols, 4.2% hydroxytyrosol, 2.2% tyrosol and 9.2% oleuropeine, inhibited gelatinases involved in the pathogenesis of MS (Liuzzi *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 patients suffering from CNS autoimmunity.

In summary, we conclude that prophylactic treatment with oleanolic acid or erythrodiol 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 actions of triterpenes might involve regulatory mechanisms related to effector functions of microglial cells. Therefore, we propose oleanolic acid and erythrodiol as compounds with promising multi-level immunomodulating characteristics, useful for therapeutic intervention in MS and other autoimmune and/or neuroinflammatory diseases.

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Conflict of interest

The authors have no conflicts of interest to report.

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