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Euphol prevents experimental autoimmune encephalomyelitis in mice: Evidence for the underlying mechanisms

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ABSTRACT

Multiple sclerosis (MS) is a severe chronic T cell-mediated autoimmune inflammatory disease of the central nervous system (CNS), the existing therapy of which is only partially effective and is associated with undesirable side effects. Euphol, an alcohol tetracyclic triterpene, has a wide range of pharmacological properties and is considered to have anti-inflammatory action. However there are no reports about the effects and mechanisms of euphol in experimental autoimmune encephalomyelitis (EAE), an established model of MS. Here we report the effects and the underlying mechanisms of action of euphol in EAE. Euphol (1–10 mg/kg) was administered orally at different time-points of EAE. Immunological and inflammatory responses were evaluated by real-time PCR, Western blot and flow cytometry assays. We provide evidence that euphol significantly attenuates neurological signs of EAE. These beneficial effects of euphol seem to be associated with the down-regulation of mRNA and protein expression of some pro-inflammatory mediators such as TNF-α, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the CNS. Furthermore, *in vitro*, euphol consistently inhibited the T cell-mediated immune response including the production of T_H1 and T_H17 cytokines in spleen cells of untreated EAE animals. Likewise, oral euphol treatment inhibited the infiltration of T_H17 myelin-specific cells into the CNS through the adhesion molecule, lymphocyte function-associated antigen 1 (LFA-1). Our findings reveal that oral administration of euphol consistently reduces and limits the severity and development of EAE. Therefore, euphol might represent a potential molecule of interest for the treatment of MS and other T_H17 cell-mediated inflammatory diseases.

1. Introduction

Multiple sclerosis (MS) and its murine model, experimental autoimmune encephalomyelitis (EAE), are characterized by an autoimmune response against central nervous system (CNS) proteins, which culminates in an inflammatory infiltrate, gliosis, damage to the myelin sheath and neuronal death [1–4]. The pathogenesis of the disease is characterized by the activation of mononuclear cells, predominantly Ag-specific $CD4^+$ and $CD8^+$ T cells, which migrate across the blood brain barrier (BBB) into the CNS, and are reactivated by resident Ag-presenting brain–spinal cord glial cells. This cell trafficking is a tightly regulated process that is associated with the activation of the T cells [3,5] and governed by the BBB via the secretion of various chemokines, and the expression of cellular adhesion molecules and tight-junction proteins [6]. It is now well established that the activated T cells and brain glial cells secrete pro-inflammatory cytokines/chemokines along with the generation of inflammatory mediators including complement, which are highly reactive free radicals (reactive oxygen species) that lead to axonal loss [3,4,7]. Although genetic and environmental factors are also implicated in the pathogenesis of MS [8,9], its precise etiology remains unclear.

$CD4^+$ T_H1 cells secreting IFN-γ were long considered to be the predominant disease-inducing T-cell subset in EAE [7]. Accumulated evidence indicates that T_H1 cytokines are present in the inflammatory lesions in the CNS, whereas T_H2 cytokines are absent, suggesting that T_H1 cytokines play a role in the

Abbreviations: CNS, central nervous system; CFA, complete Freund's adjuvant; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; MOG, myelin oligodendrocyte glycoprotein; PBS, phosphate buffered saline; T_H, T helper cells; IFN-γ, interferon gamma; IL, interleukin; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; PECAM, platelet endothelial cell adhesion molecule; LFA-1, lymphocyte function-associated antigen-1; TNF-α, tumor necrosis factor alpha; TGF-β, transforming growth factor beta; RT-PCR, real-time PCR; NO, nitric oxide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; PGs, prostaglandins; ANOVA, analysis of variance; SEM, standard error mean.

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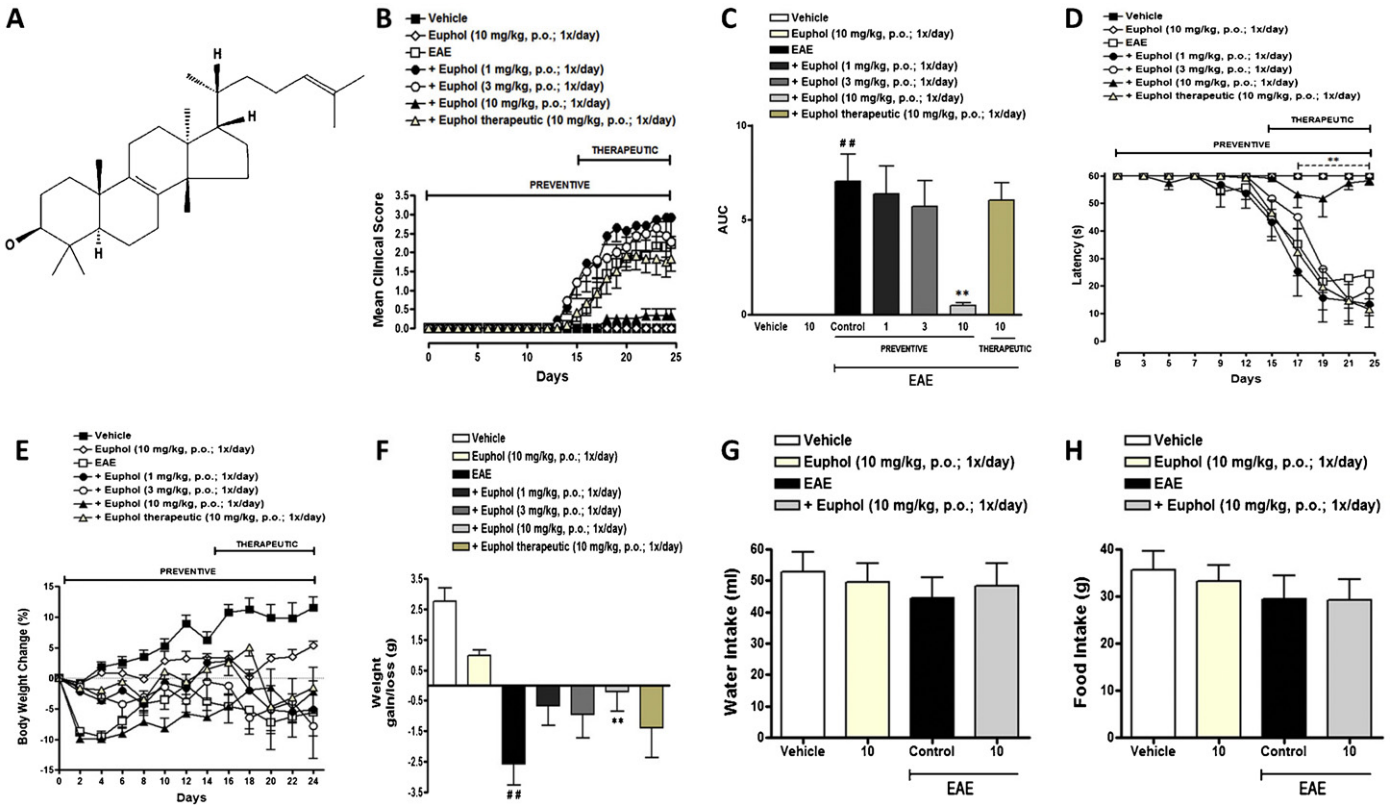

Fig. 1. Euphol attenuates the progression of EAE disease, but does not alter the amounts of water and food consumed. Chemical structure of the tetracyclic triterpene alcohol euphol (A). Active EAE was induced in C57BL/6 mice by immunization with 200 μg of peptide MOG_{35-55}/CFA on days 0 and 7. Animals were orally treated by gavage with 1, 3, or 10 mg/kg of euphol without or with EAE challenge once daily from day 0 to day 25 (preventive treatment) or with 10 mg/kg from day 15 to day 25 (therapeutic treatment, when all mice had visible clinical symptoms of disease). The clinical score (B), area under the curve (AUC) (C), locomotor activity (D), body weight change (E) and body weight gain or loss at the peak of disease (day 18 post-induction) (F) were evaluated. The clinical symptoms were scored every day in a blinded manner and expressed as the mean clinical score or as the AUC. The amounts of water (G) and food consumed (H) were measured during 25 days after EAE induction. Data are presented as mean ± SEM of 6–9 mice/group and are representative of three independent experiments. $^{\#\#}p < 0.001$ versus the control group, $^{**}p < 0.001$ versus the EAE group (one-way ANOVA with the Newmann–Keuls post-hoc test).

pathogenesis of disease [5,7]. Furthermore, recovery from EAE in mice is associated with an increase in the presence of T_H2 cytokines in the CNS [5]. Recently, IL-17-producing T_H cells (T_H17 cells), a distinct subset from T_H1 and T_H2 cells, were characterized based on the secretion of pro-inflammatory cytokines IL-17, IL-6, and TNF-α [3,5,7,10]. Evidence has also demonstrated that T_H17 cells, rather than T_H1 cells, play a role in autoimmune inflammatory conditions such as EAE [3].

The alcohol derivative tetracyclic triterpene euphol (Fig. 1A) is the main constituent found in the sap of *Euphorbia tirucalli*, a plant belonging to the family Euphorbiaceae, and known in traditional Brazilian medicine as avelóz. In the northeast of Brazil, the latex of *E. tirucalli* is used as a folk therapy against syphilis, as a laxative agent, to control intestinal parasites, and to treat asthma, cough, earache, rheumatism, cancer, chancre, epithelioma and sarcoma [11]. In addition, *E. tirucalli* showed larvicidal activity against *Aedes aegypti* and *Culex quinquefasciatus*, the most common dengue vector and lymphatic filariasis vector, respectively [12]. A recent study demonstrated that a biopolymeric fraction (BET) obtained from *E. tirucalli* showed dose-dependent anti-arthritic activity and demonstrated *in vivo* suppression of $CD4^+$ and $CD8^+$ T cells associated with the inhibition of intracellular interleukin-2 (IL-2) and interferon-γ (IFN-γ), and inhibited vascular permeability and the migration of leukocytes at the site of the insult [13]. Additional evidence has indicated that euphol isolated from the roots of *Euphorbia kansui* markedly inhibits the tumor promotion in mouse skin induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) [14] and different fractions of *Euphorbia royleana* latex (AER) showed dose-dependent anti-inflammatory, anti-arthritic and immunosuppressive properties in diverse acute and chronic test models in rats and mice [15,16]. However, there have been no reports about the effects of euphol on immune inflammatory diseases of the CNS, such as MS. In the present study, we sought to investigate the therapeutic potential of euphol on EAE disease progression, and attempted to elucidate some of the mechanisms through which euphol modulates the pro-inflammatory environment of CNS, adhesion molecule expression and T_H17 myelin-specific responses. Our results demonstrated that euphol effectively ameliorates both the clinical and pathological parameters of EAE, mainly by inhibiting the expression of pro-inflammatory mediators *in vitro* and *in vivo*. Our data also indicated that the mechanisms underlying the anti-inflammatory activity of euphol are probably associated with its ability to selectively inhibit the infiltration of T_H17 myelin-specific cells into the CNS through the modulation of adhesion molecule, such as lymphocyte function-associated antigen 1 (LFA-1).

2. Materials and methods

2.1. Animals

Female C57BL/6 mice (6–10 weeks of age) were obtained from the Laboratório de Farmacologia Experimental (LAFEX), Universidade Federal de Santa Catarina (UFSC, Florianópolis, SC, Brazil) and housed in communal cages at 22 ± 1 °C under a 12-h light/dark cycle (lights on at 07:00 h), with free access to food and tap water.

Experiments were performed during the light phase of the cycle. All experimental procedures were previously approved by UFSC's Committee on the Ethical Use of Animals and were carried out in accordance with Brazilian regulations on animal welfare (CEUA/UFSC protocol number 23080.030926/2010-62).

2.2. Active induction of EAE

C57BL/6 mice were immunized by subcutaneous (s.c) immunization into the flanks with 200 μg MOG_{35-55} dissolved in phosphate buffered saline (PBS) and emulsified with an equal volume of complete Freund's adjuvant (CFA) supplemented with 500 μg of *Mycobacterium tuberculosis* extract H37Ra. This procedure was repeated after 7 days in order to increase the incidence of EAE, as previously described [17]. All animals were also injected intraperitoneally on days 0 and 2 with 300 ng of pertussis toxin (PTX). Non-immunized (naive) and EAE animals were used as the control groups. Animals were weighed daily to evaluate any effect of the euphol administration (before, during and after) on body weight (as a parameter of health) and assessed for clinical signs of EAE by two independent observers. In addition, the food intake and water consumption were measured every two days during 25 days. We used a clinical EAE scoring system to assess neurological deficit in our mouse EAE model according to the following scale: score 0, no disease; score 1, loss of weight and tail weakness; score 2, weakness in hind limb; score 3, complete hindlimb paralysis; score 4, hind limb paralysis with fore limb weakness or paralysis; and score 5, moribund or deceased.

2.3. Treatment protocol

The compound euphol was dissolved in 5% Tween 80 solution made in saline (0.9% NaCl solution) and administered orally by gavage (p.o.). Mice received euphol (1, 3 and 10 mg/kg, p.o.) or vehicle once daily from day 0 to day 25 (preventive treatment) or euphol 10 mg/kg from day 15 to day 25 (therapeutic treatment), and were sacrificed at day 25 post-immunization. Therapeutic treatment began after the onset of symptoms in animals with EAE (animals with clinical score ≥ 0.5). Vehicle solution (5% Tween 80 + 0.9% NaCl solution) was used in control experiments.

2.4. Rotarod test

In order to assess the effect of euphol on locomotor activity and coordination, the mice were placed on a rotarod apparatus at a fixed rotational speed of 4 rpm. The maximum time for each trial was set at 60 s. Rotarod training was performed prior to disease induction and consisted of three consecutive trials in which the animals became familiar with the task. After disease induction, the mice were tested every two/three days until day 25 post-immunization.

2.5. Measurement of cytokine production in splenic lymphocytes

The 6- to 10-wk-old female EAE mice were killed at the peak of disease (day 18 post-immunization) and spleens were removed. Each spleen was individually macerated in RPMI 1640 medium supplemented with 10% fetal bovine serum, HEPES (20 nM), 2-mercapto ethanol, penicillin (100 U/ml) and streptomycin (100 μg/ml) and the cell suspension was filtered through a 70 μm filter. The resulting suspension was centrifuged at 1500 × *g* for 7 min at 4 °C. After centrifugation the supernatant was discarded and the cell pellet resuspended in ammonium chloride potassium carbonate buffer (ACK lysis buffer) using 1 ml per donor mouse to lyse red blood cells, and then incubated on ice for 5 min. After incubation, 9 ml of the medium was added to stop cell lysis. The cell debris was allowed to settle on the bottom of the tube for 2 min before being transferred to a new 15 ml conical tube and centrifuged for 5 min at 500 × *g* and 4 °C. Finally, the supernatant was discarded and the cells were resuspended in 2 ml of the medium. The splenocytes (1×10^6/well) were pre-treated with different concentrations of euphol (1–100 μg/ml) for 1 h followed by stimulation with MOG_{35-55} (10 μg/ml) for 48 h at 37 °C in a humidified 5% CO_2 atmosphere. After stimulation, the plate was centrifuged (200 × *g*/10 min) and the cell-free supernatant was collected and stored at −70 °C for cytokine determination. A cytokine bead array Mouse $T_H1/T_H2/T_H17$ Kit (BD Biosciences, San Diego, CA, USA) was used to measure IL-17A, IFN-γ, TNF-α, IL-6 and IL-2 secretion in the supernatant. The data were acquired using BD FACSCanto II (BD Biosciences, San Diego, CA, USA) and analyzed using FCAP Array Software (BD Biosciences, San Diego, CA, USA).

2.6. Real-time quantitative PCR

Lumbar spinal cord tissues were removed 25 days post-immunization from six to nine animals/group and the total RNA was extracted using the Trizol protocol. The reverse transcription assay was carried out as described in the M-MLV Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Real-time quantitative PCR analysis was performed in StepOnePlus™ using the TaqMan® Universal PCR Master Mix Kit (Applied Biosystems, Foster City, CA, USA) for quantification of the amplicons. Fifty nanogram of total RNA was used for cDNA synthesis. The cDNA was amplified in duplicate using specific TaqMan® Gene Expression target genes, the 3′ quencher MGB and FAM-labelled probes for TNF-α (Mm00443258_m1), iNOS (Mm01309898_m1), COX-2 (Mm01307334_g1), interleukin-17 (IL-17) (Mm00439618_m1), ROR-γT (Mm00441144_g1), IFN-γ (Mm99999071_m1), T-bet (Mm00450960_m1), occludin (Mm00500912_m1), claudin-4 (Mm00515514_s1), cadherin-7 (Mm00556135_m1), ICAM-1 (Mm005616024_g1), LFA1 (Mm01278854_m1), VCAM-1 (Mm01320970_m1), PECAM-1 (Mm01242584_m1) and GAPDH (NM_008084.2), the latter being used as an endogenous control for normalization. The PCRs were performed in a 96-well Optical Reaction Plate (Applied Biosystems, Foster City, CA). The thermocycler parameters were as follows: 50 °C for 2 min, 95 °C for 10 min, 50 cycles of 95 °C for 15 s, and 60 °C for 1 min. Expression of the target genes was calibrated against conditions found in control animals (i.e., animals that received vehicle).

2.7. Western blot assay

Spinal cord tissue samples were removed 25 days post-immunization and homogenized in complete radio immunoprecipitation lysis buffer (RIPA). Equal amounts of protein for each sample (30 μg) were loaded per lane and electrophoretically separated using 10% denaturing polyacrylamide gel electrophoresis (SDS-PAGE). Afterward, the proteins were transferred to nitrocellulose membranes using a Mini Trans-Blot Cell System (Bio-Rad Laboratories Inc., Hercules, CA, USA) following the manufacturer's protocol. Western blot analysis was carried out using polyclonal mouse anti-TNF-α (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal mouse anti-iNOS (1:1000) (Sigma–Aldrich, St. Louis, MO, USA) and polyclonal rabbit anti-COX-2 (1:1000, Cell Signaling Technology, Danvers, MA, USA), all incubated overnight. Following washing, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (1:25,000, Cell Signaling Technology, Danvers, MA, USA). The immunocomplexes were visualized using SuperSignal West Femto Chemiluminescent Substrate Detection System (Thermo Fischer Scientific, Rockford, IL, USA) and densitometric

values were normalized using monoclonal mouse β-actin antibody (1:500, Cell Signaling Technology, Danvers, MA, USA). Protein levels were quantified by optical density using Image-J Software and expressed as the ratio to β-actin represented by arbitrary units.

2.8. Drugs and reagents

The sap from *E. tirucalli* was initially extracted with hexane and the resulting precipitate was extracted with n-butanol. The most lipophilic compounds present in the butanol fraction were purified by means of high performance liquid chromatography analysis (HPLC). Further purification of the compounds was carried out using a Sephadex G75 column in a mixture of hexane–ethyl acetate. Recrystallization of the acetate fraction from butanol gave 3.5 g crystals, comprising euphol acetate and filtrate (1.5 g). The chemical structure of the euphol was determined by elemental analyses of ^{1}H NMR and ^{13}C NMR spectral data, and by comparison with their respective authentic compounds [14,18]. ^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra were recorded on a Bruker 500 MHz instrument. The MS spectra were recorded on a Perkin Elmer instrument, model API 150 and run in ES-MS positive mode: MH+ 427 m/e, MH+ $-H_2O$ 409 m/e. The NMR parameters were ^{13}C NMR ($CDCl_3$): 15.7 (C29), 15.8 (C18), 17.9 (C26), 19.1 (C21), 19.2 (C6), 20.4 (C19), 21.7 (C11), 24.9 (C30), 25.0 (C23), 25.9 (C27), 28.0 (C2,C7), 28.2 (C28), 28.3 (C16), 30.0 (C15), 31.1 (C12), 35.4 (C1), 35.7 (C22), 36.1 (C20), 37.5 (C10), 39.2 (C4), 44.3 (C13), 49.9 (C17), 50.2 (C14), 51.2 (C5), 79.2 (C3), 125.4 (C24), 131.1 (C25), 133.8 (C9), 134.3 (C8) and ^{1}H NMR ($CDCl_3$): 0.75 (^{3}H, s, H-18), 0.80 (^{3}H, s, H-29), 0.85 (^{3}H, d, H-21), 0.87 (^{3}H, s, H-30), 0.95 (^{3}H, s, H-19), 1.00 (^{3}H, s, H-28), 1.50 (^{3}H, s, H-26), 1.68 (^{3}H, s, H-27), 3.23 (^{1}H, dd, H-3), 5.09 (^{1}H, bt, H-24). Further details regarding euphol isolation, purification and chemical determination will be published elsewhere. The tetracyclic triterpene euphol used in this study showed >95% purity. Pertussis toxin, PBS, H&E, H_2O_2, Incomplete Freund's adjuvant oil, penicillin, streptomycin, Na-EDTA, trypsin, deoxyribonuclease I (DNase) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK) was obtained from EZBiolab, Carmel, IN 46032, USA; and *M. tuberculosis* extract H37Ra from Difco Laboratories, Detroit, MI, USA. The RPMI 1640 and fetal bovine serum were purchased from GIBCO (Carlsbad, CA, USA). The cytokine bead array (CBA) Mouse $T_H1/T_H2/T_H17$ Kit was purchased from BD Biosciences (San Diego, CA, USA). Trizol and M-MLV reverse transcriptases were purchased from Invitrogen (Carlsbad, CA, USA). The primers and probes for mouse TNF-α, iNOS, COX-2, IL-17, ROR-γT, IFN-γ, T-bet, occludin, claudin-4, cadherin-7, ICAM-1, LFA1, VCAM-1, PECAM-1 and GAPDH were purchased from Applied Biosystems (Foster City, CA, USA). The polyclonal mouse anti-TNF-α was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), monoclonal mouse anti-iNOS from Sigma–Aldrich (St. Louis, MO, USA), polyclonal rabbit anti-COX-2 and β-actin from Cell Signaling Technology (Danvers, MA, USA). Anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase were purchased from DakoCytomation (Carpinteria, CA, USA). The other reagents used were of analytical grade and were obtained from different commercial sources.

2.9. Statistical analysis

All data are presented as mean ± SEM of six to nine mice/group and are representative of two/three independent experiments. A statistical comparison of the data was performed by one-way ANOVA followed by Newmann–Keuls's test. *p*-Values less than 0.05 ($p < 0.05$ or less) were considered significant. The statistical analyses were performed using GraphPad Prism 4 Software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Euphol attenuates the clinical signs of EAE

To evaluate the efficacy of euphol, EAE was induced in C57BL/6 mice using myelin MOG_{35-55} peptide (200 μg/mice). As expected, clinical symptoms, such as tail atony and clumsy gait, appeared on day 10 post-MOG-injections and peaked around day 18, after which they were stable up to day 25, the final day of evaluation (Fig. 1B). There were no deaths during the course of EAE development. To test the preventive effect of euphol in the EAE model, this compound was given orally as 1, 3 and 10 mg/kg from day 0 of immunization to the end of the analysis. Compared with the untreated EAE group, euphol treatment with 1 and 3 mg/kg provided no significant protection against disease induction (Fig. 1B–F). However, 10 mg/kg euphol significantly blocked the clinical manifestations associated with EAE (Fig. 1B), with inhibition of 92 ± 6%, based on the area under the curve (AUC) (Fig. 1C), as well as inhibited the locomotor deficits induced by EAE (Fig. 1D). In addition, a progressive loss of body weight paralleled the severity of the disease (Fig. 1E), and at the peak of disease (day 18 post-induction), weight loss in the control group reached approximately 2.5 g for mice weighing 20 g on day 0 (Fig. 1F). However, mice pre-treated with euphol (10 mg/kg, p.o.) were protected against marked loss of body weight and recovered a healthy appearance that was similar to that of control mice (Fig. 1E and F). In contrast, treatment with euphol at doses of 1 or 3 mg/kg, p.o., did not significantly reduce body weight loss (Fig. 1D and E).

The oral anti-inflammatory effects of euphol in the induction phase of EAE led us to investigate whether initiation of treatment after onset of disease (therapeutic scheme) is also effective in preventing EAE. To address this hypothesis, euphol was administered to ongoing active EAE in C57BL/6 mice, starting on day 15 (when all mice had visible clinical symptoms ≥ 0.5). Oral treatment with euphol at 10 mg/kg given once daily significantly delayed the onset of EAE; however euphol at the same scheme of treatment did not significantly reduce the severity of EAE (Fig. 1B–F). Moreover, we also investigated whether or not higher doses of euphol (30 mg/kg, p.o.) in the therapeutic treatment (from day 15 to day 25 post-immunization) could inhibit the clinical signs induced by EAE. Once again, the treatment with euphol at doses of 30 mg/kg, p.o., during chronic phase of EAE did not significantly reduce the clinical signs induced by EAE (data not shown).

To determine whether euphol could alter appetite and water consumption after induction of EAE, the food intake and water consumption were measured every two days in the naive group, mice treated with euphol alone (10 mg/kg, p.o.) without EAE challenge, the control group (EAE) and from mice pre-treated with euphol (10 mg/kg, p.o.) with EAE during 25 days after immunization. As shown in Fig. 1H and G mice without or with EAE and treated with euphol (10 mg/kg, p.o., during 25 days) did not exhibit any significant reduction in water (Fig. 1G) and food consumption (Fig. 1H), when compared with control groups (vehicle and EAE group). Nonetheless, euphol treatment (10 mg/kg, p.o.) in the same period significantly improved locomotor deficit induced by EAE (Fig. 1D). Together, these data consistently support our hypothesis that oral administration of euphol (10 mg/kg) did not significantly alter the appetite and water consumption, but improved the locomotor activity of animals, which may explain the gain in mouse body weight during the 25 days following EAE.

Importantly, animals treated with euphol (10 mg/kg, p.o.) during 25 days without EAE challenge, did not affect the clinical score, locomotor deficit and body weight loss, as well as change in

food/water consumption when compared with vehicle group (Fig. 1B–H).

3.2. Euphol treatment inhibits pro-inflammatory mediators in the CNS

MS lesions are associated with the expression of a wide range of pro-inflammatory cytokines (IFN-γ, TNF-α, IL-17, IL-1β and IL-6) and other inflammatory mediators, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which in turn play a critical role in the infiltration of immune cells during EAE disease progression [19]. To ascertain the status of these pro-inflammatory mediators, we examined the levels of mRNA and protein in lumbar spinal cord tissues of euphol-treated and untreated EAE animals 25 days post-immunization. A pronounced increase in TNF-α and COX-2 mRNA expression was observed in spinal cord in the EAE-control group (Fig. 2A). Surprisingly, preventive treatment with euphol (1, 3 and 10 mg/kg) markedly inhibited the up-regulation of mRNA expression of TNF-α (Fig. 2A) and COX-2 (Fig. 2B) in the CNS after EAE induction. Furthermore, the expression of the other inflammatory mediators, such as iNOS in the CNS of EAE animals was dose-related down-regulated following oral euphol treatment (Fig. 2C). Likewise, at the doses of 1 and 3 mg/kg euphol did not show satisfactory effect in inhibiting the clinical signs induced EAE *in vivo* (Fig. 1B–F). For this reason, the dose of 10 mg/kg of euphol was used in subsequent experiments to investigate some of the

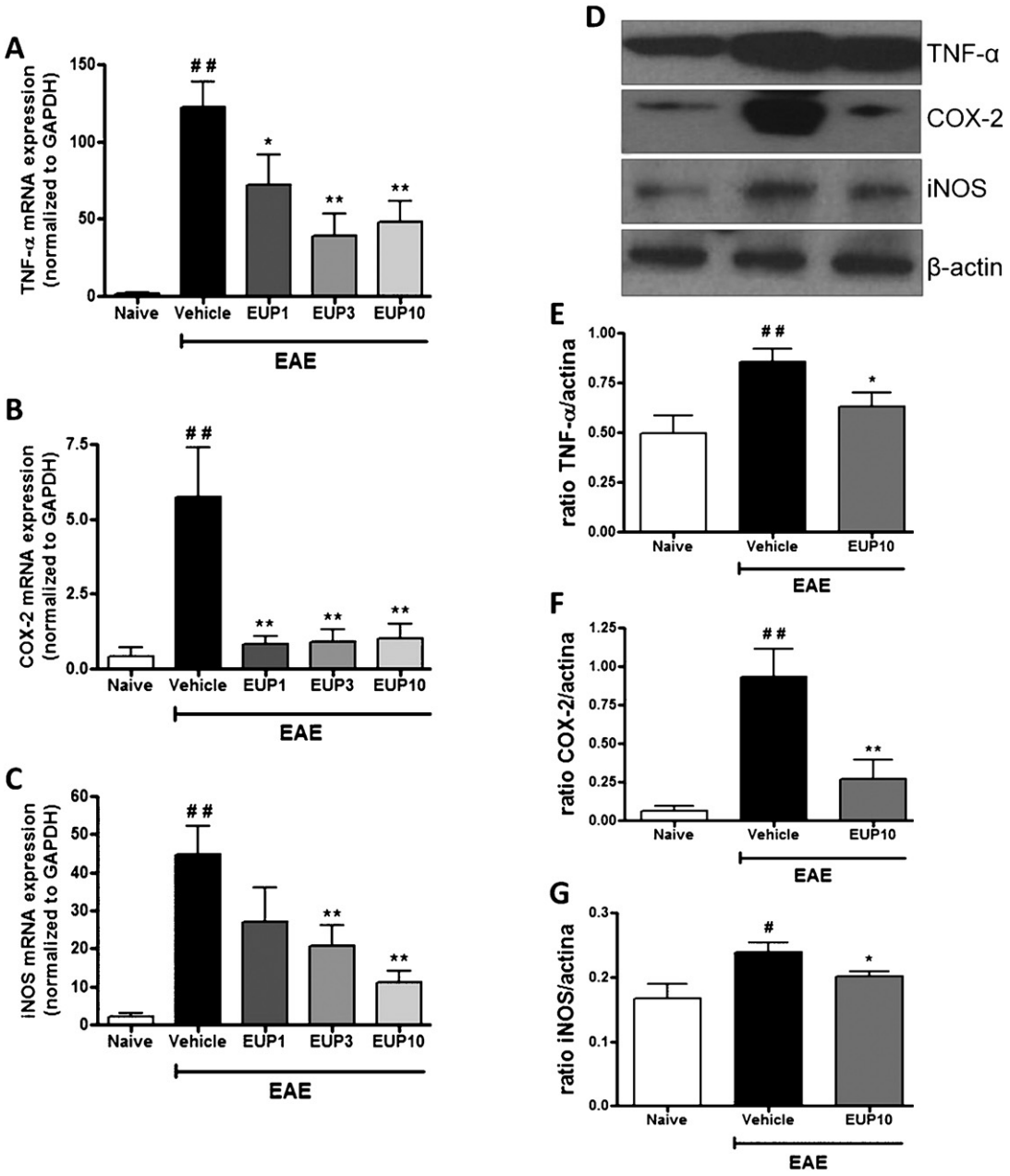

Fig. 2. Euphol treatment inhibits the expression of pro-inflammatory mediators in the CNS of EAE mice. Total mRNA and protein levels was extracted from the lumbar spinal cord of mice in the naive group, the control group (EAE) and those preventatively treated with euphol (1, 3 and 10 mg/kg, p.o.) on day 25 post-immunization. The mRNA levels of TNF-α (A), COX-2 (B) and iNOS (C) were measured by RT-PCR. The housekeeping gene GAPDH mRNA was used to normalize the relative amounts of mRNA. The protein expression levels of TNF-α (D and E), COX-2 (D and F) and iNOS (D and G) were determined by Western blot analysis, and quantified by densitometric scanning. The levels of protein were expressed as a ratio of signal intensity for the target proteins relative to that for β-actin, and their values were considered arbitrary units that represented the relative values among all samples. Data are presented as mean ± SEM of 6–9 mice/group and are representative of three independent experiments. $^{\#}p < 0.05$ and $^{\#\#}p < 0.001$ versus the naive group, $^{*}p < 0.05$ and $^{**}p < 0.001$ versus the EAE group (one-way ANOVA with the Newmann–Keuls post-hoc test).

mechanisms underlying its anti-inflammatory and immunomodulatory effects.

To further confirm the effect of euphol on TNF-α, COX-2 and iNOS protein expression, we carried out Western blot analysis in lumbar spinal cord from mice 25 days after EAE-induction. As shown in Fig. 2, only low protein levels of TNF-α (Fig. 2D and E), COX-2 (Fig. 2D and F) and iNOS (Fig. 2D and G) were detected in the naive mouse spinal cord tissue, but these values were markedly increased in EAE-untreated animals when assessed 25 days post-immunization (Fig. 2D–G). Remarkably, oral treatment with euphol (10 mg/kg) significantly inhibited the protein expression of TNF-α (Fig. 2D and E), COX-2 (Fig. 2D and F) and iNOS (Fig. 2D and G) in the spinal cord tissue after EAE immunization ($p < 0.001$).

3.3. Euphol inhibits production of T_H1 and T_H17 cytokines in MOG-primed T cells

To define the mechanism of action of antigen (Ag)-specific lymphocytes or Ag-nonspecific lymphocytes responsible for EAE, spleen cells were isolated from EAE mice on day 18 post-immunization with MOG_{35-55} peptide. The splenocytes were pre-treated with different concentrations of euphol (1–100 μg/ml) for 1 h followed by stimulation with MOG_{35-55} (10 μg/ml) for 48 h. As shown in Fig. 3, euphol markedly reduced the production of IL-17A (Fig. 3A), IFN-γ (Fig. 3B) and TNF-α (Fig. 3C); however, the production of IL-6 and IL-2 was not significantly affected by treatment with euphol *in vitro* (Fig. 3D and E). These data suggest that euphol may suppress the encephalitogenic nature of T cells, inhibiting the production of T_H1 and T_H17 inflammatory cytokines.

3.4. Euphol suppresses gene expression associated with the differentiation of T_H17 without affecting T_H1 cells in vivo

The development of clinical EAE has been associated with the production of inflammatory cytokines and signal transducers associated with the autoaggressive T_H1 and T_H17 phenotypes, including IFN-γ, T-bet, IL-17 and ROR- γT, respectively [5,7,10,20]. To determine the mechanisms involved in euphol regulation of the Ag-specific T_H1 and T_H17 response *in vivo*, we subsequently assessed whether oral pre-treatment with euphol was able to decrease the expression of T_H1 and T_H17 cytokines in the CNS induced by EAE immunization. As shown in Fig. 4, only low levels of IL-17, ROR-γT, IFN-γ and T-bet were detected in the naive mouse lumbar spinal cord; however their expression was markedly increased in the EAE-control group 25 days post-immunization (Fig. 4A–D). Euphol (10 mg/kg) given orally significantly reduced the up-regulated mRNA levels of IL-17

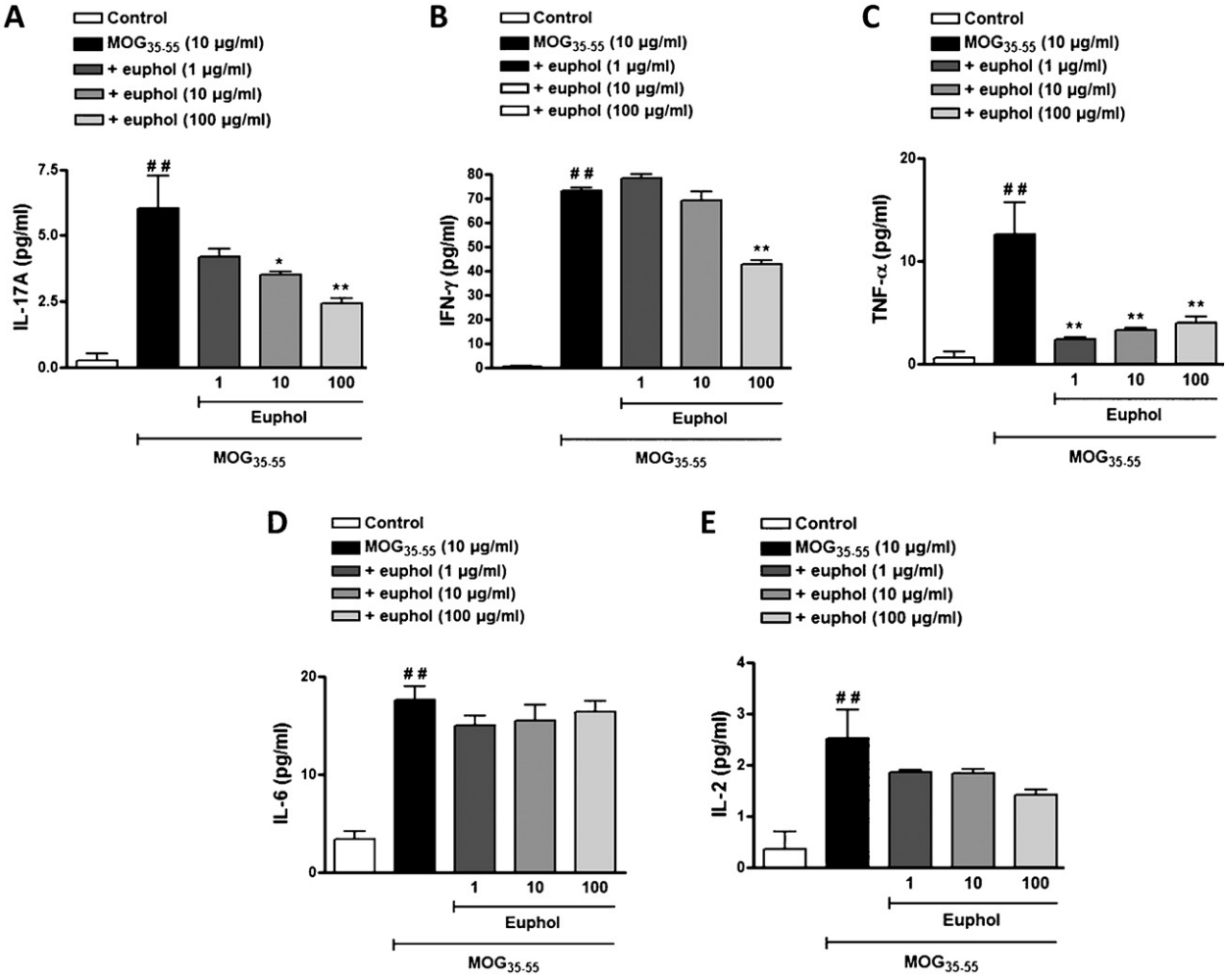

Fig. 3. Euphol treatment reduces Ag-specific T cell immune responses. The untreated group were killed at the peak of EAE disease (18th day after immunization) and spleen cells were isolated and stimulated *ex vivo* with MOG_{35-55} (10 μg/ml) peptide in the presence or absence of euphol (1–100 μg/ml) added 1 h before Ag-specific. The culture supernatants of spleen cells were collected at 48 h and analyzed for inflammatory cytokines: IL-17A (A), IFN-β (B), TNF-α (C), IL-6 (D) and IL-2 (E) using a cytokine bead array kit (CBA). Data are presented as mean ± SEM of 6 mice and are representative of two independent experiments. $^{\#\#}p < 0.001$ versus control group, $^{*}p < 0.05$ and $^{**}p < 0.001$ versus spleen cells from untreated EAE group (one-way ANOVA with the Newmann–Keuls post-hoc test).

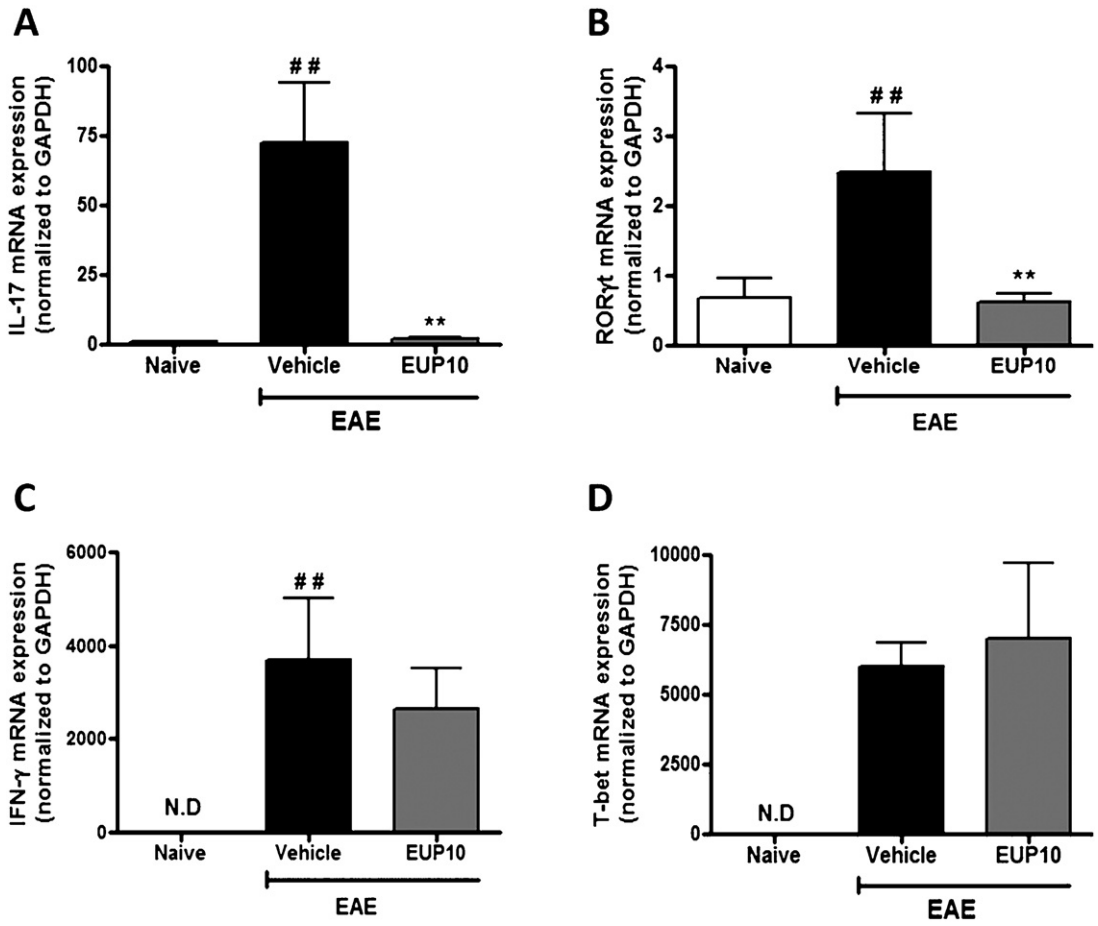

Fig. 4. Euphol treatment blocks central T_H17 myelin-specific immune responses during EAE pathology. Active EAE was induced in C57BL/6 mice with immunization of 200 μg of peptide MOG_{35-55}/CFA on days 0 and 7. The spinal lumbar cords were obtained from the naive group, the control group (EAE) and from mice pre-treated with euphol (10 mg/kg, p.o.) on the 25th day after immunization. The mRNA levels of IL-17A (A), ROR-γT (B), IFN-γ (C) and T-bet (D) were measured by RT-PCR. The housekeeping gene GAPDH mRNA was used to normalize the relative amounts of mRNA. Data are presented as mean ± SEM of 6–9 mice/group and are representative of three independent experiments. $^{\#\#}p < 0.001$ versus the naive group, $^{**}p < 0.001$ versus the EAE group (one-way ANOVA with the Newmann–Keuls post-hoc test).

(Fig. 4A) and the transcription factor ROR-γT (Fig. 4B) in the spinal cord after EAE induction. However, euphol (10 mg/kg, p.o.) failed to inhibit the increased mRNA levels of IFN-γ (Fig. 4C) and T-bet (Fig. 4D) in the spinal cord. These results suggest that euphol suppresses the expression of pro-inflammatory cytokines and the specific transcription factor associated with the differentiation and production of myelin-specific T_H17 cells, which may be one of the main mechanisms by which euphol is protective in EAE animals.

3.5. Euphol selectively blocks adhesion molecules expressed in leukocytes during the progression of EAE

The selective entry of activated leukocytes into the CNS is regulated by specific interactions at the endothelial limit [21,22]. Under physiological conditions these cells express adhesion molecules (CAMs) such as ICAM-1 and VCAM-1 at low levels, and form intercellular tight junctions, thereby limiting the

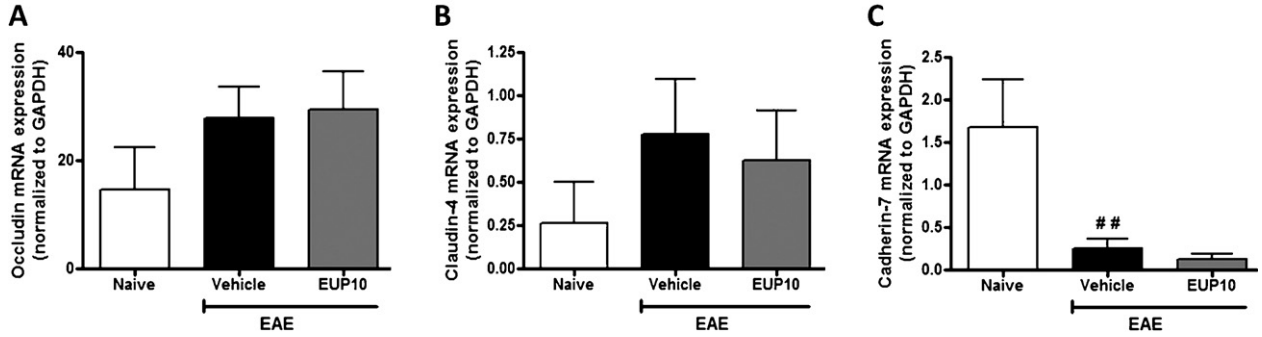

Fig. 5. Euphol treatment has no impact on tight-junction expression in the CNS during EAE progression. Active EAE was induced with MOG_{35-55} peptide/CFA and pertussis toxin. Total RNA was extracted from the lumbar spinal cord of mice in the naive group, the control group (EAE) and mice pre-treated with euphol (10 mg/kg, p.o.) on the 25th day after immunization. The mRNA levels of occludin (A), claudin-4 (B) and cadherin-7 (C) were measured by RT-PCR. The housekeeping gene GAPDH mRNA was used to normalize the relative amounts of mRNA. Data are presented as mean ± SEM of 6–9 mice/group and are representative of two independent experiments. $^{\#\#}p < 0.001$ versus the naive group (one-way ANOVA with the Newmann–Keuls post-hoc test).

trans- and paracellular movement of molecules and cells [23]. Neuroinflammatory conditions such as the binding of immune cells or the release of soluble mediators, however, can rearrange the normal assembly of tight-junction proteins and cause further up-regulation of adhesion molecules. As a consequence, the structural integrity of the BBB is disrupted and transendothelial trafficking increases [23–25]. In order to further investigate the mechanisms of action of euphol, the expression of tight-junction proteins and CAMs was evaluated in the lumbar spinal cord of EAE-treated and untreated animals in a new set of experiments. In the EAE-control group, a significant increase in the expression of occludin (Fig. 5A) and claudin-4 (Fig. 5B), as well as a decrease in cadherin-7 (Fig. 5C), was observed in the lumbar spinal cord. Unexpectedly, euphol (10 mg/kg), given orally for 25 days, did not inhibit the up-regulated mRNA levels of these tight-junction proteins in the CNS (Fig. 5). Furthermore, the expression of adhesion molecules ICAM-1 (Fig. 6A), LFA-1 (Fig. 6B), VCAM-1 (Fig. 6C) and PECAM-1 (Fig. 6C) was up-regulated by EAE immunization in the control group. However, euphol (10 mg/kg, p.o.) failed to inhibit the increased mRNA levels of ICAM-1 (Fig. 6A), VCAM-1 (Fig. 6C) and PECAM-1 (Fig. 6C) in the spinal cord. Interestingly, euphol (10 mg/kg, p.o.) treatment selectively down-regulated the mRNA levels of LFA-1 in the CNS (Fig. 6B). Taken together, our data suggest that euphol appears to selectively inhibit the differentiation/infiltration of T_H17 myelin-specific cells into the CNS through the adhesion molecule, lymphocyte function-associated antigen 1 (LFA-1).

4. Discussion

Multiple sclerosis is a multiphasic autoimmune CNS disease for which no cure is presently known. Nowadays the most widely used drugs for the treatment of MS include IFN-β1a, IFN-β1b, glatiramer acetate – Copaxone®, mitaxantrone, and more recently monoclonal antibodies such as natalizumab [26] and rituximab [27], and new oral medications such as fingolimod (FTY720) [27], cladribine and laquinimod [27]. However, these therapies are frequently associated with adverse effects to the body, and most importantly, do not effectively change the course of the disease. Thus, alternative and less detrimental drugs are urgently required. In this context, medicinal plants are important sources in the search for new drugs that are potentially anti-inflammatory and control autoimmunity [28–32]. The results reported herein demonstrate the effectiveness of the natural alcohol tetracyclic triterpene euphol in blocking EAE development in C57BL/6 mice. Euphol, given orally, exerted an anti-inflammatory effect by inhibiting the mRNA and protein expression of pro-inflammatory mediators (TNF-α, iNOS and COX-2), as well as by reducing the differentiation and activation of T_H17 myelin-specific cells in both peripheral lymphoid tissue and diseased EAE spinal cord, through

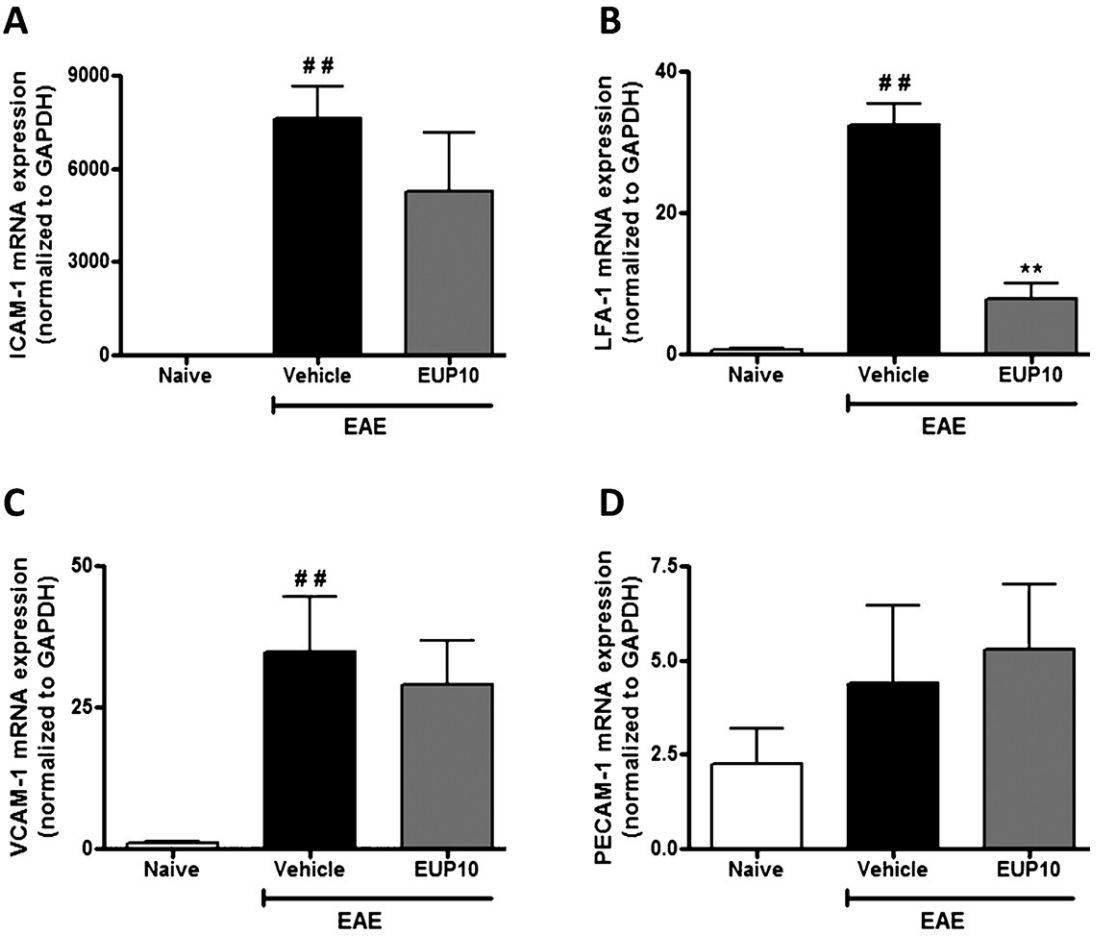

Fig. 6. Euphol selectively blocks lymphocyte adhesion molecule (LFA-1) expression in the CNS of EAE-diseased animals. Active EAE was induced with MOG_{35-55} peptide/CFA and pertussis toxin. The spinal lumbar cords were obtained from the naive group, the control group (EAE) and from mice pre-treated with euphol (10 mg/kg, p.o.) on the 25th day after immunization. The mRNA levels of ICAM-1 (A), LFA-1 (B), VCAM-1 (C) and PECAM-1 (D) were measured by RT-PCR. The housekeeping gene GAPDH mRNA was used to normalize the relative amounts of mRNA. Data are presented as mean ± SEM of 6–9 mice/group and are representative of three independent experiments. $^{\#\#}p < 0.001$ versus the naive group, $^{**}p < 0.001$ versus the EAE group (one-way ANOVA with the Newmann–Keuls post-hoc test).

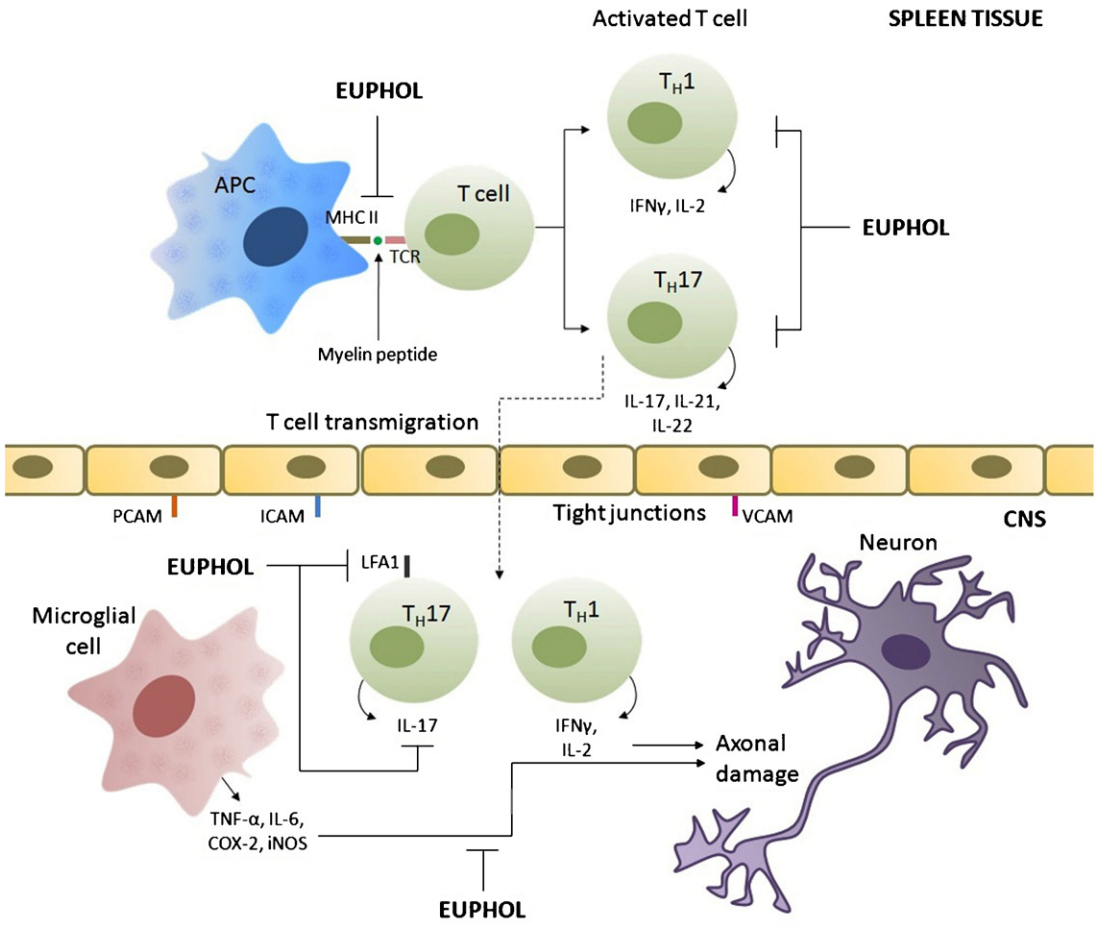

Fig. 7. Schematic representation of the mechanism via which euphol regulates the physiopathology of the EAE model. The oral euphol treatment in the induction phase of EAE reduces the expression of pro-inflammatory mediators (TNF-α, iNOS and COX-2), as well as inhibits the differentiation and activation of T_H17 myelin-specific cells in both peripheral lymphoid tissue and diseased EAE spinal cord, through the modulation of adhesion molecules such as LFA-1, which is mainly expressed in lymphocytes. APC: antigen-presenting cell; MHC II: major histocompatibility complex class II molecules; TCR: T cell receptor; T_H cell: precursor T helper cell; IL: interleukin; TNF-α; tumor necrosis factor-alpha; IFN-γ: interferon-gamma; CNS, central nervous system; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; PECAM, platelet endothelial cell adhesion molecule; LFA-1, lymphocyte function-associated antigen-1; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2. (⊣), inhibition; (→), stimulation.

the modulation of adhesion molecules such as LFA-1, which is mainly expressed in lymphocytes (see proposed scheme in Fig. 7).

These observations are consistent with a recent report that demonstrated that a biopolymeric fraction (BET) from *E. tirucalli* showed dose-dependent anti-arthritic activity in a *M. tuberculosis*-induced adjuvant arthritis test in rats through suppression of $CD4^+$ and $CD8^+$ T cells, mainly of the T_H1 subtype [13]. Moreover, recent data from our group established that preventive and therapeutic oral administration of euphol attenuated both DSS- and TNBS-induced acute colitis in mice. Likewise, euphol treatment also inhibited colon tissue levels and expression of IL-1β, CXCL1/KC, MCP-1, MIP-2, TNF-α and IL-6, while reduced NOS2, VEGF and Ki67 expression in colonic tissue. Importantly, this action was associated with inhibition of activation of nuclear factor-κB (NF-κB). Finally, euphol, at the same schedule of treatment, markedly inhibited both selectin (P- and E-selectin) and integrin (ICAM-1, VCAM-1 and LFA-1) expression in colonic tissue [33].

The pathogenesis of MS/EAE is a complex process, involving autoaggressive T_H1 and T_H17 cells, macrophages, dendritic cells, astrocytes and microglia, as well as a range of inflammatory mediators produced by these cells [3,4,34–38]. In the present study, we used the EAE model of MS to test the potential therapeutic effects of euphol in the treatment of MS. Our data showed that euphol delayed the onset and clearly reduced the clinical severity of EAE *in vivo*. In addition, the same treatment of animals with euphol significantly down-regulated the mRNA and protein expression of relevant pro-inflammatory mediators, namely TNF-α, iNOS and COX-2. Herein, dissociation of euphol-mediated inhibition of expression of these mediators from preventive benefit at lower doses, and with higher dose, suggests that these mediators are not required or not sufficient in mediating euphol-induced preventive action. For this reason, understanding the differential effects on mediator expression and preventive action between lower and higher doses could be mechanistically important and should be further investigated.

As previously reported, the occurrence of high levels of TNF-α is mainly associated with changes in blood brain barrier (BBB) permeability, as well as with the presence of inflammatory mediators and cells infiltrated in CNS tissues both in MS patients [39] and in EAE models [40]. In addition, TNF-α production is associated with the T_H1 response and classically induces activation of a variety of cell types and expression of adhesion molecules, cytokines and chemokines in the CNS, which leads to neuroinflammation [41]. Consistent with high TNF-α level during EAE progression, we also found an up-regulated expression of iNOS and COX-2 in the CNS. iNOS was recently found to be a major contributor to the initiation/exacerbation of inflammatory/degenerative conditions in the CNS through the production of excessive nitric oxide (NO) [42], and NO derived from activated microglia and astrocytes has been implicated in the damage of myelin-producing oligodendrocytes in the EAE model [43–45]. Moreover, COX-2 immunoreactivity has been found in EAE [19], and the

expression of COX-2 is frequently associated with iNOS, suggesting that both enzymes could contribute to the progression of MS, through their ability to produce free radicals such as superoxide anion and NO, which may combine to form the more toxic free radical species peroxynitrite [46]. In fact, another report has shown that colocalization of COX-2 and iNOS may be functionally linked to oligodendroglial excitotoxic death in MS [47]. Interestingly, our data demonstrate that euphol-induced suppression of clinical signs of EAE correlates with the profile of its actions in down-regulating the inflammatory mediators in the CNS.

Until recently, the primary effector T cell in the pathology of both EAE and MS was thought to be a $CD4^+$ T_H1 cell, which requires IL-12 for its differentiation and is characterized by the secretion of IFN-γ, IL-2 and TNF-α [3]. This belief was overturned in part by the finding that increased clinical activity in multiple sclerosis correlated well with the expression of IFN-γ and IL-12 in the CNS [48], and, in fact, that MS was exacerbated by the administration of IFN-γ [49]. However, numerous observations have led several research groups to question this simple paradigm. Most striking were the discoveries that mice deficient in IL-12, IFN-γ or TNF-α develop severe EAE [5]. In contrast, IL-$23^{-/-}$ mice are completely resistant to EAE [50]. IL-23 is crucial for the development of pathogenic T_H17 cells [51], a distinct T cell lineage that is characterized by the production of IL-17A, IL-17F and IL-22 [52]; transfer of T_H17 cells induces more severe EAE compared with the transfer of T_H1 cells. Studies have shown that T_H17 cell differentiation is independent of IL-23 and is induced by transforming growth factor beta (TGF-β) plus IL-6 [53], but that maintenance of pro-inflammatory T_H17 cells requires the presence of IL-23 [54]. In addition, both *in vitro* and *in vivo* differentiation of the T_H17 cell lineage is required in the activation of STAT3, which is activated by IL-6, and subsequently up-regulation of transcription factor ROR-γT [53], which serves as the master switch in the differentiation of T_H17 cells [55–57]. Our *in vitro* and *in vivo* data suggest that euphol significantly reduces IL-17A and ROR-γT expression in both the periphery and in the CNS. Taken together, a less severe inflammatory environment in peripheral lymphoid tissue as well as in the CNS after euphol treatment may prevent significant T_H17 activation and migration into the CNS, thereby delaying disease onset and inducing less severe CNS pathology.

There is an extensive body of knowledge about the molecular trafficking signals involved in the migration of neuroantigen-specific autoaggressive T lymphocytes into the CNS [58–60]. During the course of EAE, autoaggressive $CD4^+$ T lymphocytes are activated outside the CNS and accumulate behind the blood–CNS barrier, where they initiate the cellular events leading to inflammatory tissue destruction [59]. This process involves sequential capture on, rolling along and firm adhesion to the microvascular endothelium, followed by transmigration through the vessel wall and further migration into the extravascular tissue [61]. Moreover, all steps in the recruitment cascade are orchestrated by cell adhesion molecules (CAMs) on both leukocytes and endothelial cells, and different subsets of CAMs are responsible for the different steps in extravasation, such as tight-junction proteins, selectins and integrins [62]. Integrins are expressed constitutively on leukocytes and many other cell types, and are activated rapidly from a low-affinity to a high-affinity state following cell activation and ligand binding [63]. In leukocyte recruitment, interactions between the β_2 integrins $\alpha_L\beta_2$ (CD11a/CD18, also known as lymphocyte function antigen 1 (LFA-1)) or $\alpha_M\beta_2$ (CD11b/CD18, also known as Mac-1) and the intercellular adhesion molecules ICAM-1 and ICAM-2 are important in firmly anchoring the leukocytes to the endothelium [64]. Recent reports suggest that β_2-integrin and their ligands are critical for autoaggressive lymphocyte trafficking into the brain and spinal cord during the development and progression of MS and the EAE model [58]. For instance, antibodies to the α-chains of LFA-1 and Mac-1 provide protection from disease, primarily by attenuating disease severity rather than completely blocking disease development [65–67]. In this context, to gain further insight into the mechanisms through which euphol modulates cell migration and improves the clinical symptoms induced by EAE in C57BL/6 mice, we assessed the levels of chemotactic factors and the expression of adhesion molecules after induction of EAE. Our results demonstrated that euphol selectively suppressed the up-regulation of LFA-1 into the CNS when assessed 25 days post-immunization, and suggest that euphol appears to selectively inhibit the differentiation/infiltration of T_H17 myelin-specific cells into the CNS through the modulation of LFA-1.

Together these findings provide new insights into the neuroprotective actions of euphol, a natural alcohol-derived tetracyclic triterpene. However, the precise mechanisms responsible for such actions and the major signaling molecules involved remain unclear and will need to be determined. In this context, it is interesting to note that the immunomodulatory effect of euphol is similar to the effects demonstrated by cannabinoid (CB) agonists, especially agonists selective for the CB_2 cannabinoid receptor [68–71]. Furthermore, euphol has been found to inhibit monoacylglycerol lipase (MGL) activity with high potency (IC_{50} = 315 nM), MGL being a serine hydrolase that is involved in the biological deactivation of the endocannabinoid 2-arachidonoylglycerol (2-AG) [72]. In addition, recent published data from our group demonstrated that euphol binds with high affinity to CB_2 and, to a lesser extent, CB_1 receptors [73]. Therefore further experiments are required to confirm whether or not euphol modulates the development of EAE through interaction with endogenous CB ligands or with its receptors.

In summary, the natural alcohol derivative tetracyclic triterpene euphol significantly prevented the development of neurological signs of EAE by either mediating inhibition of the T_H17 pathway, which would reduce pro-inflammatory mediators and ameliorate disease activity, or by direct down-regulation of adhesion molecules such as LFA-1, resulting in a corresponding decrease in the migration of autoaggressive T cells to the CNS, which may in turn directly reduce IL-17 expression and polarization (Fig. 7). Therefore, our data suggest that euphol might constitute an attractive therapeutic molecule for the treatment of MS, as well as other T_H17 autoimmune diseases.

Conflict of interest

We declare that Rafael C. Dutra, Paula R.C. Souza, Allisson F. Bento, Rodrigo Marcon and Maíra A. Bicca have no conflict of interest. Luiz F. Pianowski is the proprietor of the contract research organization (CRO) Pianowski & Pianowski Ltda. He has received consulting fees from Amazônia Fitomedicamentos and was responsible for the chemical characterization of euphol and for providing it for this study. Luiz F. Pianowski and João B. Calixto are assigned as inventors in the patent on the analgesic action of euphol. However, this does not alter our adherence to all the Biochemical Pharmacology policies on sharing data and materials.

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