ORIGINAL ARTICLE

Palmitoylethanolamide improves colon inflammation through an enteric glia/toll like receptor 4-dependent PPAR-α activation

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ABSTRACT

Objective Enteric glia activation has been reported to amplify intestinal inflammation via the enteroglial-specific S100B protein. This neurotrophin promotes macrophage recruitment in the mucosa, amplify colonic inflammation and interacts with toll-like receptors (TLR). Molecules inhibiting S100B-driven enteric activation might mitigate the course of ulcerative colitis (UC). This study aims to investigate the effects of palmitoylethanolammide (PEA), a drug able to counteract astroglial activation in the central nervous system, on intestinal inflammation, in humans and mice.

Design Mouse models of dextran sodium sulphate (DSS)-induced colitis, colonic biopsies deriving from UC patients and primary cultures of mouse and human enteric glial cells (EGC), have been used to assess the effects of PEA, alone or in the presence of specific PPARα or PPARγ antagonists, on: macroscopic signs of UC (DAI score, colon length, spleen weight, macrophages/neutrophils infiltration); the expression and release of proinflammatory markers typical of UC; TLR pathway in EGCs.

Results PEA treatment improves all macroscopic signs of UC and decreases the expression and release of all the proinflammatory markers tested. PEA anti-inflammatory effects are mediated by the selective targeting of the S100B/TLR4 axis on ECG, causing a downstream inhibition of nuclear factor kappa B (NF-kB)-dependent inflammation. Antagonists at PPARα, but not PPARγ, abolished PEA effects, in mice and in humans.

Conclusions Because of its lack of toxicity, its ability in reducing inflammation and its selective PPARα action, PEA might be an innovative molecule to broaden pharmacological strategies against UC.

Significance of this study

What is already known about this subject?

- Enteric glial cells actively participate in regulating intestinal pathophysiology.
- Enteric glial cells overexpress S100B and are activated during ulcerative colitis.
- PEA is able to counteract astroglial activation in a model of Alzheimer disease.

What are the new findings?

- The expression of TLR4/S100B proteins, together with p38/p-ERK/pJNK-pathway signalling molecules and NF-κB expression, are upregulated in EGCs deriving from DSS-treated mice and UC patients.
- PEA is able to counteract enteroglial activation, to inhibit macrophages and neutrophils infiltration in colonic mucosa and to downregulate the expression and release of all the proinflammatory markers typical of UC, in mice and humans.
- PEA anti-inflammatory effects are mediated mainly by the selective targeting of PPARα site in gut mucosa.

How might it impact on clinical practice in the foreseeable future?

- This study, by in vitro, ex vivo and in vivo analysis, adds further data on the role of EGCs in intestinal pathophysiology. Additionally, since PEA can counteract inflammatory signals in mice and humans, it might represent a novel and potential pharmacological tool to modulate inflammation during ulcerative colitis.

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INTRODUCTION

Massive and persistent mucosal infiltration of macrophages and neutrophils in the large intestine,[1] together with the release of cytokines, interleukins and proinflammatory signalling molecules by immune cells, represent the most evident features of ulcerative colitis (UC).[2] Recently, other cell types have been reported to substantially contribute to the onset/progression of UC and of other inflammatory conditions.[3] Enteric glial cells (EGCs) exert a key role in the maintenance of gut homeostasis cooperating with surrounding cells. Specifically, EGCs assure the correct trophism of neurones in the enteric nervous system (ENS),[4] protect enteric neurones from oxidative stress,[5] control epithelial barrier functions by reducing epithelial permeability and actively participate in the course of intestinal inflammation acting as the first defensive line of the ENS.[6–8]

In pathological conditions, EGCs activity is profoundly altered and, following injury and inflammation, these cells are activated and undergo a dynamic process associated with increased proliferation and a proinflammatory phenotype.[9] Enteroglial activation is characterised by the over-release of neurotrophins, growth factors and cytokines that, in turn, recruit infiltrating immune cells

such as macrophages, neutrophils and mast cells in the colonic mucosa.[7 10 11] Abnormalities in the enteroglial network were described in patients with inflammatory bowel diseases (IBD), where inflammatory processes induce a massive overexpression and secretion of the S100B protein from EGCs.[12]

S100B protein, a Ca^{+2}/Zn^{+2}-binding protein specifically expressed by EGCs in the gastrointestinal tract,[13] emerges as a pivotal signalling molecule that participates in the onset and progression of the inflammatory status, as it orchestrates a wide range of signal activation pathways, directly correlated with the severity of gut degenerative processes.[12 14] EGCs-derived S100B protein regulates nitric oxide (NO) production in intestinal mucosa of patients with UC through the interaction with the receptor for advanced glycation endproducts (RAGE).[6 13] Recent evidences indicate that RAGE is also involved in the enteroglial toll-like receptors (TLR) signalling pathway.[15] Taken together, these studies suggest that S100B overproduction may regulate the expression of different members of the TLR family in EGCs. Molecules able to counteract intestinal inflammation targeting EGCs and S100B signalling could represent a novel approach to strengthen current pharmacological strategies to treat gut inflammatory diseases, including UC.

Along this line, palmitoylethanolamide (PEA) is an endogenous N-acylethanolamide that is thought to be involved in protective mechanisms activated as a result of the stimulation of the anti-inflammatory response.[16] PEA belongs to a group of Autacoid Local Inflammation Antagonism amides (ALIAmides) involved in many pathophysiological processes, including pain, convulsion, neurotoxicity and inflammation.[17–19] Anti-inflammatory effects of PEA depend upon its capability to activate peroxisome-proliferator-activated receptor-α (PPARα),[20] a member of nuclear hormone receptor superfamily of ligand-activated transcription factors. PEA-mediated activation of PPARα decreases NO production, neutrophil influx and the expression of proinflammatory proteins, such as inducible NO synthase (iNOS), cyclo-oxygenase-2 (COX2) and tumour necrosis factor-α (TNFα).[21–24] To date, whether PEA is able to counteract enteroglial activation occurring during UC remains uninvestigated.

The present study aimed to evaluate the beneficial effect of PEA administration in (1) mouse models of dextran sulphate sodium (DSS)-induced colitis (2) mucosal biopsies from patients with UC; (3) primary cultures of EGCs derived from mice and humans. Specifically, we aim to evaluate: (1) the protective and anti-inflammatory effect of PEA by testing its ability to modulate enteroglial-derived S100B protein expression; (2) the efficacy of PEA to prevent S100B-mediated amplification of inflammatory responses through the involvement of the TLR pathways and (3) the role of PPARα as the specific receptor responsible for PEA action.

MATERIALS AND METHODS

Animals and experimental design

Six-weeks-old wild-type (WT) male CD-1 mice (Harlan Laboratories, Udine, Italy) or PPARα null (KO) mice (Taconic, Germantown, New York, USA) were used for experiments. All procedures were approved by La Sapienza University's Ethics Committee. Animal care was in compliance with the IASP and European Community (EC L358/1 18/12/86) guidelines on the use and protection of animals in experimental research. Animals were randomly divided into the following groups (n=10 each): non-colitic control group; colitic group; colitic groups receiving daily PEA 2, 10 or 50 mg/kg, respectively[25 26]; colitic group receiving daily PEA (10 mg/kg) and selective PPARα antagonist MK866 (10 mg/kg); colitic group receiving daily PEA and selective PPARγ antagonist GW9662 (1 mg/kg); internal control colitic group receiving daily PPARα or PPARγ antagonist, respectively[27]; non-colitic group receiving daily PEA 10 mg/kg (as drug internal control). Colitis was induced by administrating 4% DSS (MP Biomedicals, Solon, Ohio, USA) in drinking water for six consecutive days, as described in figure 1A. PEA alone, or combined with PPAR antagonists, was given through intraperitoneal administration from day 2 to day 6. At day 7, animals were sacrificed, spleens were isolated to measure their weight and colons were isolated to measure their length and to perform macroscopic, histochemical and biochemical analyses as described below.

Disease activity index (DAI) in mice

The DAI scale is based on the evaluation of different parameters characterising experimental colitis induction and progression. Body weight, presence of gross blood in the faeces and stool consistency were recorded daily (from day 0 to 7) by an observer blinded to the treatment. According to Cooper *et al*,[28] the DAI was determined by scoring changes in: weight loss (0=none; 1=1 to 5%; 2=5 to 10%; 3=10 to 20%; 4=>20%); stool consistency (0=normal; 2=loose; 4=diarrhoea) and rectal bleeding (0=normal; 2=occult bleeding; 4=gross bleeding).

Whole-mount-cultured human intestinal biopsies

The experimental group comprised 8 patients diagnosed with UC (5 women; mean age 47 years) and eight control undergoing colonoscopy for colon cancer screening, (2 women; mean age 50 years). All persons received and signed an informed consent. All procedures were approved by the ethical committee of the University of Naples Federico II. In all individuals, four mucosal biopsies from the rectosigmoid region were collected and cultured in FBS-supplemented Dulbecco Modified Eagle's Medium (DMEM) at 37°C in 5% CO_2/95% air.[7] All biopsies were cultured, for 24 h, with or without PEA at the following concentrations 0.001, 0.01, 0.1 or 1 μM. Biopsies were then homogenised and analysed by western blot as described below. In the same experimental conditions, some samples were fixed in PFA and used for immunohistochemical analysis.

Protein extraction and western blot analysis

Mouse colonic tissues and human biopsies were homogenised in ice-cold hypotonic lysis buffer to obtain cytosolic extracts. To test the nuclear translocation of p50 and p60 subunits, as markers of NF-κB activation, we used nuclear extracts prepared according to a method previously published by our group.[15] Extracts underwent electrophoresis through a polyacrilamide minigel. Proteins were transferred onto nitrocellulose membrane that were saturated with non-fat dry milk and then incubated with either mouse anti-S100B (Neo-Marker, Milan, Italy), mouse anti-iNOS, mouse anti-CD283 (TLR-3), rabbit anti-COX2 (all BD Biosciences, Milan, Italy), rabbit anti-GFAP, rabbit anti-TLR4, rabbit anti-PPARα (all Abcam, Cambridge, UK), mouse anti-NF-κBp50 (p50), mouse anti- NF-κBp65 (p65), mouse anti-pERK, rabbit anti-TLR2, rabbit anti-TLR7, mouse anti pJNK, mouse anti-β-actin (all Santa Cruz Biotechnology, Santa Cruz, California, USA). Membranes were then incubated with the specific secondary antibodies conjugated to horseradish peroxidase (HRP) (Dako, Milan, Italy). Immune complexes were revealed by enhanced chemiluminescence detection reagents (Amersham Biosciences, Milan, Italy). Blots were analysed by scanning densitometry (GS-700 imaging densitometer; Bio-Rad). Results were expressed as OD (arbitrary

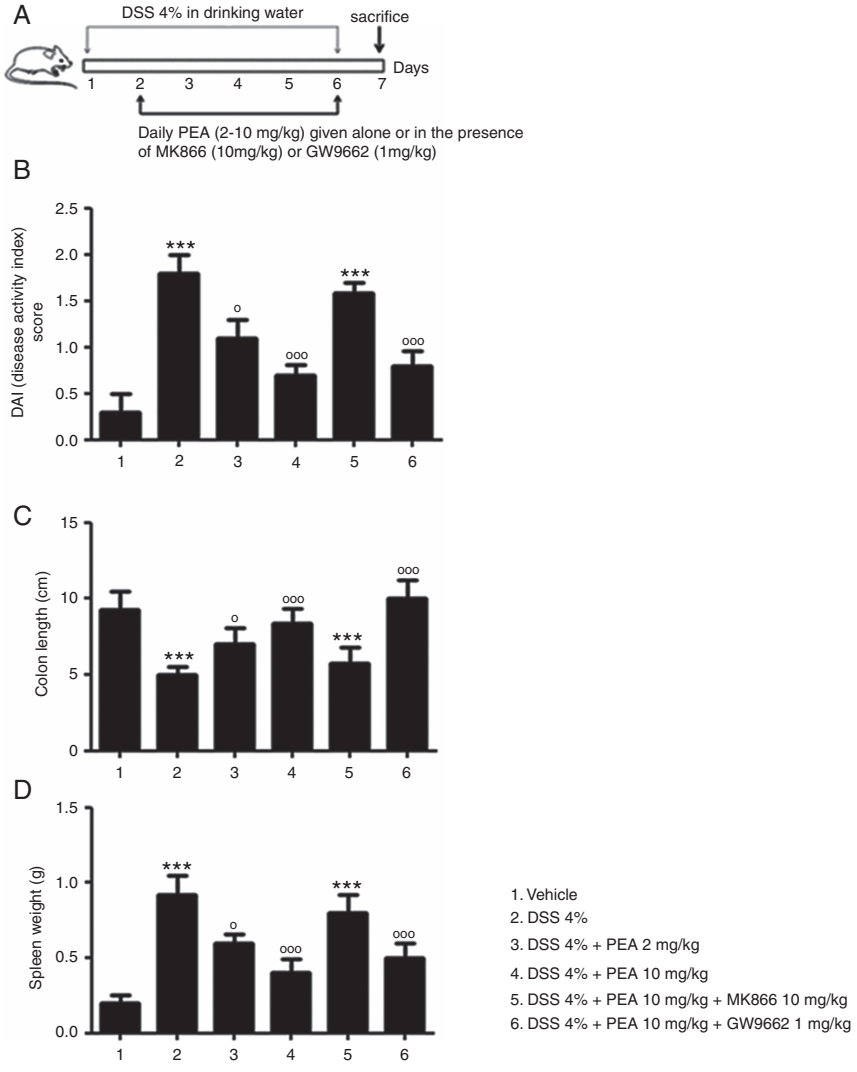

Figure 1 Experimental plan and positive effects of palmitoylethanolammide (PEA) on macroscopic signs of inflammation in mice. (A) Timetable scheme showing dextran sodium sulphate (DSS) and PEA administration in CD-1 mice: DSS-exposed (4%) mice were treated daily with 2 mg/kg or 10 mg/kg PEA given intraperitoneally alone or in the presence of MK866 (10 mg/kg) or GW9662 (1 mg/kg), (respectively, PPARα and PPARγ antagonists). (B) Effect of PEA treatment in DDS exposed mice on DAI score, (C) on colonic length and (D) on spleen weight. Results are expressed as mean ±SEM of n=5 experiments. ***p<0.001 versus vehicle (saline); °p<0.05; °°°p<0.001 versus DSS-treated mice.

units; mm^2) and normalised on the expression of the housekeeping protein β-actin.

Preparation of blood samples from mice

Before being sacrificed, mice were deeply anaesthetised and the blood was drawn by cardiac puncture and collected in 5% EDTA vials. To determine NO, prostaglandin E2 (PGE2), interleukin-1β (IL-1β), TNFα, and S100B levels, plasma was isolated from the blood, immediately frozen, and stored at −80°C until the assays.

NO quantification

NO was measured as nitrite (NO_2^-) accumulation in plasma of mice and in human biopsies supernatants by a spectrophotometer assay based on the Griess reaction.[29] Briefly, Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine in H_3PO_4) was added to an equal volume of plasma or supernatant and the absorbance was measured at 550 nm. Nitrite concentration (nM) was thus determined using a standard curve of $NaNO_2$.

Enzyme-linked immunosorbent assay for PGE2, IL-1β, TNFα and S100B

Enzyme-linked immunosorbent assay (ELISA) for PGE2, IL-1β, TNFα (R&D Systems, Minneapolis, Minnesota, USA) and S100B (Biovendor R&D, Brno, Czech Republic) was carried out on plasma of mice and on human biopsies supernatants according to the manufacturer's protocol. Absorbance was measured on a microtitre plate reader. PGE2, IL-1β, TNFα and S100B levels were determined using standard curves method.

Determination of macrophages infiltration in the colonic mucosa of mice and humans

Samples for immunohistochemical assessment of macrophages were isolated from mouse distal colon and human colonic surgical specimens. Tissues were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, sectioned in 15 μm slices and processed for immunohistochemistry. Slices were pretreated using heat-mediated antigen retrieval with a sodium citrate buffer, incubated with MAC387 (Abcam) at RT,[30] and detected using HRP-conjugated compact polymer system. 3,3′-Diaminobenzidine (DAB) was used as the chromogen. Slices were then counterstained with haematoxylin, mounted with Eukitt and analysed with a microscope (Nikon Eclipse 80i by Nikon Instruments Europe, Amstelveen, The Netherlands). Images were captured by a high-resolution digital camera (Nikon Digital Sight DS-U1).

Myeloperoxidase assay

MPO (MPO), a marker of polymorphonuclear leukocyte accumulation and general inflammation occurring in colonic tissues, was determined as previously described.[31] After removal, colonic tissues from mice and humans were rinsed with a cold saline solution, opened and deprived of the mucosa using a

glass slide. The resulting layer was then homogenised in a solution containing 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich) dissolved in 10 mM potassium phosphate buffer and centrifuged for 30 min at 20 000×g at 37°C. An aliquot of the supernatant was mixed with a solution of tetramethylbenzidine (1.6 mM; Sigma-Aldrich) and 0.1 mM hydrogen peroxide (Sigma-Aldrich). The absorbance was then spectrophotometrically measured at 650 nm. MPO activity was determined as the amount of enzyme degrading 1 mmol/min of peroxide at 37°C and was expressed in milliunits per 100 mg of wet tissue weight.

Enteric glia isolation from colonic tissues of mice and humans

Human colonic sample tissues were obtained from patients undergoing surgery. The non-UC group (control group) comprised 7 adult patients (3 women; mean age 66 years; range: 43–82) undergoing surgery for left colon carcinoma and colonic polyps (3 and 4 patients, respectively). All the resected tissues were macroscopically identified as normal segments from uninvolved resection margins.

The UC group was represented by surgical specimens of the sigmoid colon from patients with refractory UC, obtained from 9 patients (5 women; mean age 48 years; range: 17–62). Surgical specimens (stripes of at least 1×1 cm) were processed for the isolation of EGCs according the procedure described by Cirillo *et al*.[13] The resulting EGC-enriched cultures (500×10^3 cells/mL) were treated with PEA and/or PPARα or PPARγ antagonists, for 24 h.

In parallel, using the above described protocol, in another set of experiments, EGCs were isolated from the left colons of DSS-treated (in the presence or absence of PEA and PPAR antagonists) and control mice. Human and mouse EGCs were used for immunofluorescence and immunoblot analysis.

PPARα silencing in human and mouse EGCs

EGCs were silenced using the RNAifect kit (Qiagen, Valencia, California, USA), according to the manufacturer's instructions. Briefly, cells were seeded at 1.6×10^5 cells/well in a six-wells plate. After 24 h, cells were treated with control siRNA (150 nM) or PPARα siRNA (50 or 150 nM; Dharmacon, Lafayette, Colorado, USA) plus RNAiFect Transfection Reagent (Quiagen). To evaluate the effectiveness of the silencing, 96 h after transfection, total RNA was isolated using the Tri-Reagent kit (Molecular Res., Cincinnati, Ohio, USA) and the amount of PPARα RNA determined by QuantiGene assay (Panomics, Freemont, California, USA), according to the manufacturer's protocol. PPARα expression was evaluated, in non-silenced and silenced EGCs, by immunofluorescence analysis and by western blotting, using a specific rabbit anti-PPARα antibody (Abcam). Moreover, PPARα-silenced EGCs were treated with various concentrations of PEA, as previously described and in presence or absence of exogenous S100B (5 μM, Abcam). In these cells, TLR4, S100B, iNOS and COX2 expression and TNFα, PGE2, NO and S100B release was assessed as previously described. S100B and TLR4 expression was also evaluated by immunofluorescence, as described below.

Immunofluorescence analysis of S100B and TLR4 expression in human and mouse EGCs

EGCs derived from control and treated mice, as well as EGCs isolated from UC and non-UC patients (treated as previously indicated), were blocked with bovine serum albumin and subsequently stained with rabbit anti-S100B antibody (Santa Cruz Biotechnology) and mouse anti-TLR4 (AbCam). Nuclei were stained with Hoechst. Negative controls were carried out by omitting the primary antibodies. To test any non-specific antigen-binding sites, additional experiments were performed using specific isotype antibody controls (Abcam), at the same concentration as the primary antibodies. Cells were then incubated in the dark with the proper secondary antibody: fluorescein isothiocyanate-conjugated anti-rabbit (Abcam) or Texas Red-conjugated anti-mouse (Abcam), respectively. Cells were analysed with a microscope (Nikon Eclipse 80i), and images were captured by a high-resolution digital camera (Nikon Digital Sight DS-U1).

Measurement of PEA levels in human and mouse tissues

Description of the measurement of PEA levels in human and mouse tissues is reported in the online supplementary data (available online only).

Statistical analysis

Results were expressed as mean±SEM of *n* experiments. Statistical analysis were performed using parametric one-way analysis of variance (ANOVA) and multiple comparisons were performed by Bonferroni's posthoc test; p values <0.05 were considered significant.

RESULTS

PEA, dose-dependently, ameliorates colitis in wild-type mice

PEA treatment significantly ameliorates DAI score, preserves colonic length and reduces splenomegaly induced by DSS, in a dose-dependent fashion.

Starting from day 4 after DSS administration, DAI score was significantly increased in DSS groups ($p<0.01$ vs control), together with a consistent increase in bloody diarrhoea, a significant loss of body weight and a significant shortening of colon and a marked increase of spleen weight (figure 1).

PEA treatment significantly inhibited the increase in DAI score in a dose-dependent fashion ($p<0.05$ for PEA 2 mg/kg and $p<0.001$ for PEA 10 mg/kg vs DSS-treated mice; figure 1B), suggesting an overall improvement of intestinal symptoms associated with DSS-induced colitis; such effect was accompanied by a reduction of all parameters used to define the DAI score (see Materials and Methods; figure 1B). PEA was able to preserve colonic length and to prevent splenomegaly in DSS-treated mice (figure 1C and D). Ameliorative effects exerted by PEA are the result of a PPARα activation. Indeed, when PEA was coadministered with the selective PPARα antagonist MK866, its ameliorative effect was almost completely abolished (figure 1B, C and D). Conversely, PEA efficacy was unaffected in the presence of the selective PPARγ antagonist GW9662. MK886 and GW9962 given alone, in the absence of PEA, did not show any of the described effects on colitic groups (data not shown).

PEA decreases enteric activation and inflammatory markers expression and release in DSS-treated mice and in human UC samples

The administration of DSS in mice caused a significant increase of the iNOS, COX2, S100B and GFAP protein expression, compared with control (vehicle; $p<0.001$; figure 2A and B); similarly iNOS, COX2, S100B and GFAP protein expression was significantly upregulated in UC-derived versus non-colitic biopsies ($p<0.001$; figure 2C and D).

PEA treatment resulted in a significant dose-dependent decrease of iNOS, COX-2, GFAP and S100B protein overexpression, in

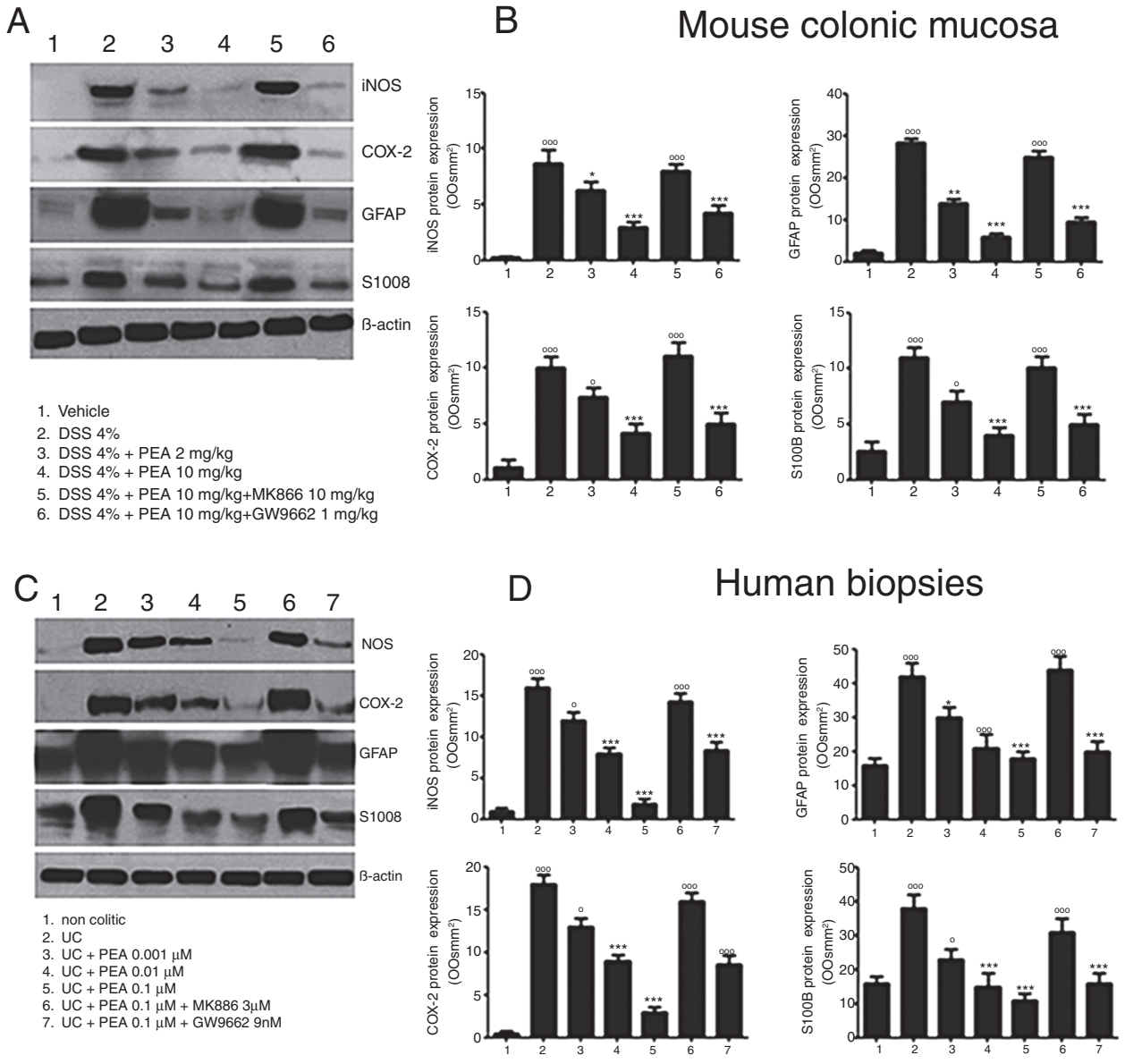

Figure 2 Palmitoylethanolammide (PEA) dose-dependently decreases the expression of inflammatory mediators in mice and humans. (A) Western blot analysis and (B) densitometric analysis (arbitrary units normalised on the expression of the housekeeping protein β-actin) showing the effects of PEA (2 mg/kg or 10 mg/kg), given alone or in the presence of MK866 (10 mg/kg) or GW9662 (1 mg/kg), on iNOS, COX-2, GFAP and S100B protein expression in colonic tissue of DSS-treated mice. Results are expressed as mean±SEM of n=5 experiments performed in triplicate. *** p<0.001 versus vehicle (saline); °p<0.05 and °°°p<0.001 versus DSS-treated mice. (C) Western blot analysis and (D) densitometric analysis (arbitrary units normalised on the expression of the housekeeping protein β-actin) showing the effect of PEA (0.001 μM, 0.01 μM and 0.1 μM) on iNOS, COX2, GFAP and S100B protein expression in human UC-cultured biopsies in the presence or absence of MK866 3 μM or GW9662 9 nM. Results are expressed as mean±SEM of n=5 experiments performed in triplicate. *** p<0.001 versus Non-colitic (control); °p<0.05 and °°°p<0.001 versus UC patients.

mice colonic homogenates ($p<0.05$ for PEA 2 mg/kg and $p<0.001$ for PEA 10 mg/kg vs DSS-mice; figure 2A and B) and human samples ($p<0.05$ for PEA 0.001 μM and $p<0.001$ for PEA 0.01 μM and 0.1 μM vs UC samples; figure 2C and D).

Griess reaction and ELISA assays showed a significant increase of NO, PGE2, IL-1β, TNFα and S100B release in plasma of DSS-treated mice ($p<0.001$ vs vehicle; figure 3A) and in supernatants of cultured bioptic samples derived from UC patients ($p<0.001$ vs non-colitic; figure 3B). Also in this case, PEA treatment caused a significant and dose-dependent decrease of all the inflammatory mediators (figure 3A and B).

PEA inhibitory effects on the expression and release of inflammatory mediators typical of UC depended upon the involvement of PPARα. Indeed, the coadministration of PEA with the selective PPARα antagonist MK866 did not result in a significant decrease of inflammatory mediators, in DSS-treated mice versus control (figure 3A) and in UC-derived biopsies versus non-colitic biopsies (figure 3B). Conversely, PEA coadministered with the selective PPARγ antagonist GW9662, continued to exert anti-inflammatory effects in DSS-mice and in UC samples (figure 3A and B), confirming that by blocking PPARα receptors, PEA-mediated effects are abolished.

PEA reduces macrophage and neutrophil infiltration in experimental mouse colitis and in human UC samples

As expected, compared with control mice (figure 4A panel1), colonic mucosa of DSS-treated mice was extensively infiltrated by macrophages (figure 4A panel 2); also UC-colonic samples

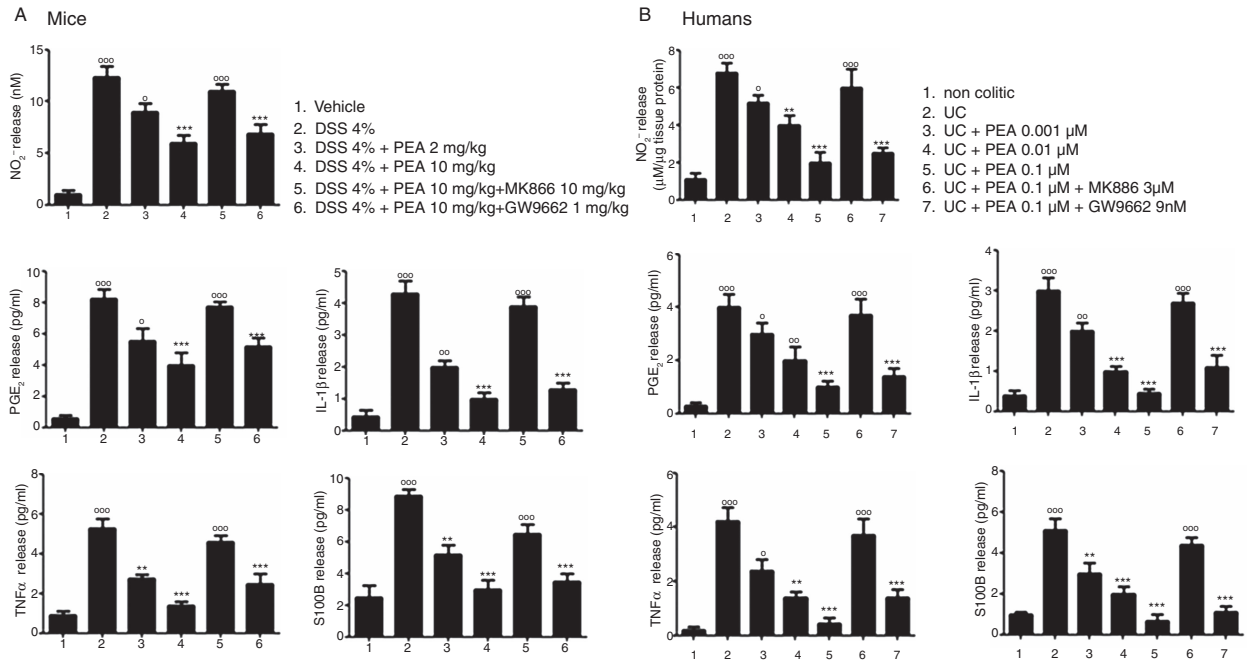

Figure 3 Palmitoylethanolammide (PEA) dose-dependently decreases the release of inflammatory mediators in mice and humans (A) Effect of PEA (2 mg/kg or 10 mg/kg), given alone or in the presence of MK866 (10 mg/kg) or GW9662 (1 mg/kg), on the release of NO_2^-, PGE2, IL-1β, TNFα and S100B in plasma of DSS-treated mice. Results are expressed as mean±SEM of n=5 experiments performed in triplicate. ***p<0.001 versus vehicle (saline); ° p<0.05 °°p<0.01 and °°°p<0.001 versus DSS-treated mice. (B) effect of PEA (0.001 μM, 0.01 μM and 0.1 μM) on the release of NO_2^-, PGE2, IL-1β, TNFα and S100B in the supernatant of whole-mount UC-cultured biopsies in the presence or absence of MK866 3 μM or GW9662 9 nM; ***p<0.001 versus Non-colitic (control); ° p<0.05; °°p<0.01 and °°°p<0.001 versus UC patients.

showed a broad infiltration of macrophages (figure 4B panel 2) compared with control samples (figure 4B panel 1). Increased macrophage infiltration was accompanied by a marked neutrophil infiltration, as shown by the enhanced MPO activity in DSS-treated mice (p<0.001 vs vehicle; figure 4C) and in UC biopsies (p<0.001 vs non-colitic samples; figure 4F). In mice, PEA treatments caused a significant and dose-dependent inhibition of macrophage infiltration (p<0.05 for PEA 2 mg/kg and p<0.001 for PEA 10 mg/kg vs DSS-mice; figure 4B and A panels 3 and 4) and MPO activity (p<0.05 for PEA 2 mg/kg and p<0.001 for PEA 10 mg/kg vs DSS-mice; figure 4C), indicating that PEA is able to control immune cells infiltration in mouse colonic tissues.

Similar results were obtained in human biopsies, where PEA treatment induced a significant overall reduction of MAC387-positive macrophages infiltration (p<0.05 for PEA 0.01 μM and p<0.001 for PEA 0.1 μM vs non-colitic; figure 4E and D panels 3 and 4) and MPO accumulation (p<0.05 for PEA 0.01 μM and p<0.001 for PEA 0.1 μM vs non-colitic; figure 4F). Additionally, and according to the previous results, we confirmed that PPARα antagonists, but not PPARγ antagonists, abolished PEA effects, in mice (figure 4A panels 5 and 6, 4B and 4C) and in human colonic inflamed tissues (figure 4D panels 5 and 6, 4C; 4E and 4F).

TLR2, TLR3 and TLR7 expression was unaffected by PEA in mouse and human tissues

Data on TLR2, TLR3 and TLR7 expression are reported in online supplementary material (available online only).

PEA decreases enteric glial response trough TLR4/S100B inhibition in mouse and human EGCs, via PPARα activation

Immunofluorescence analysis revealed a significant increase in TLR4 and S100B expression in EGCs isolated from DSS-treated mice (p<0.001 vs vehicle; figure 5A and B) and from UC patients (figure 5C and D). PEA administration was able to significantly reduce TLR4 and S100B expression in mouse EGCs (p<0.01 for PEA 2 mg/kg and p<0.001 for PEA 10 mg/kg vs DSS-mice; figure 5A and B) and human EGCs (p<0.05 for PEA 0.01 μM and p<0.001 for PEA 0.1 μM and vs non-colitic EGCs; figure 5C and D). Again, PEA effects were selectively inhibited by coadministration of MK886, but not by GW9662 (figure 5), further demonstrating the involvement of PPARα as a key factor in determining PEA effects.

Interestingly, stimulation of human and mouse EGCs with exogenous S100B (5 μM), induced a significant increase in the expression and release of proinflammatory markers (NO, TNFα and PGE2), that was dose-dependently inhibited by PEA (figures 6F and 7G; all p<0.001 vs vehicle).

In the colonic mucosa of UC patients and DSS-treated mice, PPARα expression was significantly increased compared with control conditions (p<0.05; figure 6A), and to further determine whether PPARα was directly responsible for PEA pharmacological activity, we performed PPARα-silencing in human EGCs. Effectiveness of PPARα silencing is shown in figure 6B, where immunofluorescence and western blot analysis revealed that PPARα expression is decreased in silenced EGCs compared with non-silenced cells.

As compared with PPARα expressing cells, TLR4 and S100B expression was increased in PPARα-silenced EGCs deriving from UC patients (versus non-colitic patients), and it was unaffected by treatment with PEA (p=NS vs colitic; figure 6C and D). Moreover, in PPARα-silenced EGCs, compared to non-silenced cells, the expression of iNOS and COX2 proteins and the release of TNFα, PGE2, S100B and NO were unaffected by PEA (p=NS vs colitic; figure 6C). Most interestingly, in PPARα-silenced EGCs, differently from non-silenced EGCs,

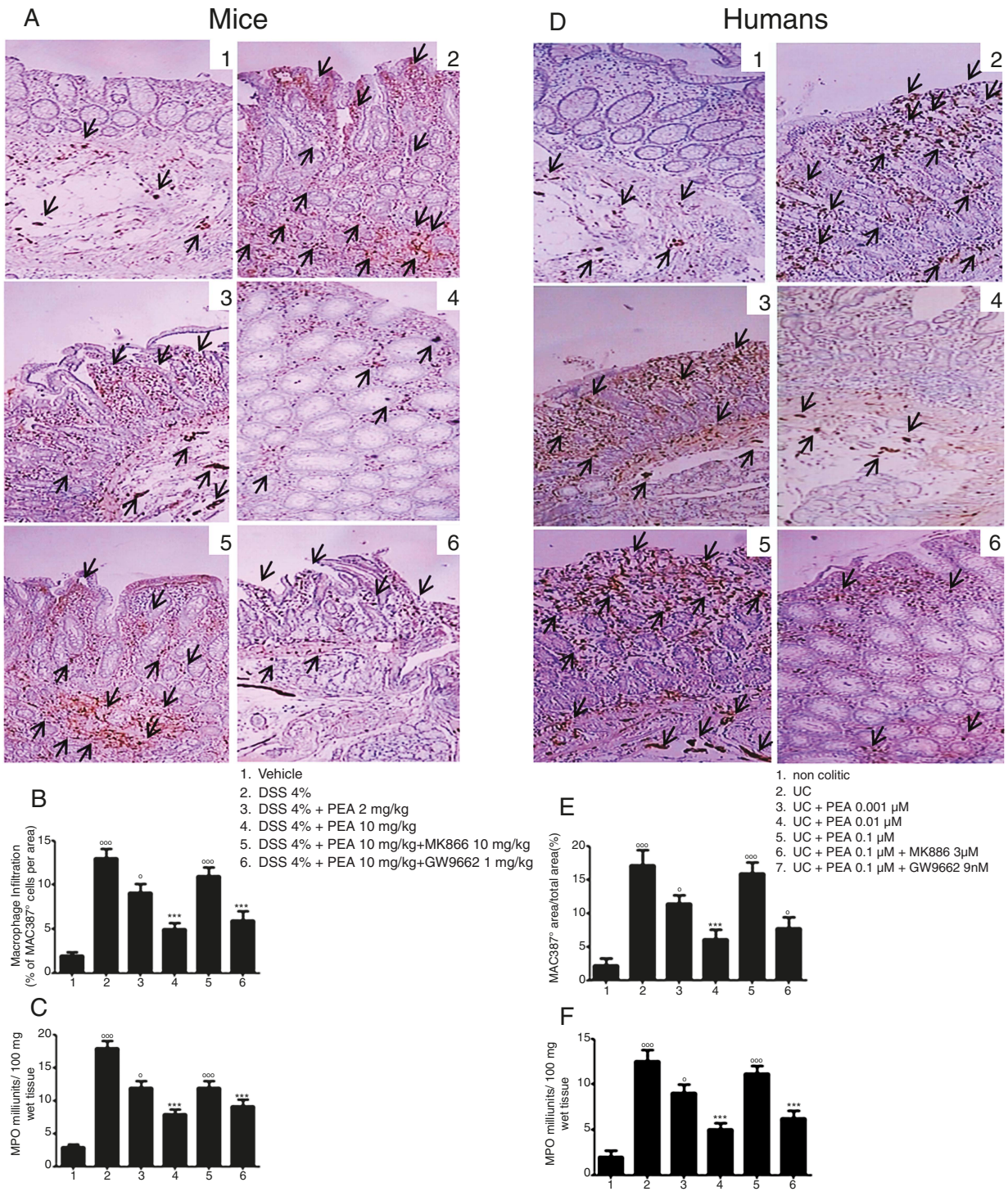

Figure 4 Palmitoylethanolammide (PEA) reduces immune cells infiltration in mice and human colonic criptae through PPARα-dependent involvement. (A) Immunohistochemical images showing untreated (vehicle) mice colonic mucosa (panel 1), DSS-treated mice colonic mucosa (panel 2), and DSS-treated mice colonic mucosa in the presence of: PEA 2 mg/kg (panel 3), PEA 10 mg/kg (panel 4), PEA 10 mg/kg plus MK866 10 mg/kg (panel 5), and PEA 10 mg/kg plus GW9662 1 mg/kg (panel 6); arrows indicate MAC387 (macrophages infiltration marker) immunopositive colon criptae; magnification 10X. (B) Quantification of the effects of PEA on MAC387 immunopositive cells in mice colon criptae; data shows the number of MAC387 immunopositive cells per area unit and are expressed as mean±SEM of n=3 experiments. (C) Quantification of the effects of PEA on myeloperoxidase (MPO) levels, as a marker of neutrophils infiltration, in untreated and DSS-treated mice colonic tissues. Results are expressed as mean ±SEM of n=6 experiments. ***p<0.001 versus vehicle (saline); °p<0.05 and °°°p<0.001 versus DSS-treated mice. (D) Immunohistochemical images showing non-colitic human colonic mucosa (panel 1) and untreated colonic mucosa derived from UC (panel 2) or UC-derived colonic mucosa treated with: PEA 0.01 μM (panel 3), PEA 0.1 μM (panel 4), PEA 0.1 μM plus MK866 3 μM (panel 5) and PEA 0.1 μM plus GW9662 9 nM (panel 6); arrows indicate MAC387 (macrophages infiltration marker) immunopositive colon criptae; magnification 10X. (E) Quantification of MAC387 immunopositive macrophages in colon criptae; data shows the number of MAC387 immunopositive cells per area unit and are expressed as mean±SEM of n=3 experiments; (F) Quantification of the effects of PEA on MPO levels in non-colitic and PEA-treated UC-derived samples. Results are expressed as mean ±SEM of n=6 experiments. ***p<0.001 versus non-colitic (control); °p<0.05 and °°°p<0.001 versus UC-derived samples.

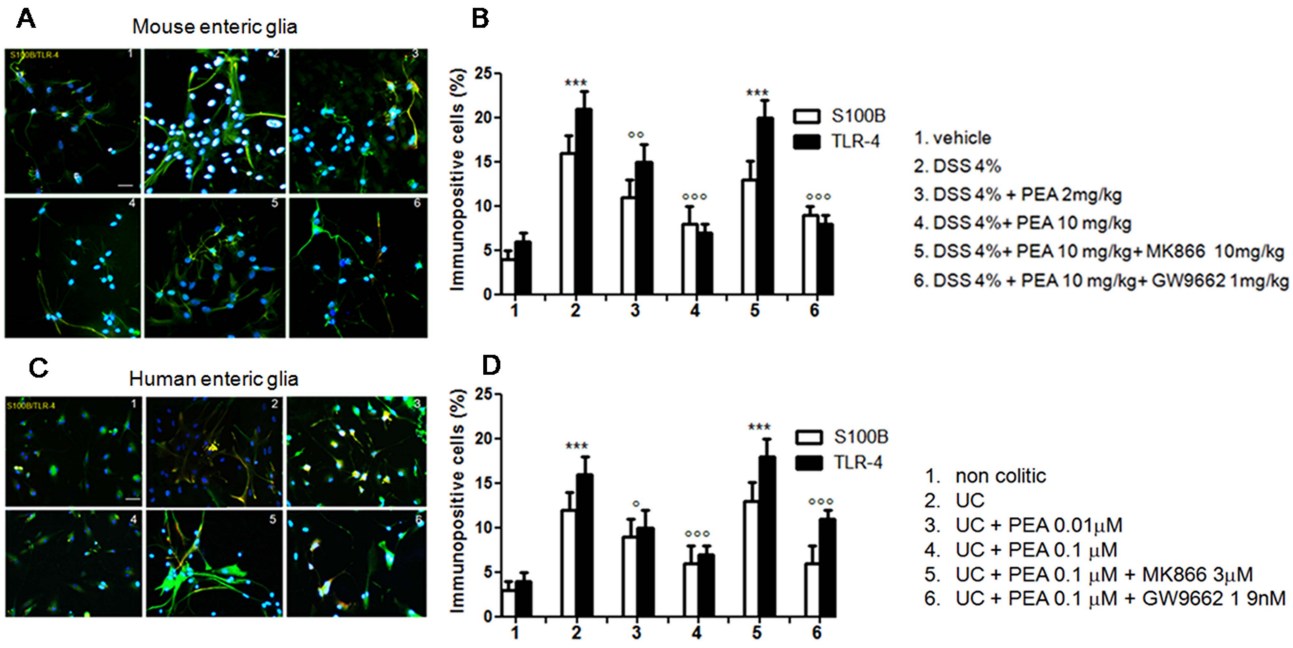

Figure 5 Palmitoylethanolammide (PEA), via PPARα activation, reduces enteric abnormal activation in mice and human enteric glial cells (EGC). (A) Immunofluorescence staining of S100B (green) and TLR4 (red) expression in EGCs isolated from untreated mice (1), in EGCs isolated from DSS-treated mice (2), in EGCs isolated from DSS-treated mice in the presence of: PEA 2 mg/kg (3), PEA 10 mg/kg (4), PEA 10 mg/kg plus MK866 10 mg/kg (5), PEA 10 mg/kg plus GW9662 1 mg/kg. All panels are merged images representative of n=5 experiments showing S100B/TLR4 colocalisation. Nuclei were stained with Hoechst. Scale bars: 100 mm. (B) Relative quantification of S100B/TLR4 immunopositive cells. Results are expressed as mean±SEM of n=5 experiments each performed in triplicate. ***p<0.001 versus vehicle (saline); °p<0.05 and °° p<0.01; °°°p<0.001 versus DSS-treated mice. (C) Immunofluorescence staining of S100B (green) and TLR4 (red) expression in EGCs isolated from non-colitic patients (1), in EGCs isolated from UC patients (2), in EGCs isolated from UC patients and treated with: PEA 0.01 μM (3), PEA 0.1 μM (4), PEA 0.1 μM plus MK866 3 μM (5) and PEA 0.1 μM plus GW9662 9 nM (6). All panels are merged images representative of n=5 experiments showing S100B/TLR4 colocalisation. Nuclei were stained with Hoechst. Scale bars: 100 mm. (D) Relative quantification of S100B/TLR4 immunopositive cell. Results are expressed as mean±SEM of n=5 experiments each performed in triplicate. ***p<0.001 versus non-colitic (control); °p<0.05; °°°p<0.001 versus UC.

PEA treatment failed to significantly affect S100B-induced increase of NO, TNFα and PGE2, respectively (figures 6E, F).

PPARα mediates PEA-induced anti-inflammatory effects in mouse colon and EGCs

In order to demonstrate the pivotal role played by PPARα in mediating PEA anti-inflammatory effects also in vivo, we used a PPARα KO mouse. As compared with WT mice, in DSS-treated PPARα KO mice, PEA (2, 10 and 50 mg/kg) failed to significantly inhibit the increase in DAI score, in preserving colonic length and in preventing splenomegaly ($p<0.001$ vs vehicle; figure 7A, B and C). Similarly, PEA treatment was ineffective in reducing S100B, TLR4, iNOS and COX2 expression as well as S100B, NO, PGE2 and TNFα release in colonic tissues ($p<0.001$ vs vehicle; figure 7D and E).

We evaluated the effects of PEA also in EGCs derived from PPARα KO mice and, also in this case, differently from EGCs obtained by WT mice, PEA treatment did not affect the DSS-induced increase in S100B and TLR4 expression (figure 7F); similarly PEA treatment was not able to significantly reduce the release of inflammatory markers as NO, TNFα and PGE2 (figure 7G and H).

PPARα activation and relative S100B/TLR4 inhibition by PEA leads to the inhibition of NF-κB through the p38/p-ERK/pJNK pathway

As previously shown, TLR4 expression was significantly increased in EGCs derived from DSS-treated mice and patients with UC (figure 5). Such increase leaded to the canonical TLR4-dependent MAP-kinases pathway activation, characterised by the sequential increase of phosphorilated p38, pERK and pJNK intracellular signalling, converging in p50 and p65 upregulation, as markers of NF-κB activation, in mice (figure 8A and B) and in humans (figure 8C and D).

In DSS-treated mice and in human EGCs isolated from UC patients, PEA dose dependently inhibited TLR4 expression, and this effect was followed by a consequent decrease of all the MAP kinases pathways and a downstream inhibition of p50 and p65 protein expression (figure 8). According to the previous results, PEA-dependent TLR4 signalling down-regulation was due to a PPARα activation, as shown by the inhibitory effect on PEA activity exerted by the PPARα antagonist MK866, but not by the PPARγ antagonist GW9662, in mice and humans (figure 8).

Quantisation of endogenous levels of PEA in humans and mice

Data on endogenous levels of PEA are reported as online supplementary material (available online only).

DISCUSSION

Current therapies for UC are represented mainly by chronic administration of mesalamine, glucocorticosteroids or other immunosuppressive drugs.[32 33] These drugs possess, however, a short-term efficacy and they are not suitable as a maintenance therapy due to a variety of systemic adverse reactions.[34] Drugs like sulfasalazine and its derivative, 5-aminosalicylic acid, are effective only in mild-to-moderate acute phase of the disease and in preventing relapse. Biological drugs as monoclonal anti-TNFα antibody (infliximab and adalimumab) have been

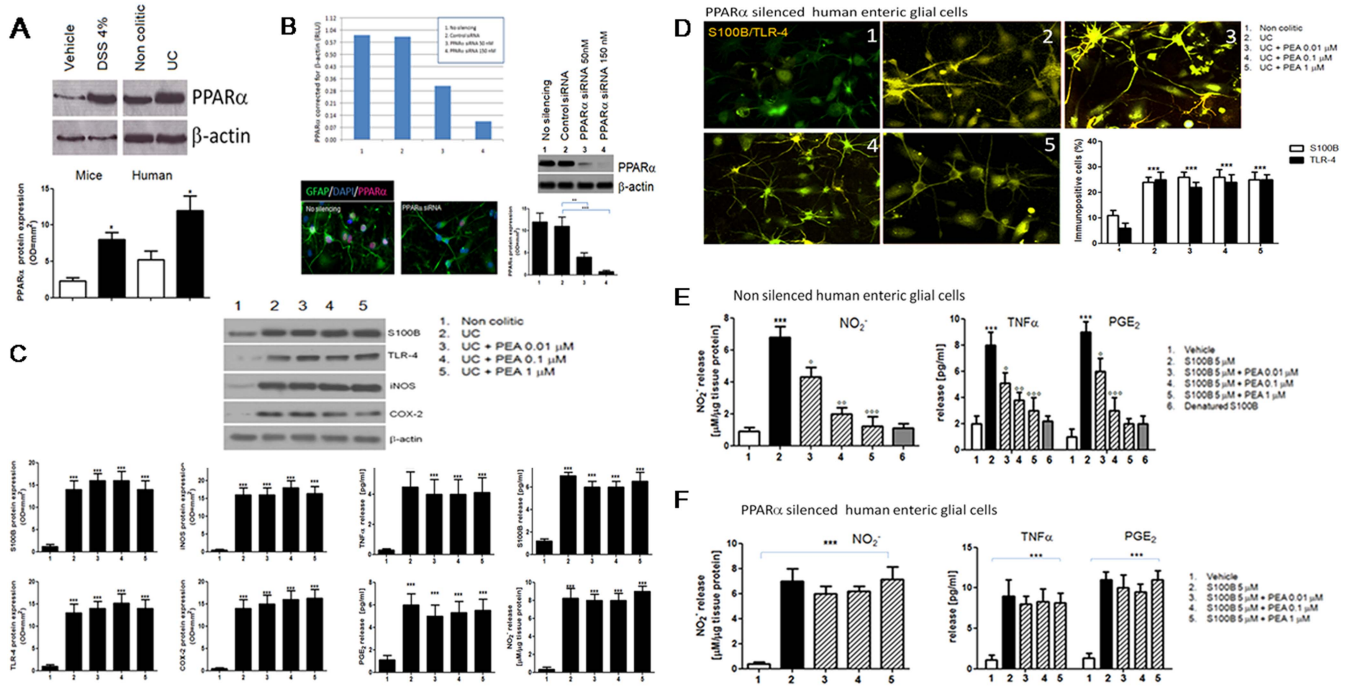

Figure 6 Effects of PEA on PPARα-silenced human and mouse enteric glial cells (EGC). (A) Western blot and densitometric analysis (arbitrary units normalised on the expression of the housekeeping protein β-actin) showing the levels of PPARα in DSS-treated mice versus vehicle and in UC patients versus non colitics. Data are expressed as mean ± SEM of n=3 experiments performed in triplicate. *p<0.05. (B) Effectiveness of PPARα silencing in human EGC; upper left panel shows PPARα mRNA expression (normalised on β-actin) in non-silenced EGC (1), in EGC treated with control siRNA (2), in EGC treated with PPARα siRNA 50 nM (3), and in EGC treated with PPARα 150 nM (4); upper and lower right panels show Western blot and densitometric analysis(normalised on β-actin) of PPARα expression in human EGC; lower left panel shows immunofluorescence analysis of PPARα expression in silenced and non-silenced human EGC. (C) Western blot and densitometric analysis (arbitrary units normalised on the expression of the housekeeping protein β-actin) showing the effects of PEA on expression and release of inflammatory markers in EGC derived from UC patients versus non colitic EGC. Results are expressed as mean ± SEM of n=3 experiments performed in triplicate. ***p<0.001 versus non colitic. (D) Immunofluorescence staining of S100B (green) and TLR4 (red) expression and relative quantification of immunopositive cells in PPARα-silenced human EGC. All panels are merged images representative of n=3 experiments. Nuclei were stained with Hoechst. Scale bars: 100 mm. (E) Effects of exogenous S100B (5μM), given alone or in presence of various concentrations of PEA, on the release of NO, TNFα and PGE2 in the supernatant of non-silenced and in (F) PPARα-silenced human EGC.S100B protein, previously degraded through high temperature (100°C for 3 min), was unable to induce any significant pro-inflammatory response in human EGC (graph E, grey bar). Results are expressed as mean ± SEM of n=3 experiments performed in triplicate. ***p<0.001 versus vehicle (saline); °p<0.05, °°p <0.01 and °°°p <0.001 versus S100B 5μM.

recently introduced in the therapy of relapsing IBD with encouraging results in the maintenance of remission.[35] However, the long-term jeopardy of these drugs, the possibility to induce severe side effects, together with the high costs of the therapy for the patients warrant novel and alternative pharmacological approaches.[36–38] The research of new drugs and new therapeutic targets for IBD treatments, thus, represents an outstanding challenge for gastroenterologists and pharmacologists.

Beside their supportive role, nowadays EGCs represent a very extensively studied cell population in the etiopathogenesis of IBD.[8 11 39] Specifically targeting the EGC-driven neuroinflammation and the relative enteric neuropathy may account for a novel rationale for drug design, enlarging the current therapeutic strategies for IBD. Here, we propose PEA as a new pharmaceutical tool that, by counteracting mucosal immune cells infiltration, enteric abnormal activation and release of proinflammatory mediators occurring during UC, may ameliorate the course of the disease. We also show how the anti-inflammatory effects exerted by PEA in the intestinal mucosa are abolished by antagonists at PPARα, but not at PPARγ site.

In the present study, we provide evidence that PEA efficiently and dose-dependently improved DSS-induced colitis in mice, as demonstrated by the attenuation of the DAI score, the preservation of colonic length and the reduction of splenomegaly. Treatment with PEA also resulted in microscopic amelioration of intestinal inflammation, as demonstrated by the reduction of MPO activity, a marker of mucosal neutrophils activation, and the rate of mucosal macrophage infiltration in mice and human colon ex vivo cultures.

We showed that PEA activity is directly dependent upon the activation of PPARα, since in PPARα-silenced EGCs and in PPARα-KO mice, PEA is not able to exert anti-inflammatory effect. This is in agreement with previous studies demonstrating that this ALIAmide significantly reduces reactive astrogliosis in the central nervous system.[20 26 27] We also demonstrated that PEA caused a significant decrease of colitis-related inflammatory mediators, such as iNOS and COX2 protein expression and NO, PGE2 and TNFα release, in colonic tissues and in blood samples deriving from treated mice, as well as in the supernatant and in tissue homogenates of biopsies deriving from UC patients.

Beyond the description of an overall anti-inflammatory effect exerted by PEA, our study shows that this molecule specifically decrease enteroglial-derived S100B protein expression, confirming that its anti-inflammatory and protective effects could be closely associated with the specific reduction of EGC activation during colitis. In this context, the PPARα-mediated inhibition of S100B expression and release by PEA appears as a novel and

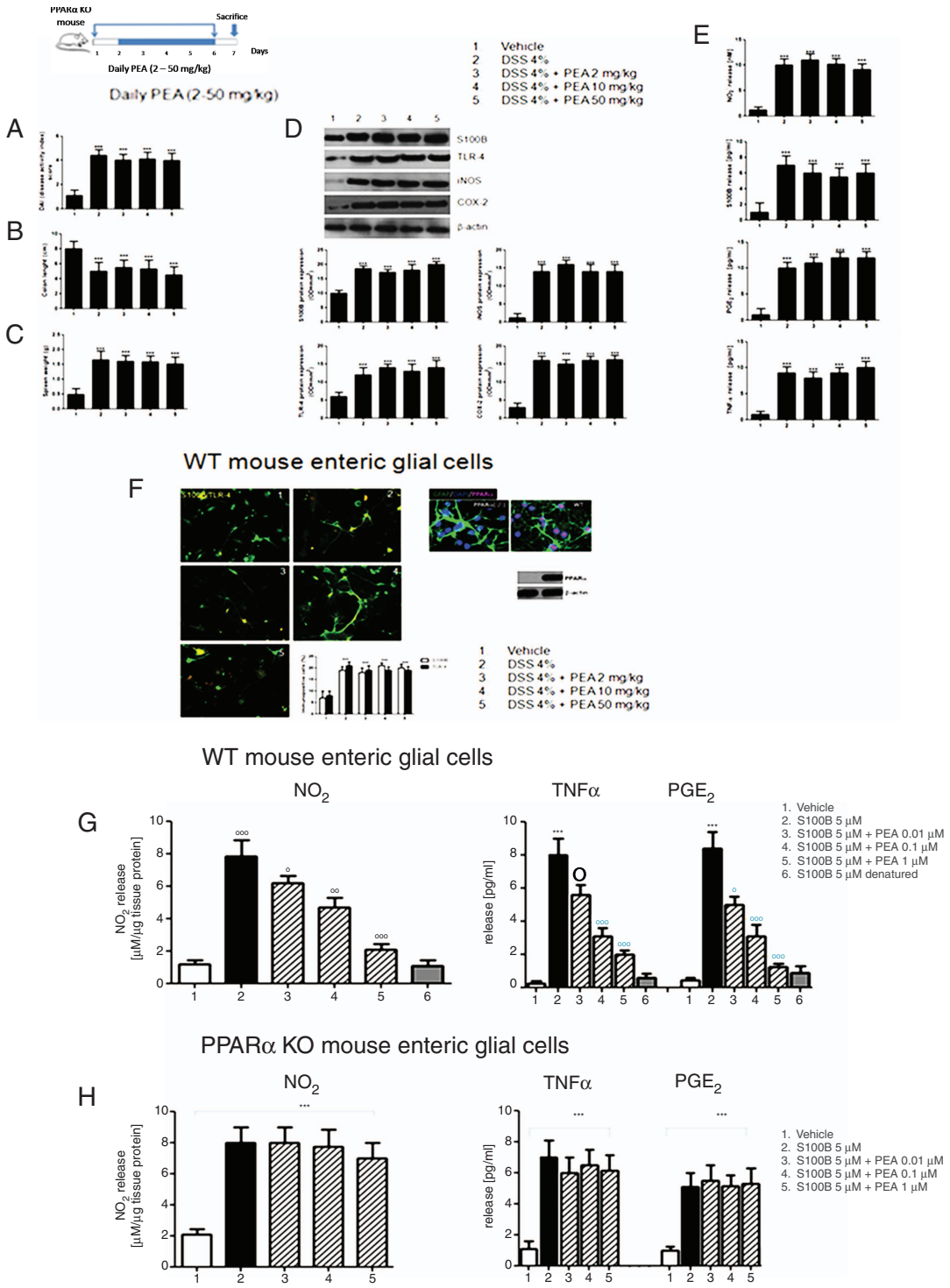

Figure 7 Experimental plan and inhibition of PEA effects in PPARα null (KO) mice. (A) Effect of PEA treatment in DDS exposed mice on DAI score, (B) on colonic length and (C) on spleen weight. Results are expressed as mean±SEM of n=5 experiments. ***p<0.001 versus vehicle. (D) Western blot and densitometric analysis (arbitrary units normalised on the expression of the housekeeping protein β-actin) showing the effects of PEA on iNOS, COX2, GFAP and S100B protein expression in colonic tissue of DSS-treated PPARα KO mice. Results are expressed as mean ± SEM of n = 3 experiments performed in triplicate. ***p<0.001 versus vehicle. (E) Effect of PEA on the release of NO, PGE2, IL-1β, TNFα and S100B in plasma DSS-treated PPARα KO mice. Results are expressed as mean±SEM of n=3 experiments performed in triplicate. ***p<0.001 versus vehicle. (F) Upper right panels: Immunofluorescence and Western blot analysis showing the effectiveness of PPARα knok down in EGC isolated from PPARα KO mice; left panels: immunofluorescence staining of S100B (green) and TLR4 (red) expression and relative quantification of immunopositive cells in EGC isolated from PPARα KO mice. All panels are merged images representative of n=3 experiments. Nuclei were stained with Hoechst. Scale bars: 100 mm. (G) Effects of exogenous S100B (5μM), given alone or in presence of various concentrations of PEA, on the release of NO, TNFα and PGE2 in the supernatant of EGC isolated from wild type mice or from (H) PPARα KO mice. S100B protein, previously degraded through high temperature (100°C for 3 min), was unable to induce any significant pro-inflammatory response in mouse EGC (graph G, grey bar). Results are expressed as mean±SEM of n=3 experiments performed in triplicate. ***p<0.001 versus vehicle (saline); °p<0.05, °°p <0.01 and °°°p <0.001 versus S100B 5μM.

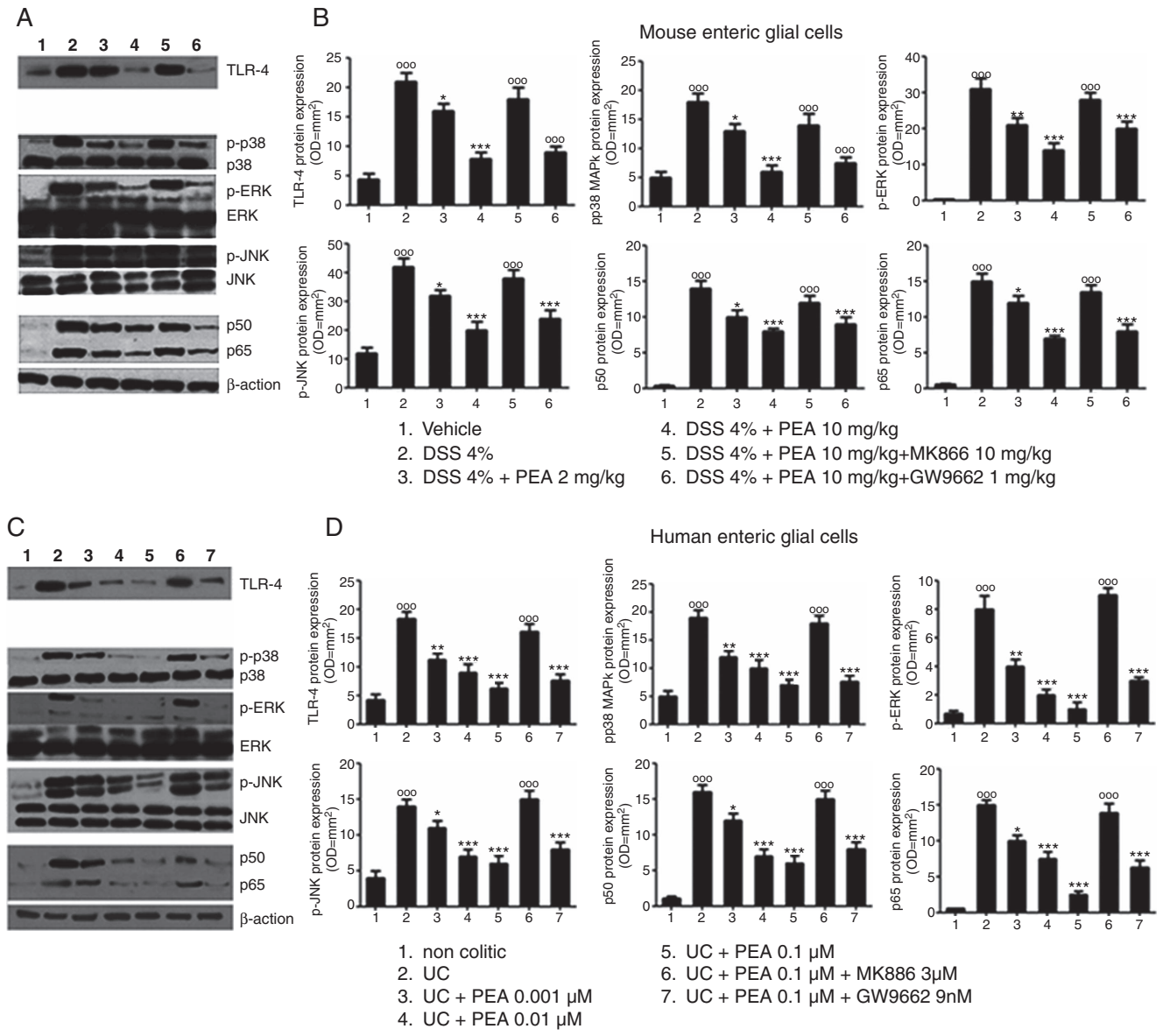

Figure 8 PEA, interacting with PPARα, reduces the expression of the downstream mediators of the TLR4 pathway, both in mouse and human enteric glial cells (EGC). (A) Western blot analysis and (B) densitometric analysis (arbitrary units normalised on the expression of the housekeeping protein β-actin) showing the effect of PEA (2–10 mg/kg) given alone or in the presence of MK866 (10mg/kg) or GW9662 (1mg/kg) on TLR4, p-p38, p-ERK, p-JNK, NF-kBp50 and NF-kBp65 protein expression in mice EGC; data are expressed as mean±SEM of n=5 experiments performed in triplicate. ***p<0.001 versus vehicle (saline); °p<0.05, °°p<0.01 and °°°p<0.001 versus DSS-treated mice. (C) Western blot analysis and (D) densitometric analysis (arbitrary units normalised on the expression of the housekeeping protein β-actin) showing the effect of PEA (0.001–0.1M) given alone or in the presence or absence of MK866 3M or GW9662 9nM on TLR4, p-p38, p-ERK, p-JNK, NF-kBp50 and NF-kBp65 protein expression in human EGC. Results are expressed as mean±SEM of n=5 experiments performed in triplicate. ***p<0.001 versus non colitic (control) patients-derived EGC; °p<0.05, °°p<0.01 and °°°p<0.001 versus UC patients-derived EGC.

critical step at the basis of anti-inflammatory effects exerted by this ALIAmide. This is confirmed by the observation that PEA is able to counteract the expression and release of proinflammatory markers from EGCs stimulated with exogenous S100B. Since enteroglial S100B can also increase macrophage activity causing a massive proinflammatory response during colitis,[40] specific inhibition of S100B release may be a key event in the effort to block gut inflammation.

Although the exact mechanism by which S100B may orchestrate a so complex proinflammatory scenario is not fully understood, recently, a close relationship with TLR activation has been proposed.[15] The direct interaction between S100B and RAGE receptors during colitis has to be considered an initial event of a deeper activation of a more specific pathway involved in the triggering and the maintenance of a persistent enteroglial-sustained inflammation in the gut.[13]

In order to gain more mechanistic insights into S100B and TLR response, we evaluated the effect of PEA on the expression of different subtypes of TLR, distributed on enteric glia.[15 41] Our results demonstrated that PEA selectively, and mainly via a PPARα-dependent mechanism, reduces the expression of S100B and TLR4 in EGCs derived from DSS-treated mice and UC patients, without affecting the expression of other TLRs. This data is parallel with the evidence that PPARα agonists may mediate TLR4 down-regulation as it has been previously observed in vascular smooth cells.[42] Additionally, we showed

that S100B-mediated proinflammatory effects are abolished in PPARα-silenced EGCs, and in EGCs isolated from PPARα KO mice. Our results confirmed the importance of the role played by EGCs, by S100B and by PPARα in mediating the anti-inflammatory effects exerted by PEA.

Such pharmacological upstream interaction leads to the downstream decrease of all the sequel of events triggered by the TLR4 activation, including the decrease of p38-pERK-pJNK expression. As a consequence of this pharmacological modulation, PEA is able to inhibit NF-kB activation, as demonstrated by the decrease of p50 and p65 subunits expression induced by PEA in mice and human EGCs.

Since its discovery, PEA was considered as an endogenous compound able to efficiently suppress the inflammatory process in in vitro and in clinical studies.[43] It has been previously demonstrated that PEA administration resulted in a potent inhibition of intestinal motility in mice and it has been suggested to introduce this compound for the treatment of IBD.[25] The observation that PEA level is significantly increased in UC patients versus controls, confirming data present in literature,[44] and in DSS-treated mice versus controls, might give strength to the hypothesis that, on the basis of our results, this compound may putatively act as on-demand modulator of chronic inflammation of the gut.

In summary, we propose PEA as a new drug able to control the acute phase of intestinal inflammation occurring in UC, profoundly and beneficially impacting on abnormal EGCs activation and, mainly via PPARα, inhibiting the S100B/TLR4 axis. Because of its well-known pharmacological and toxicological profile, PEA might be regarded as a potential, innovative, manageable and low-cost tool against colitis. Very interestingly, PEA could be very quickly moved to clinic, since it is currently administrated orally as a dietary supplement, in anti-inflammatory and analgesic preparations in dermatology and gynaecology (eg, Normast, Pelvilen).[16] Moreover, since the magnitude and duration of PEA signalling is principally regulated in vivo by the enzyme fatty acid amide hydrolase (FAAH), it is reasonable to suggest that inhibition of this enzyme may also potentiate the pharmacological effects of PEA.[45] Based on our present and previous studies, it could be of extreme interest to test pre-clinical PEA efficacy also in clinical trials involving UC patients.

In conclusion, although further studies are required to better define the PEA anti-inflammatory effects and the involvement of EGC in UC onset/progression, considering the PEA-related good compliance and its relative lack of toxicity in humans even at high dosage, this study lays the basis to further considering PEA as a new pharmaceutical tool for UC treatment.

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