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RESEARCH PAPER

Inhibitor of PI3Kγ ameliorates TNBS-induced colitis in mice by affecting the functional activity of $CD4^+CD25^+FoxP3^+$ regulatory T cells

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Keywords
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BACKGROUND AND PURPOSE
Phosphoinositide 3-kinase-γ (PI3Kγ) is implicated in many pathophysiological conditions, and recent evidence has suggested its involvement in colitis. In the present study, we investigated the effects of AS605240, a relatively selective PI3Kγ inhibitor, in experimental colitis and its underlying mechanisms.

EXPERIMENTAL APPROACH
Acute colitis was induced in mice by treatment with trinitrobenzene sulphonic acid (TNBS), and the effect of AS605240 on colonic injury was assessed. Pro-inflammatory mediators and cytokines were measured by immunohistochemistry, ELISA, real time-polymerase chain reaction and flow cytometry.

KEY RESULTS
Oral administration of AS605240 significantly attenuated TNBS-induced acute colitis and diminished the expression of matrix metalloproteinase-9 and vascular endothelial growth factor. The colonic levels and expression of IL-1β, CXCL-1/KC, MIP-2 and TNF-α were also reduced following therapeutic treatment with AS605240. Moreover, AS605240 reduced MIP-2 levels in a culture of neutrophils stimulated with lipopolysaccharide. The mechanisms underlying these actions of AS605240 are related to nuclear factor-κ (NF-κB) inhibition. Importantly, the PI3Kγ inhibitor also up-regulated IL-10, CD25 and FoxP3 expression. In addition, a significant increase in CD25 and FoxP3 expression was found in isolated lamina propria $CD4^+$ T cells of AS605240-treated mice. The effect of AS605240 on Treg induction was further confirmed by showing that concomitant *in vivo* blockade of IL-10R significantly attenuated its therapeutic activity.

CONCLUSIONS AND IMPLICATIONS
These results suggest that AS605240 protects mice against TNBS-induced colitis by inhibiting multiple inflammatory components through the NF-κB pathway while simultaneously inducing an increase in the functional activity of $CD4^+CD25^+$ Treg. Thus, AS605240 may offer a promising new therapeutic strategy for the treatment of inflammatory bowel diseases.

Abbreviations
AS605240, 5-quinoxilin- 6-methylene-1,3-thiazolidine-2,4-dione; CXC, chemokine ligand; DSS, dextran sulphate sodium; FoxP3, transcription factor forkhead box P3; IBD, inflammatory bowel disease; IL, interleukin; KC, keratinocyte-derived chemokine; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage-inflammatory protein-2; MMP-9, matrix metalloproteinase-9; MPO, myeloperoxidase; NF-κB, nuclear factor-κB; PI3Kγ, phosphoinositide 3-kinase γ; TGF-β, transforming growth factor-β; TNBS, 2,4,6-trinitrobenzene sulphonic acid; TNF-α, tumour necrosis factor- α; Treg, regulatory T cell; VEGF, vascular endothelial growth factor

Introduction

Human inflammatory bowel disease (IBD), which includes ulcerative colitis and Crohn's disease (CD), is an idiopathic and chronic inflammation of the gastrointestinal tract that is characterized by a dysfunction of mucosal T cells, imbalanced cytokine production and cellular inflammation leading to damage of the intestinal mucosa (Korzenik *et al.*, 2006). Although the precise mechanisms underlying the inflammatory reaction and the immune responses observed in IBD have not been fully elucidated, it has been suggested that various inflammatory mediators are involved in this disease. In fact, an array of therapeutic approaches targeting chemotactic peptides, such as keratinocyte-derived chemokine (KC), macrophage inflammatory protein-2 (MIP-2), matrix metalloproteinase (MMPs; namely MMP-2, MMP-3 and MMP-9), growth factors [vascular endothelial growth factor (VEGF)] and pro-inflammatory cytokines [tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)], have been extensively investigated (Podolsky, 1991; Medina *et al.*, 2006; Huang *et al.*, 2009; Tolstanova *et al.*, 2009).

An increased influx of neutrophils and macrophages accompanied by the secretion of pro-inflammatory cytokines is considered to exacerbate IBD (Medina *et al.*, 2006). The uncontrolled immune response in inflamed mucosa is associated with a deficiency of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Treg) (Izcue *et al.*, 2008). These cells prevent the proliferation and activation of inflammatory $CD4^+$ or $CD8^+$ T cells via cell–cell contact-dependent mechanisms and also by inducing the production of suppressor cytokines such as IL-10 and/or transforming growth factor-β (TGF-β) (Maloy and Powrie, 2001; Boden and Snapper, 2008). Notably, these Treg subsets, often referred to as 'inducible Tregs', are not found in the thymic environment but seem to be induced in peripheral tissues, such as the gut. Suppression of T_H1 and T_H17 cell response by Tregs confirm these special T-cell populations as a new therapeutic target to treat severe inflammatory colitis (Read *et al.*, 2000; Liu *et al.*, 2003).

Phosphoinositide 3-kinase-γ (PI3Kγ), which belongs to the class I PI3 kinase family, plays a critical role in inflammatory cell activation and recruitment. PI3Kγ, mostly activated by G-protein-coupled receptors (GPCRs) and mainly located in haematopoietic cells (Cantley, 2002), leads to the formation of phosphatidylinositol-(3, 4, 5)-triphosphate (PIP3) and consequently phospho-Akt. The interaction of phospho-Akt with PIP3 at the cell membrane stimulates phosphorylation of downstream targets, which regulate several inflammatory and immune functions, including the recruitment of macrophages and neutrophils, and T-cell activation (Ruckle *et al.*, 2006). Inhibition of PI3Kγ is expected to offer an innovative rationale-based therapeutic strategy for inflammatory diseases. Leucocytes, such as neutrophils, defected by PI3Kγ or inhibited with a PI3Kγ inhibitor display a reduction in chemotaxis in response to chemokines (Van Dop *et al.*, 2010). T cells from PI3Kγ-null mice show impaired proliferation and cytokine production of interferon γ (INF-γ) and IL-2 when stimulated with concanavalin A (ConA) (Sasaki *et al.*, 2000). In addition, there is genetic and pharmacological evidence indicating that the PI3K–Akt–mTOR signalling network interferes with FoxP3 induction *in vitro*, as well as *in vivo* (Haxhinasto *et al.*, 2008; Sauer *et al.*, 2008), but the mechanisms that link PI3K–Akt–mTOR signalling to FoxP3 expression have not been fully elucidated.

The PI3Kγ inhibitor, AS605240, by inhibiting the recruitment of inflammatory cells and suppressing the progression of inflammation, was found to be effective in the treatment of autoimmune diseases including systemic lupus, rheumatoid arthritis and arteriosclerosis (Barber *et al.*, 2005; Camps *et al.*, 2005). Recently, it was reported that AS605240 ameliorates dextran sodium sulphate (DSS)–induced colitis in mice (Peng *et al.*, 2010). However, the cellular mechanisms of these effects of the PI3K-γ inhibitor were not evaluated. Susceptibility to trinitrobenzene sulphonic acid (TNBS) varied in each mouse, but some developed hapten-induced delayed-type hypersensitivity and proceeded to develop chronic colitis. Granulomas with infiltration of inflammatory cells in all the layers were seen in the intestine of this model. The isolated macrophages produced large amounts of IL-12 and the lymphocytes produced large amounts of IFN-γ and IL-2. This suggests that the colitis seen in this model was induced by a T_H type-1 response, constituting a CD model (Elson *et al.*, 1995; Mane *et al.*, 2001; McCartney *et al.*, 2002).

In the present study, we used pharmacological and molecular approaches to examine the role of PI3Kγ in the TNBS mouse model of colitis. We also assessed the molecular mechanisms involved in the PI3Kγ pathway in this paradigm. Herein, we report for the first time that pharmacological blockade of PI3Kγ, using a relatively selective PI3Kγ inhibitor, markedly improves TNBS-induced colitis by inhibiting pro-inflammatory mediators through the nuclear factor-κB (NF-κB) pathways and inducing functional activity of $CD4^+CD25^+$ Treg in intestinal tissues.

Methods

Animals

All animal care and experimental protocols used in this study were approved by the local Ethics Committee from Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil. The experiments were conducted using male CD1 mice (8–10 weeks of age) obtained from Universidade Federal de Santa Catarina. Animals were kept in a 12 h light/dark cycle, with controlled humidity (60–80%) and temperature (22 ± 1°C). Food and water were freely available. Experiments were performed during the light phase of the cycle. The animals were acclimatized to the experimental laboratory for at least 1 h before testing.

Administration of AS605240 and study design

AS605240 (PI3Kγ inhibitor, 1-5-quinoxilin-6-methylene-1,3-thiazolidine-2,4-dione) was synthesized by Dr Paulo C. Leal, according to patent WO 2004/007491 A1. Pharmacokinetic parameters corresponding to the administration of 50 $mg \cdot kg^{-1}$ p.o. resulted in a plasma concentration of 3 mM, $t_{1/2}$ of 2.2 h, Fz of 27%, C_{max} of 979 $ng \cdot mL^{-1}$, clearance of 2.86 $L \cdot kg^{-1} \cdot h^{-1}$ and V_d of 1.1 $L \cdot kg^{-1}$ and the solubility of the AS605240-salt in water/saline is 10 $mg \cdot mL^{-1}$ (Camps *et al.*, 2005). To evaluate the potential therapeutic effect of

AS605240 in experimental colitis, animals were orally treated by gavage with different doses of AS605240 twice a day (3, 10 and 30 mg·kg^{-1}) 24 h after TNBS instillation. The dose of 30 mg·kg^{-1} (p.o.) AS605240 was used in subsequent experiments. A similar treatment protocol was carried out using the positive control drug dexamethasone (1 mg·kg^{-1}, s.c.). To block IL-10R function, an ultra-purified IL-10R mAb (1B1.3a; BD Pharmingen, San Diego, CA, USA) was injected at 100 μg·kg^{-1} (i.p.), for 7 days before and after TNBS administration until the end of the experiments. Control mice received normal IgG (anti-mouse IgG; R&D Systems, Minneapolis, MN, USA) at the same dose. AS605240 was dissolved in 0.9% NaCl solution just before use, and control mice were treated with this vehicle.

Induction and assessment of TNBS-induced colitis

Colitis was induced according to the methodology described previously (Wallace *et al.*, 1989) and adapted recently (Hara *et al.*, 2008). After being deprived of food for 18–24 h with free access to a 5% glucose solution, mice were randomly divided into control and colitis groups. Briefly, mice deprived of food for 1 day were lightly anaesthetized by administration of xylazine (10 mg·kg^{-1}, i.p.) and ketamine (80 mg·kg^{-1}, i.p.), and then a catheter (polyethylene PE-50) was carefully inserted into the colon (4 cm proximal to the anus). To induce colitis, TNBS (2 mg in 100 μL of 35% ethanol solution) was slowly administered. To assure the distribution of TNBS within the entire colon, mice were carefully maintained at a 45° angle (head down position) for 2 min and then returned to their cages. Four hours later, the animals were given free access to food and water. Throughout the experiments, mice were monitored for body weight loss and overall mortality. At 72 h following TNBS administration, the animals were killed, and the colon was removed, dissected and opened lengthwise. The severity of colon damage was macroscopically assessed using the criteria previously established for TNBS-induced colitis (Wallace *et al.*, 1989). A score ranging from 0 to 10 was employed, as follows: 0, no damage; 1, hyperaemia without ulcers; 2, hyperaemia and wall thickening without ulcers; 3, one ulceration site without wall thickening; 4, two or more ulceration sites; 5, 0.5 cm extension of inflammation or major damage; 6–10, 1 cm extension of inflammation or severe damage. The score was increased by 1 for every 0.5 cm of damage up to a maximal score of 10; by 0 or 1 for absence or presence of diarrhoea; 0 or 1, absence or presence of stricture and by 0, 1 or 2 for absence, presence of mild or severe adhesion respectively.

Myeloperoxidase (MPO) assay

Neutrophil infiltration into the colon was assessed indirectly by using the MPO activity assay. Colon segments were homogenized at 5% in EDTA/NaCl buffer (pH 4.7) and centrifuged at 10 000× *g* for 15 min at 4°C. The pellet was resuspended in 0.5% hexadecyl trimethyl ammonium bromide buffer (pH 5.4), and the samples were frozen in liquid nitrogen. Upon thawing, the samples were similarly centrifuged, and 25 μL of the supernatant was used for the MPO assay. The MPO enzymatic reaction was assessed by the addition of 1.6 mM tetramethylbenzidine (TMB), 80 mM NaPO4 and 0.3 mM hydrogen peroxide (H_2O_2). The absorbance was measured spectrophotometrically at 690 nm for MPO, and the results are expressed in optical density (OD) mg^{-1} tissue.

Histological analysis and evaluation of microscopic damage

In another set of experiments, each excised portion of distal colon was immediately fixed in 10% formaldehyde solution, embedded in paraffin, sectioned at 5 μm thicknesses, mounted on glass slides and then deparaffinized. For histological analysis, slices were stained using haematoxylin-eosin standard techniques. Distal portions of colon were examined in cross sections at ×20, ×200 and ×400 magnifications. In each specimen, six random fields of view were analysed by two double-blind observers, using Sight DS-5 M-L1 digital camera connected to an Eclipse 50i light microscope (both from Nikon, Melville, NY, USA). Histological changes in each sample were graded from 0 to 4: 0, no inflammation; 1, very low level of leucocyte infiltration; 2, low level of leucocyte infiltration; 3, high level of leucocyte infiltration, high vascular density and thickening of the colon wall; 4, transmural infiltration, loss of goblet cells, high vascular density and thickening of the colon wall.

Cytokine assays

Briefly, colon segments were homogenized in phosphate buffer containing 0.05% Tween 20, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 UI aprotinin A. The homogenate was centrifuged at 3000× *g* for 10 min and supernatants were stored at –70°C until further analysis. IL-1β, KC, MIP-2, IL-10 and CINC-1 levels were evaluated using ELISA kits from R&D Systems, according to the manufacturer's instructions. The amount of protein in each sample was measured using the Bradford method (Bradford, 1976).

Primary cultures of peritoneal neutrophils

Naïve mice received an i.p. injection of 1 mL of 3% thioglycollate (BD, Franklin Lakes, NJ, USA). Four hours later, peritoneal exudate cells were harvested by three consecutive lavages of the peritoneal cavity with 7 mL of cold phosphate-buffered saline, interspersed with gentle massages of the abdomen. The recovered lavage samples were centrifuged at 200× *g* for 10 min at 4°C and subjected to hypotonic lysis to eliminate red blood cells, regardless of the presence of a red pellet. Centrifugation was repeated and after an additional wash, the cells were resuspended in complete Dulbecco's modified Eagle medium (DMEM) (supplemented with 10% heat-inactivated calf serum, glucose, 2 mM L-glutamine, 10 mM HEPES, 100 μg·mL^{-1} streptomycin and 100 U·mL^{-1} penicillin). Exudate cell numbers and populations were determined and confirmed by Poch haemocytometry (Sysmex Corporation, São Paulo, SP, Brazil). Neutrophils comprised 85% to 90% of the cells present in the exudate samples collected at 4 h following thioglycollate injection. Peritoneal neutrophils (2 × 10^6 cells mL^{-1}) were stimulated with lypopolysaccharide (LPS, 100 ng·mL^{-1}) for 4 h, either in the presence or absence of AS605240 (3, 10 and 30 μg·mL^{-1}) for 30 min before stimulation. Control cells were incubated with the corresponding vehicles. After stimulation, the plate was

centrifuged (200× *g*, 10 min), and cell-free supernatant was collected and stored at −70°C until the determination of cytokine (IL-1β and MIP-2) levels as described above.

Intestinal epithelial cell-6 culture

Intestinal epithelial cell-6 (IEC-6), a non-transformed rat intestinal epithelia-derived cell line, was maintained in DMEM (containing glucose, supplemented with 2 mM L-glutamine, 10 mM HEPES, 100 μg·mL^{-1} streptomycin and 100 U·mL^{-1} penicillin). Cells (2×10^5 cells per well) were distributed in 24-well plates, and all experiments were performed after cultures reached confluency (2–3 days after plating). After confluency, the cell culture medium was replaced. Cells were treated with AS605240 (3 and 10 μg·mL^{-1}) for 30 min before stimulation. Cells were then stimulated with LPS (100 ng·mL^{-1}) for 4 h in the presence or absence of AS605240 (3 and 10 μg·mL^{-1}) or vehicle. Control cells were incubated with the appropriate corresponding vehicles. After stimulation, the plate was centrifuged (200× *g*, 10 min), and the cell-free supernatant was collected and stored at −70°C for the determination of the CINC-1 levels as described above.

Immunohistochemical analysis

Immunohistochemical analysis was performed on paraffin-embedded colonic tissue sections (5 μm) using monoclonal mouse anti-VEGF (1:200), polyclonal rabbit anti-caspase-3-cleaved (1:250), polyclonal rabbit anti-MMP-9 (1:200) and monoclonal mouse anti-phospho-p65 NF-κB (1:50), according to the method described previously (Medeiros *et al.*, 2007). After quenching endogenous peroxidase with 1.5% hydrogen peroxide in methanol (v/v) for 20 min, high-temperature antigen retrieval was performed by immersion of the slides in a water bath at 95 to 98°C in 10 mmol·L^{-1} trisodium citrate buffers, pH 6.0, for 45 min. The slides were then processed using the Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, CA), according to the manufacturer's instructions. After the appropriate biotinylated secondary antibody, immune complexes were visualized with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB: Dako Cytomation, Glostrup, Denmark) + 0.03% H_2O_2 in phosphate buffered saline (PBS) (for the exact amount of time: 1 min). The reaction was stopped by thorough washing in water and counterstained with Harris's haematoxylin. Besides staining untreated animals as negative controls, sections were incubated with isotype-matched primary antibodies of irrelevant specificity or the primary antibody was omitted. Despite antigen retrieval, these controls resulted in little or no staining, principally due to the fact that peroxide pretreatment (inactivation of endogenous peroxidase) appears to destroy the epitopes to which anti-mouse (secondary) antibody otherwise binds. Images were obtained by using a Sight DS-5M-L1 digital camera connected to an Eclipse 50i light microscope (both from Nikon, Melville, NY, USA). Settings for image acquisition were identical for control and experimental tissues. Four ocular fields per section (8–10 mice per group) were captured and a threshold optical density that best discriminated staining from the background was obtained using the NIH ImageJ 1.36b imaging software (NIH, Bethesda, MD, USA). The total pixel intensity was determined and data were expressed as OD, using a counting grid at ×200 and ×400 magnification.

Real-time quantitative PCR

Total RNA from colons was extracted using the TRizol® protocol and its concentration was determined by NanoDrop 1100 (NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription assays were carried out as described in the M-MLV Reverse Transcriptase protocol according to the manufacturer's instructions. cDNA (100 ng) was amplified in triplicate using TaqMan® Universal PCR Master Mix Kit with specific TaqMan Gene Expression target genes, the 3′ quencher MGB and FAM-labelled probes for mouse CD25 (Mm01340213), FoxP3 (Mm0475165_m1), TNF-α (Mm00443258_m1), IL-10 (Mm00439614_m1) and VIC-labelled probe glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NM_008084.2), which was used as an endogenous control for normalization. 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. those animals administered vehicle.

Lamina propria lymphocyte extraction

Lamina propria lymphocytes (LPLs) were prepared from mice 72 h after TNBS administration. For the LPL extraction, the colon was carefully cleaned of the mesentery and residual fat and cut open longitudinally and subsequently cut into large fragments (1 to 1.5 cm). Fragments were placed in a 50 mL conical flask and rinsed well with ice-cold PBS (Sigma-Aldrich, St. Louis, MO, USA.). Cleaned intestinal fragments were placed in 15 mL of epithelial cell dissociation solution (Ca^{2+} and Mg^{2+} free Hank's buffered saline with 5 mM EDTA and 10 mM HEPES) at 37°C for 15 min with gentle agitation and subsequently vortexed for 15 s. This step was repeated once more. The fragments were then digested for 45 min at 37°C with 300 U·mL^{-1} Clostridial collagenase type I (Gibco®, Carlsbad, CA, USA) and 1 mg·mL^{-1} DNase (Sigma-Aldrich) with slow agitation and then vortexed well. Supernatants were collected by filtering through a 70 μm cell strainer (BD Biosciences, San Diego, CA, USA). Digestions were repeated two more times until the material was completely digested. LPLs were resuspended in 8 mL of 40% Percoll and layered on top of 5 mL of 80% Percoll (Sigma-Aldrich). LPLs were recovered from the interface of the 40/80% gradient after centrifugation (500× *g* for 5 min at 4°C), washed and used in flow cytometry assays as describe below.

Flow cytometry assay

The LPLs (1×10^6 cells) were permeabilized with 0.25% of Triton X-100 for 5 min at 4°C and then fixed in 200 μL of ice-cold 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) (Merck, Whitehouse Station, NJ, USA). Antibodies directed against the following mouse cell surface and intracellular staining were used: anti-CD4-APC (eBioscience, San Diego, CA, USA), anti-CD25-PE (Caltag Laboratories, Burlingame, CA, USA) and FoxP3-FITC (eBioscience). Data were collected using FACSCanto II (BD Biosciences) and analysed using FlowJo software (Tree Star, Inc., Ashland, OR, USA.).

Statistical analysis

All data are expressed as means ± SEM (n = 8 to10 animals/group). For nonparametric data, Kruskal–Wallis followed by

Dunn's test was used. For parametric data, statistical comparison of the data was performed by one-way ANOVA followed by the Student Newman–Keuls *post hoc* test. *P*-values less than 0.05 ($P < 0.05$) were considered significant. Statistical analyses were performed using GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA, USA).

Materials

Dexamethasone, H_2O_2, Tween 20, Tween 80, EDTA, aprotinin, PBS, haematoxylin, eosin, TMB, H_2O_2, OPD, TNBS, DNase and Percoll were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Formaldehyde was obtained from Merck (Frankfurt, Darmstadt, Germany). Bradford reagent was purchased from Bio-Rad Laboratories (Richmond, CA, USA). Anti-mouse KC and the DuoSet kits for MIP-2, IL-10, IL-1β/IL-1F2 and CINC-1 were obtained from R&D Systems. The monoclonal antibody against IL-10R (1B1.3a) was purchased from BD Pharmingen. Trizol® and M-MLV reverse transcriptase were purchased from Invitrogen (Carlsbad). Primers and probes for mouse CD25 (Mm01340213), FoxP3 (Mm0475165_m1), TNF-α (Mm00443258_m1), IL-10 (Mm00439614_m1), GAPDH (NM_008084.2) and TaqMan® Universal PCR Master Mix Kit were purchased from Applied Biosystems (Foster City, CA, USA). Monoclonal mouse anti-VEGF was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal rabbit anti-MMP-9 was purchased from Abcam® (Cambridge, MA, USA). Monoclonal mouse anti-phospho-p65 NF-κB and polyclonal rabbit anti-caspase-3-cleaved antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Secondary antibody Envision Plus, streptavidin–HRP reagent and 3,3-diaminobenzidine chromogen were purchased from Dako Cytomation (Carpinteria, CA, USA). Anti-CD4-APC and FoxP3-FITC antibodies were purchased from eBioscience and anti-CD25-PE from Caltag Laboratories.

Results

AS605240 treatment attenuates the severity of TNBS-induced colitis

Recently, our group has shown that 24 h after TNBS administration, colitis was already established, as revealed by severe macroscopic damage allied to increased MPO activity (Bento *et al.*, 2008). At later time points up to 72 h, TNBS-treated mice developed severe diarrhoea, striking hyperaemia, necrosis and inflammation accompanied by an extensive wasting disease, rectal prolapse and sustained weight loss. To evaluate the potential therapeutic effect of AS605240 in TNBS-induced colitis, animals received different doses of AS605240 twice a day (3, 10 and 30 mg·kg^{-1}, p.o.), 24 h following colitis induction. Three days after TNBS administration, animals were killed, and colon length and damage were analysed. Treatment with AS605240 (Figure 1A) significantly decreased macroscopic damage in a dose-related manner (Figure 1B). In addition, data from Figure 1B show that dexamethasone (1 mg·kg^{-1}), the reference corticosteroid drug used in clinical practice, significantly reduced the macroscopic score, exhibiting essentially the same percentage of inhibition observed for AS605240 (30 mg·kg^{-1}, p.o.): 92% and 90% respectively ($P < 0.01$). AS605240 treatment also significantly restored colon length (Figure 1C, D). However, this PI3Kγ inhibitor, as well as dexamethasone, failed to recover weight loss ($P > 0.05$) (Figure 1E).

AS605240 treatment diminishes neutrophil influx in the colon tissue

Several reports have suggested that tissue damage and inflammatory signs in experimental colitis are mainly mediated by neutrophils (Wallace *et al.*, 1998; Bento *et al.*, 2008). To further investigate whether the effects of AS605240 were linked to the inhibition of neutrophil influx, we measured MPO activity. As demonstrated in Figure 2A, at 72 h after colitis induction, the TNBS-treated group displayed a marked increase in colonic MPO levels when compared with the vehicle group. Notably, oral treatment with AS605240 (10 and 30 mg·kg^{-1}) significantly reduced MPO levels in mouse colon tissue.

The severity of colon inflammation was further evaluated by histological examinations (Figure 2B, C). Three days following TNBS administration, colons exhibited disruption of the epithelial barrier and transmural inflammation characterized by intense infiltration of inflammatory cells (predominantly neutrophils), corroborating the MPO assay. This cell influx was associated with ulcerations, loss of globule cells, marked destruction in the crypts and fibrosis throughout the colon. Of note, oral treatment with AS605240 (30 mg·kg^{-1}) or with dexamethasone markedly improved these signs, restoring the histological appearance of the mucosa and submucosa and decreasing the loss of epithelial cells and mucosal ulceration, when compared with the TNBS-treated group (Figure 2B, C).

AS605240 administration suppresses the levels and expression of colonic pro-inflammatory cytokines in mice with TNBS-induced colitis

Characteristically, innate immune responses are activated during the progression of IBD and up-regulate the expression of pro-inflammatory cytokines and chemokines such as IL-1β, IL-6, TNF-α, CXCR2 and CXCL1/KC (Ferretti *et al.*, 1994; Bento *et al.*, 2008). Since AS605240 decreased cell infiltration and colitis-induced damage, we investigated whether oral treatment with this PI3Kγ inhibitor could also modulate the levels and expression of IL-1R, CXCL1/KC, CXCR2/MIP-2 or TNF-α in colonic tissue. Seventy-two hours following colitis induction, the levels of IL-1β, KC and MIP-2 were markedly increased (Figure 3A to C) in the colonic tissue from the TNBS-treated group. AS605240 treatment (30 mg·kg^{-1}, p.o.) significantly diminished IL-1β, CXCL1/KC and MIP-2 levels (Figure 3A–C). Similar results were observed in colons from dexamethasone-treated animals (Figure 3A to C). We investigated further, using real-time quantitative PCR, whether the anti-inflammatory effects of AS605240 were also linked to the decrease in TNF-α expression in colonic tissue. As shown in Figure 3D, low expression of TNF-α was detected in the colonic tissue of the vehicle control group; however, this value was markedly increased after TNBS administration. AS605240 (30 mg·kg^{-1}), given orally following TNBS administration, significantly inhibited the up-regulated mRNA level of TNF-α (Figure 3D).

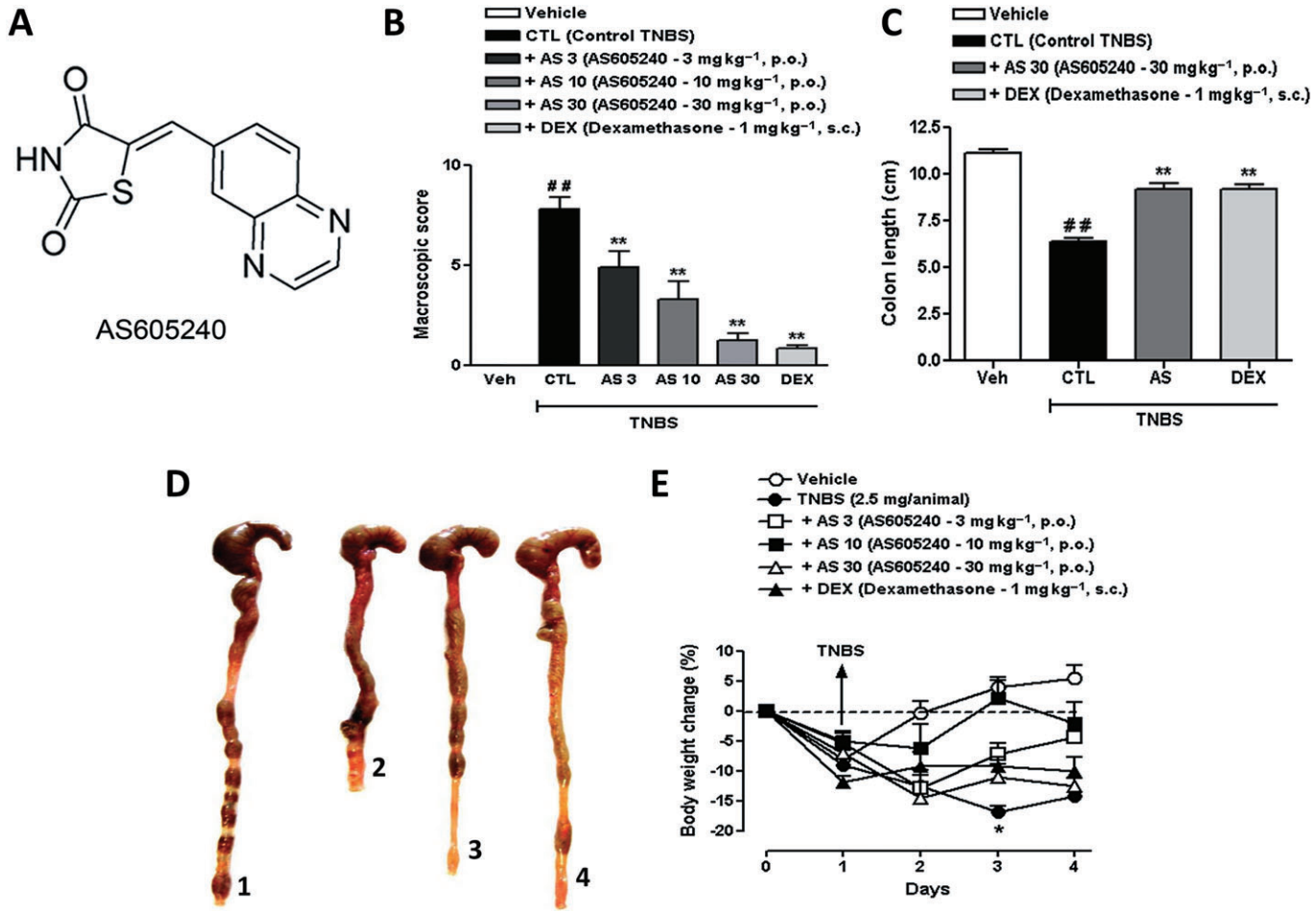

Figure 1

Therapeutic treatment with AS605240 protects CD1 mice against TNBS-induced acute colitis. (A) Chemical structure of AS605240, a relatively selective PI3Kγ inhibitor. Mice were given 100 μL of the TNBS (in 35% ethanol) and after 24 h, treated with AS605240 (3, 10 and 30 mg·kg^{-1}, p.o.) or with vehicle or dexamethasone (1 mg·kg^{-1}, s.c.). (B) Macroscopic score and (C) colon length of colitis. (D) Representative photograph of colons from day 3 after the induction of TNBS-colitis. 1, Vehicle-treated control; 2, TNBS-treated; 3, TNBS plus AS605240 (30 mg·kg^{-1}, p.o.); 4, TNBS plus dexamethasone (1 mg·kg^{-1}, s.c.). (E) The time-course of body weight changes on day 3 after TNBS-induced colitis. Each column represents the mean ± SEM of 8 to 10 mice per group. ##$P < 0.01$ versus vehicle-treated control group; *$P < 0.05$; **$P < 0.01$ versus TNBS-treated group. PI3Kγ, phosphoinositide 3-kinase-γ; TNBS, trinitrobenzene sulphonic acid.

AS605240 treatment inhibits inflammatory cytokine release from primary cultures of neutrophils without interfering with intestinal epithelial cells

The inflammatory cells present in the colon after administration of TNBS produce cytokines and chemokines; hence, any observed reduction of these colonic mediators could be just a consequence of decreased cell migration. To clarify whether AS605240 also diminishes the production of inflammatory mediators or modifies the profile of the cytokines produced by the inflammatory and intestinal epithelial cells, we used a primary culture of neutrophils and intestinal epithelial cells and evaluated the levels of IL-1β, MIP-2 and CINC-1 after *in vitro* stimulation with LPS (100 ng·mL^{-1}), an important component of colitis-induced damage. Cultured neutrophils stimulated with LPS (100 ng·mL^{-1}) displayed a marked increase in IL-1β and MIP-2 production compared to basal levels after 4 h following inflammatory stimulation (Figure 3E). AS605240 (10 and 30 μg·mL^{-1}) significantly inhibited MIP-2 production in neutrophils, without affecting the increase in IL-1β production (Figure 3E). We next investigated whether this inhibitor of PI3Kγ could alter chemokine release, such as CINC-1, in intestinal epithelial cells stimulated with LPS. To perform these studies, intestinal epithelial cells were stimulated with LPS (100 ng·mL^{-1}) for 4 h in the presence or absence of AS605240 (3 and 10 μg·mL^{-1}) or vehicle, and the CINC-1 level was measured as described above. As shown in Figure 3F, LPS-stimulation resulted in a significant increase in CINC-1 level. However, AS605240 (3 and 10 μg·mL^{-1}) failed to reduce CINC-1 levels in the intestinal epithelial cells after LPS stimulation (Figure 3F). Importantly, AS605240 (3, 10 and 30 μg·mL^{-1}) did not alter the basal production of any of these cytokines and chemokine in control neutrophils or intestinal

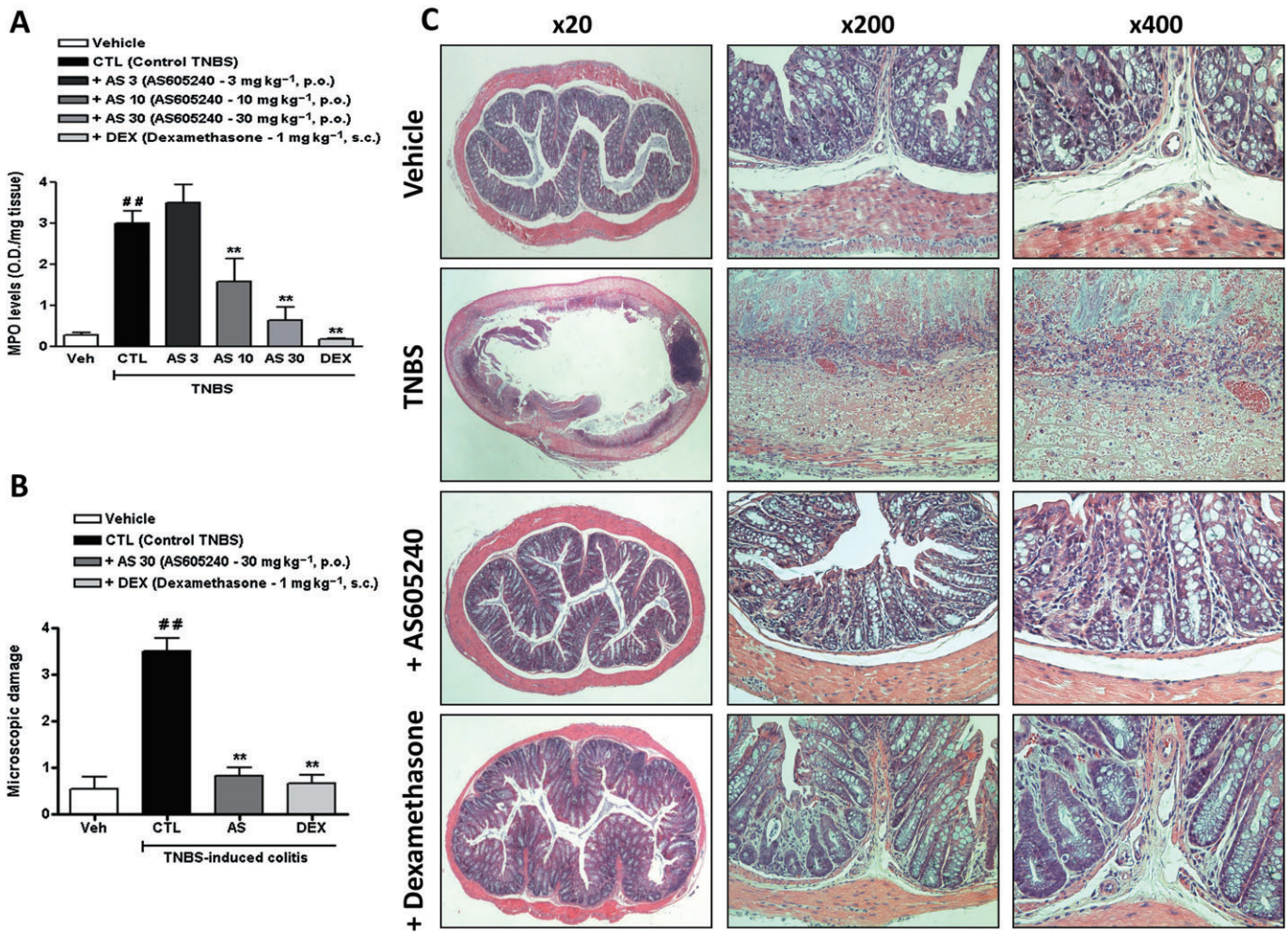

Figure 2

Therapeutic treatment with AS605240 reduces the severity of TNBS-induced acute colitis. Mice were given 100 μL of the TNBS (in 35% ethanol) and after 24 h, treated with AS605240 (3, 10 and 30 mg·kg^{-1}, p.o.) or with vehicle or dexamethasone (1 mg·kg^{-1}, s.c.). (A) The effect of the therapeutic AS605240 treatment on the infiltration of neutrophils into the colonic tissue of mice with TNBS-induced colitis, myeloperoxidase (MPO) levels were determined in colon tissue. (B) The effect of AS605240 on histological colitis score was determined. The parameters of the colitis score for each mouse were recorded on day 3 after TNBS-induced colitis. (C) Representative histological sections were examined microscopically after H&E staining with magnification ×20, ×200 and ×400. The images are representative of at least four mice per group. Each column represents the mean ± SEM of 8 to 10 mice per group. ##P < 0.01 versus vehicle-treated control group; **P < 0.01 versus TNBS-treated group. TNBS, trinitrobenzene sulphonic acid.

epithelial cells (i.e. incubated in the absence of LPS) (Figure 3E, F). These data, allied to those presented before, allow us to suggest that the anti-inflammatory effect displayed by AS605240 might be related, at least in part, to the inhibition of cytokines and chemokines produced by polymorphonuclear leucocytes (PMN) without affecting intestinal epithelial cells. However, further experiments are needed to clarify whether AS605240 treatment could have some effect on other inflammatory cells, such as macrophages.

AS605240 prevents apoptotic cell death after TNBS-induced colitis

Disruption of intestinal homeostasis involving an imbalance between proliferation and apoptosis of enterocytes has been implicated in the pathogenesis of intestinal inflammation (Martinez *et al.*, 2006). Therefore, we determined whether AS605240 affects apoptosis of cells in colon tissue in TNBS-induced acute colitis. Mucosal ulceration and the loss of epithelial cells were accompanied by increased apoptosis as demonstrated by the increased expression of caspase-3-positive cells. In the vehicle group, only low levels of apoptosis were detected by the caspase-3-cleaved antibody, in contrast to the extensive apoptosis of epithelial cells in mice with TNBS-induced colitis (Figure 4A to E). Remarkably, oral treatment with AS605240 (30 mg·kg^{-1}) significantly prevented apoptotic cell death and preserved the normal structure of the epithelial cells (Figure 4A–E).

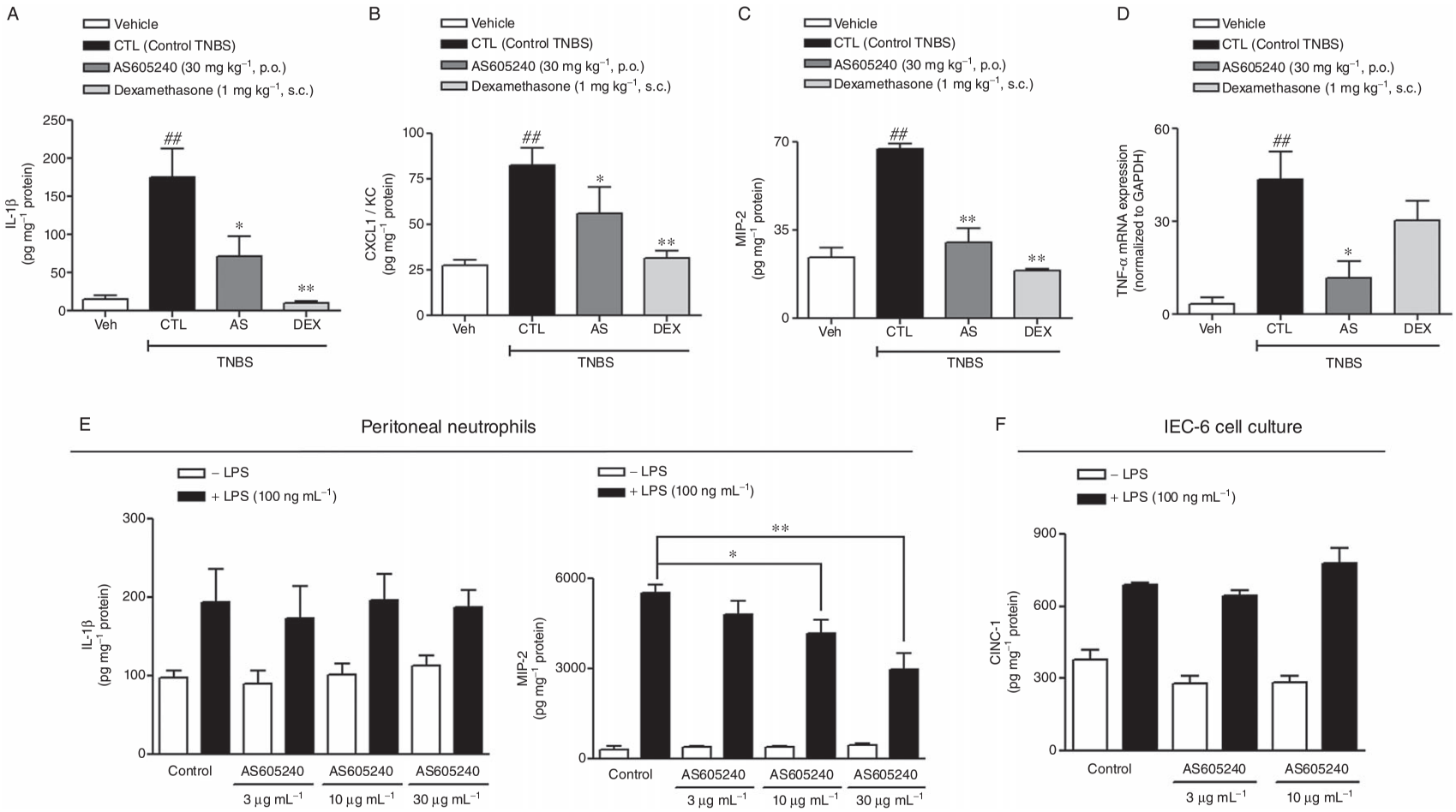

Figure 3

Treatment with AS605240 reduces pro-inflammatory cytokine and chemokine production in colonic tissue and in neutrophil cultures. Mice were given 100 μL of the TNBS (in 35% ethanol) and after 24 h, treated with AS605240 (30 mg·kg^{-1}, p.o.) or with vehicle or dexamethasone (1 mg·kg^{-1}, s.c.). (A) IL-1β, (B) CXCL-1/KC, (C) MIP-2 levels and (D) TNF-α mRNA expression were evaluated in colonic tissue at 72 h following TNBS administration. The cytokine levels were assayed by ELISA and real time-polymerase chain reaction, respectively. Real time PCR assays were performed in duplicate and with three mice per group. GAPDH mRNA was used to normalize the relative amount of mRNA. Peritoneal neutrophils and intestinal epithelial cells (IEC-6 cell culture) were stimulated *in vitro* with LPS (100 ng·mL^{-1}) for 4 h, in the presence or absence of AS605240 (3, 10 and 30 μg·mL^{-1}). (E) IL-1β and MIP-2 production by peritoneal neutrophils and (F) CINC-1 production by IEC-6 cell culture. Each column represents the mean ± SEM of 8 to 10 mice per group. ##$P < 0.01$ versus vehicle-treated control group; *$P < 0.05$; **$P < 0.01$ versus TNBS- and LPS-treated group. TNBS, trinitrobenzene sulphonic acid; IL, interleukin; TNF-α, tumour necrosis factor-α.

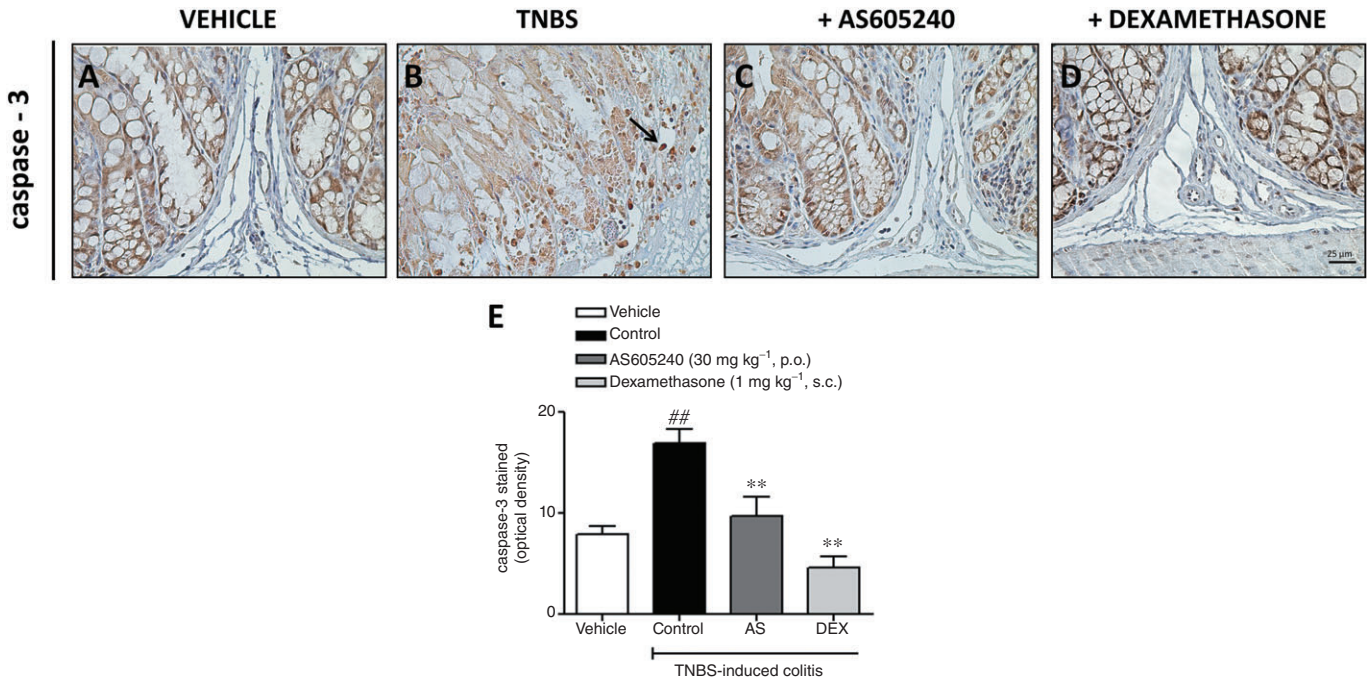

Figure 4

AS605240 inhibits apoptotic cell death in colonic tissue after TNBS-induced colitis. (A) Representative sections of colonic tissues from mice with colitis and treated with vehicle (B) TNBS alone (C) TNBS plus AS605240 (30 mg·kg^{-1}, p.o.) or (D) TNBS plus dexamethasone (1 mg·kg^{-1}, s.c.) were obtained on day 3 for immunohistochemical assays. (E) Graphical representation of the immunostaining for caspase-3-cleaved antibody expression evaluated in colon tissue. The black arrows indicate positively stained areas. Scale bar corresponds to 25 µm and applies throughout. Each column represents the mean ± SEM of 8 to 10 mice per group. ##$P < 0.01$ versus vehicle-treated control group; *$P < 0.05$; **$P < 0.01$ versus TNBS-treated group. TNBS, trinitrobenzene sulphonic acid.

AS605240 inhibits MMP-9 and VEGF expression induced by TNBS

Tissue injury observed in experimental colitis is at least in part mediated by MMP-9 (Garg *et al.*, 2009). Furthermore, increased expression of this protease has been observed in patients with IBD, where it is suggested to have an important role by inducing mucosal proteolysis, tissue ulceration and fistula formation (Naito and Yoshikawa, 2002). Our present data supported this observation, by demonstrating that TNBS-induced colitis increased MMP-9 levels (Figure 5A–I). Interestingly, therapeutic treatment with AS605240 significantly reduced this protease expression (Figure 5A–I) in colonic tissue. It has been suggested that MMP-9, by modulating VEGF, also induces angiogenesis (Arihiro *et al.*, 2001), a phenomenon that is increased in patients with IBD (Danese *et al.*, 2006). In accord with previous data from the literature (Bento *et al.*, 2008), TNBS induced a significant increase in VEGF levels (Figure 6A to E). Notably, treatment with AS605240 (30 mg·kg^{-1}, p.o.) or with dexamethasone significantly inhibited VEGF levels in colon tissue (Figure 6A–E).

AS605240 blocks TNBS-induced NF-κB activation in colonic tissue

The expression of a number of inflammatory mediators is controlled by the transcription factor NF-κB (Ledeboer *et al.*, 2005), and its inhibition is able to prevent experimental colitis (Neurath and Pettersson, 1997). To further define some of the colitis-induced signalling systems, which are modulated by AS605240, we assessed the effects of AS605240 on NF-κB activation. TNBS induced marked phosphorylation of NF-κB in the colonic tissue (Figure 7A to I), while therapeutic treatment with AS605240 significantly reduced NF-κB activation (Figure 7A–I).

AS605240 directly affects function and activity of IL-10 producing regulatory T cells

NF-κB activation can be inhibited by anti-inflammatory cytokines, namely IL-10 (Wang *et al.*, 1995), which is produced by some T-cell subsets, such as regulatory lymphocytes (Treg). Indeed, CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells have been shown to ameliorate inflammatory colitis mainly by the production of IL-10 and TGF-β (Maloy and Powrie, 2001; Liu *et al.*, 2003). Additionally, it has been suggested that reduced activity of the PI3K pathway is required for FoxP3 expression and the suppressive activity of Tregs (Crellin *et al.*, 2007; Sauer *et al.*, 2008). Hence, in this set of experiments, we investigated whether treatment with AS605240 could up-regulate IL-10 levels and expression in colonic tissue and whether this up-regulation is associated with the increase in CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells. As shown in Figure 8A and B, TNBS-treated mice showed a reduction in IL-10 protein and mRNA levels. Interestingly, AS605240 administration (30 mg·kg^{-1}, p.o.) resulted in a marked increase in this

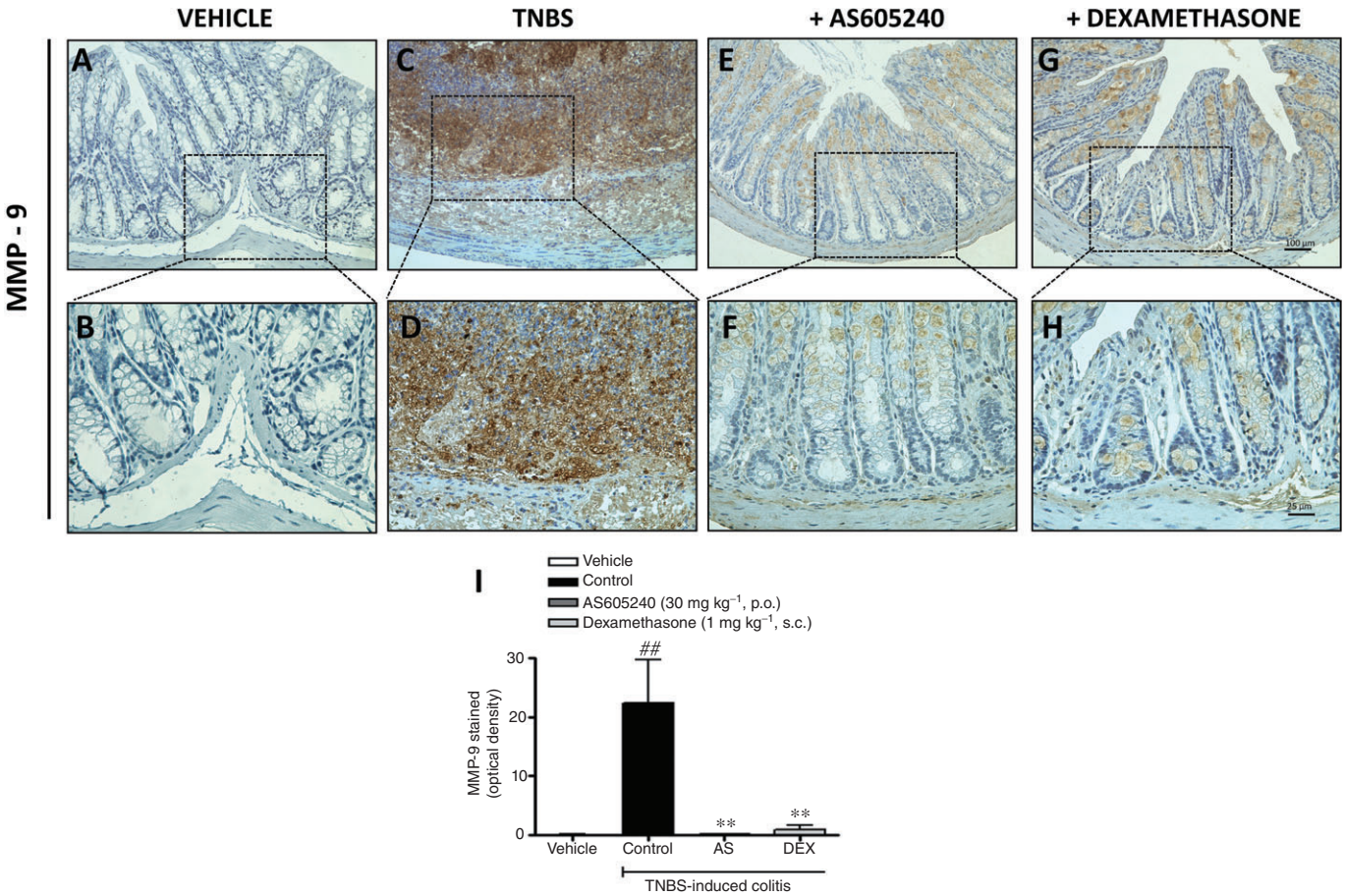

Figure 5

PI3Kγ inhibitor blocks TNBS-induced MMP-9 up-regulation. MMP-9 immunohistochemical analysis was performed 3 days after the administration of 100 µL of TNBS (in 35% ethanol) or with vehicle in colonic tissues. Vehicle, TNBS plus AS605240 (AS, 30 mg·kg^{-1}, p.o.) or TNBS plus dexamethasone (1 mg·kg^{-1}, s.c.) were administered 24 h after TNBS administration. (A–H) Representative images of MMP-9 immunoreactivity in colon tissue. Scale bar corresponds to 100 and 25 µm, respectively, and applies throughout. (I) Graphical representation of the immunostaining for MMP-9 expression evaluated in colon tissue. Each column represents the mean ± SEM of 8 to 10 mice per group. ##$P < 0.01$ versus vehicle-treated control group; **$P < 0.01$ versus TNBS-treated group. PI3Kγ, phosphoinositide 3-kinase-γ; TNBS, trinitrobenzene sulphonic acid; MMP-9, matrix metalloproteinase-9.

cytokine protein and mRNA levels in the colonic tissue after TNBS administration ($P < 0.05$) (Figure 8A, B).

Next, we investigated whether therapeutic treatment with AS605240 could increase CD25 and FoxP3 expression in colonic tissue. Only very low expression of CD25 and FoxP3 were detected in the colonic tissue of naïve and TNBS-treated groups (Figure 8C, D). Notably, oral treatment with AS605240 (30 mg·kg^{-1}) significantly increased the mRNA levels of both CD25 and FoxP3 in the colonic tissue (Figure 8C, D). To further support our hypothesis that the therapeutic effect of AS605240 might be mediated via the induction of CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells, we performed cellular analysis of the lymphocytes present in the colon tissue by flow cytometry. Hence, we isolated LPLs (CD4$^+$ T cells) from either untreated or AS605240-treated colitis mice. As shown in Figure 8E, the vehicle-treated (–TNBS) group and the TNBS control group had a similar percentage of FoxP3$^+$ Treg cells within the CD4$^+$CD25$^+$ T cells of the LPLs. Consistent with the anti-inflammatory properties of AS605240, mice given AS605240 had a reproducibly marked increase in the percentage of FoxP3$^+$ cells within the CD4$^+$CD25$^+$ compartment of the LPLs (Figure 8E). Additionally, the absolute number of CD4$^+$CD25$^+$FoxP3$^+$ cells in the colon tissue was significantly higher in AS605240-treated mice compared with the TNBS control group or the animals without colitis (Figure 8F). Recently, it was shown that the curative activity of CD4$^+$CD25$^+$ Treg is also critically dependent on IL-10. So to further support the suggested induction of CD4$^+$CD25$^+$ Treg activity by AS605240, we used an IL-10R monoclonal antibody (mAb) to block IL-10 function *in vivo*. The dose for the anti-IL-10R was optimized according to recently published studies focussing on the role of IL-10 for CD4$^+$CD25$^+$ Treg in experimental colitis and experimental allergic encephalomyelitis with some modifications (Mekala *et al.*, 2005; Bolpetti *et al.*, 2010). Treatment with control IgG or IL-10R mAb alone did not result in a significant change in disease severity and intensity of inflammation. As already indicated above, AS605240 treatment markedly reversed disease progression.

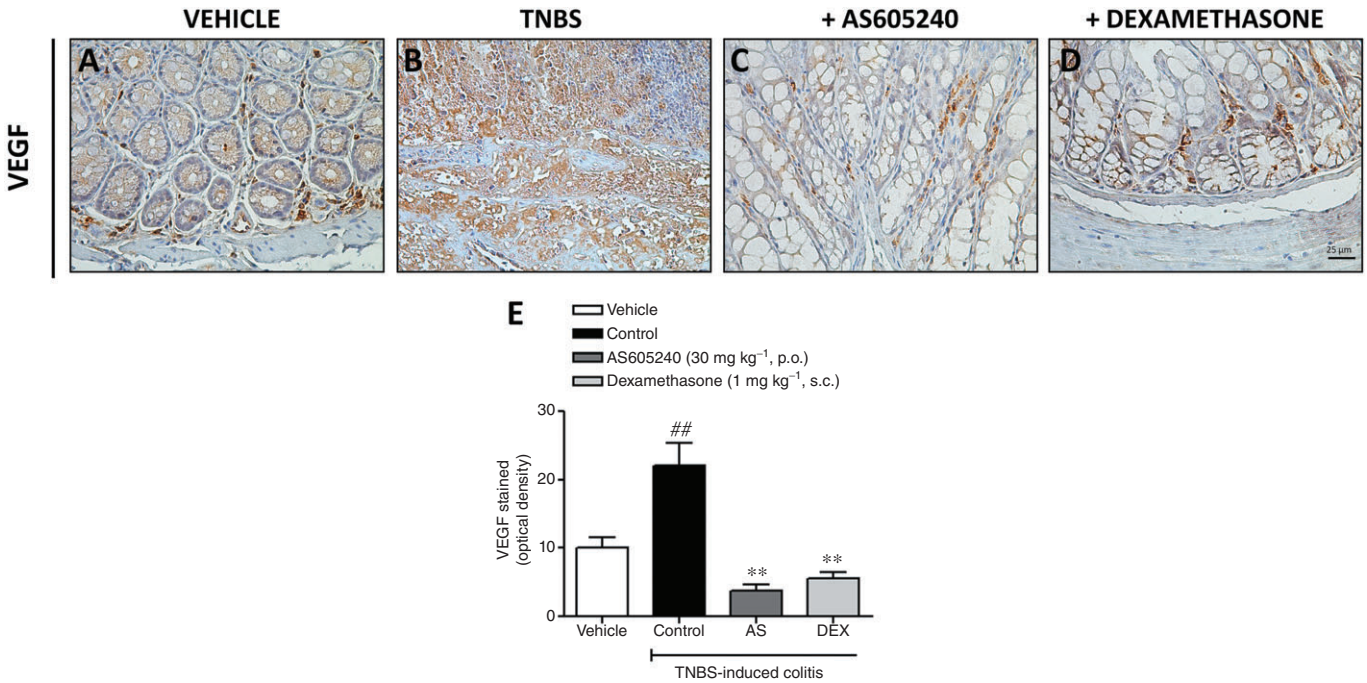

Figure 6

AS605240 treatment inhibits VEGF expression in colonic tissue after TNBS-induced colitis. Representative sections of colonic tissues from mice with colitis and treated with (A) vehicle (B) TNBS alone (C) TNBS plus AS605240 (30 mg·kg^{-1}, p.o.) or (D) TNBS plus dexamethasone (1 mg·kg^{-1}, s.c.) were obtained on day 3 for immunohistochemical assays. (E) Graphical representation of the immunostaining for VEGF expression evaluated in colon tissue. Scale bar corresponds to 25 µm and applies throughout. Each column represents the mean ± SEM of 8 to 10 mice per group. ##$P < 0.01$ versus vehicle-treated control group; *$P < 0.05$; **$P < 0.01$ versus TNBS-treated group. VEGF, vascular endothelial growth factor; TNBS, trinitrobenzene sulphonic acid.

However, the anti-IL-10R mAb evidently abolished the therapeutic potential of AS605240 in TNBS-induced colitis (Figure 8G–J). These data further support our hypothesis that AS605240 increases the function and activity of $CD4^+CD25^+FoxP3^+$ cells in the colon tissue by a mechanism that is critically dependent on IL-10.

Taken together, our data indicate that the mechanisms underlying AS605240 anti-inflammatory actions are associated with its ability to prevent NF-κB activation and increase the function and activity of $CD4^+CD25^+FoxP3^+$ regulatory T cells producing IL-10. This is in turn associated with the inhibition of apoptosis and MMP-9 and VEGF expression, as well as the attenuation of the levels and expression of pro-inflammatory mediators in the colonic tissue.

Discussion

There is now increasing evidence that PI3Kγ plays a regulatory role in immune responses (Vanhaesebroeck *et al.*, 2005). Among other factors, such as genetic susceptibility, persistent infection, defective microbial clearance and/or mucosal barrier function, altered immune responses largely contribute to the pathogenesis of IBD (Sartor, 1997). The data in this study clearly indicate that the significant anti-inflammatory properties of AS605240, a relatively selective inhibitor of PI3Kγ, in T_H1-mediated colitis resulted from a marked increase in the functional activity of IL-10-producing $CD4^+CD25^+$ Treg. Our results clearly demonstrate that AS605240 decreased VEGF and MMP-9 expression, neutrophil influx and the levels of pro-inflammatory mediators (namely IL-1β, KC and MIP-2) in colonic tissues and in primary cultures of neutrophils through the inhibition of NF-κB.

Several animal models of intestinal inflammation have been established, although only a few of them partially resemble human IBD (Elson *et al.*, 1995). TNBS-induced colitis in mice, which is typified by colonic transmural damage caused by hapten-induced delayed hypersensitivity, has been used as a model to study human Crohn's disease (Elson *et al.*, 1995). In the present study, it was found that after the establishment of colitis, the systemic curative therapeutic treatment with AS605240 administration significantly reduced colon oedema, attenuated macroscopic and histological damage and restored colon length; nonetheless, this PI3Kγ inhibitor, as well as dexamethasone, failed to recover the loss of body weight. Recent studies have suggested that the synthetic glucocorticoid dexamethasone increases leptin mRNA expression and plasma level (Lee *et al.*, 2007; Jahng *et al.*, 2008) and induces long-lasting hyperleptinaemia in rats (Caldefie-Chezet *et al.*, 2001). Furthermore, repeated treatment with dexamethasone at a dose of 1 mg·kg^{-1}·day^{-1} has been shown to increase the density of 5-hydroxytryptamine receptors (5-HT) receptors in the rat frontal cortex and decreased body weight (Katagiri *et al.*, 2001). In summary, these results suggest that dexamethasone suppressed the food intake and weight gain by modulating

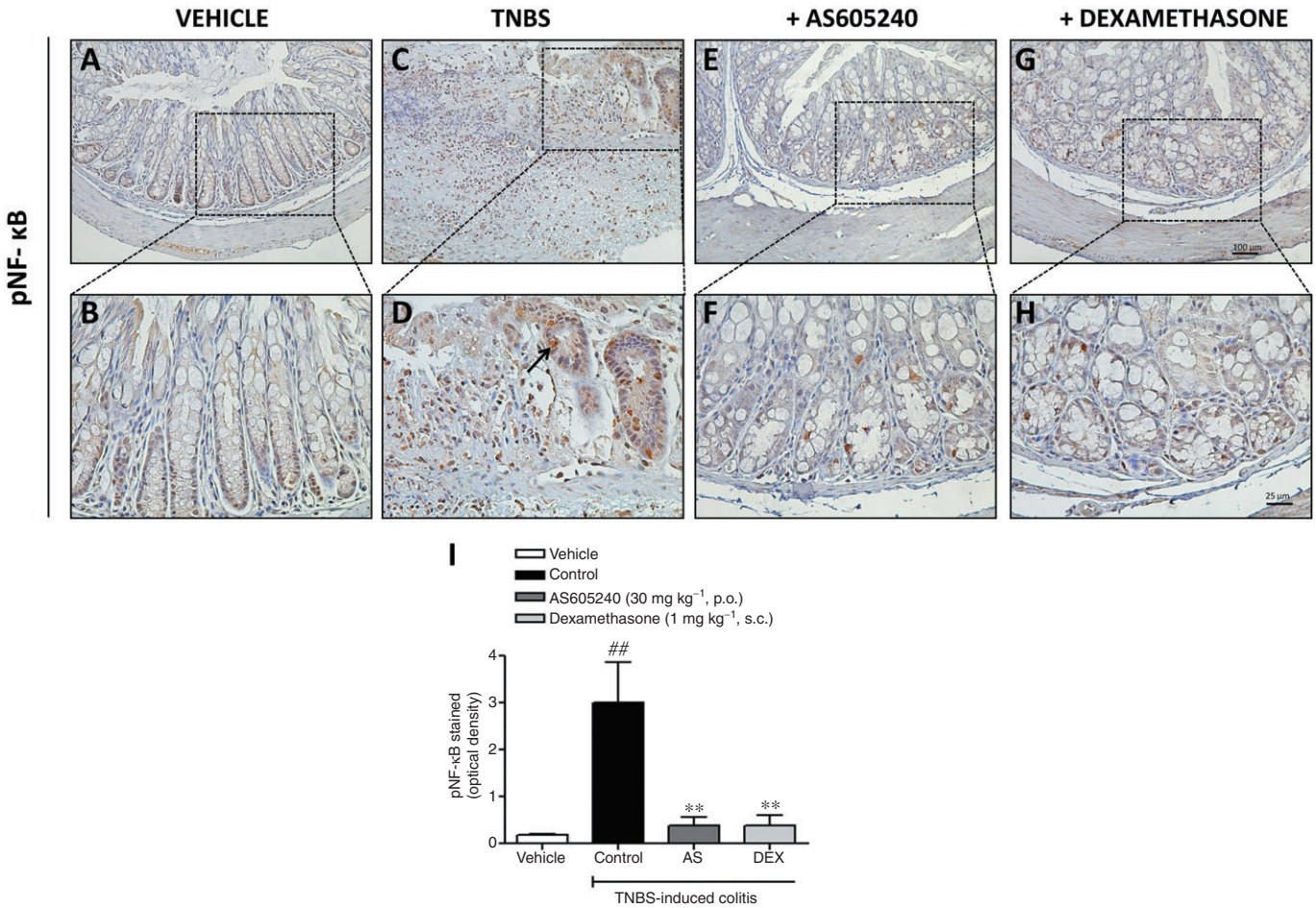

Figure 7

PI3Kγ inhibitor prevents NF-κB activation after TNBS-induced colitis. Phosphorylation of NF-κB was performed 3 days after administration of 100 μL of TNBS (in 35% ethanol) or with vehicle in colonic tissues. Vehicle, TNBS plus AS605240 (AS, 30 mg·kg^{-1}, p.o.) or TNBS plus dexamethasone (1 mg·kg^{-1}, s.c.) were administered 24 h after TNBS administration. (A-H) Representative images of phospho-NF-κB immunoreactivity in colon tissue. Scale bar corresponds to 100 and 25 μm, respectively, and applies throughout. (I) Graphical representation of the average pixel intensity of phospho-NF-κB levels evaluated in colon tissue. Each column represents the mean ± SEM of 8 to 10 mice per group. ##$P < 0.01$ versus vehicle-treated control group; **$P < 0.01$ versus TNBS-treated group. PI3Kγ, phosphoinositide 3-kinase-γ; NF-κB, nuclear factor-κB; TNBS, trinitrobenzene sulphonic acid.

leptin and the 5-HT receptors. In our study, the oral treatment with AS605240 showed a similar anti-inflammatory effect as dexamethasone, though both failed to recover the loss of body weight. This discrepancy between efficacy and failure to prevent body weight loss by AS605240 could be due, at least in part, to an effect on the leptin pathway and 5-HT receptor. However, further experiments are needed to confirm this hypothesis.

A substantial amount of evidence suggests that neutrophils have a crucial role in the pathogenesis of colitis (Wallace *et al.*, 1998). The infiltration of PMN into the colon tissue is believed to play a key role in mediating tissue damage and clinical symptoms in humans and experimental colitis, and the inhibition of neutrophil influx and/or activation might be an attractive and relevant therapeutic strategy to treat IBDs (White *et al.*, 1998). PI3Kγ plays a crucial role in the movement of leucocytes from the bloodstream to sites of injury or inflammation in response to chemokines (Van Dop *et al.*, 2010). In mouse models of several chronic inflammatory diseases, the absence or block of PI3Kγ has previously been shown to ameliorate disease activity (Camps *et al.*, 2005; Gonzalez-Garcia *et al.*, 2010). To clarify this hypothesis, we used a primary culture of neutrophils to show that AS605240 reduces only MIP-2 levels during LPS-induced inflammation without affecting intestinal epithelial cells. This indicates that AS605240 not only decreases cell migration but also modulates the activation of inflammatory cells.

Apart from the imbalance in the immune system, the process of angiogenesis has recently been demonstrated to be one of the major contributors to the pathogenesis of IBD (Danese *et al.*, 2006). Furthermore, intestinal inflammation and subsequent colitis appear to be caused by disruption of intestinal homeostasis and integrity, and up-regulated inducible nitric oxide synthase (iNOS) expression in gut mucosa has been shown to cause apoptosis of epithelial cells in the gut mucosa (Yue *et al.*, 2001). To further evaluate the

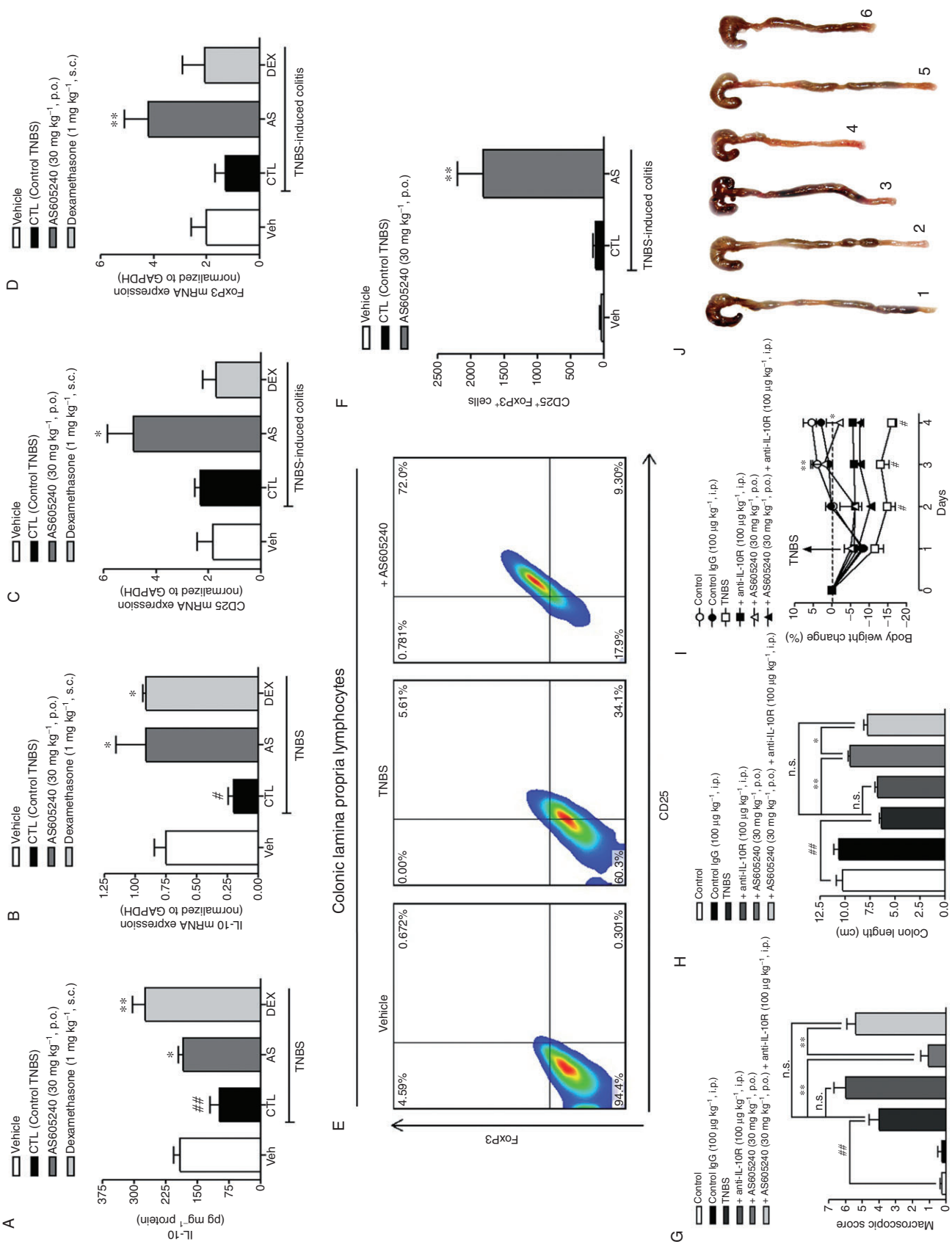
A
Vehicle
CTL (Control TNBS)
AS605240 (30 mg kg−1, p.o.)
Dexamethasone (1 mg kg−1, s.c.)
IL-10 (pg mg−1 protein)
Veh
CTL
AS
DEX
TNBS
B
IL-10 mRNA expression (normalized to GAPDH)
TNBS
C
CD25 mRNA expression (normalized to GAPDH)
TNBS-induced colitis
D
FoxP3 mRNA expression (normalized to GAPDH)
TNBS-induced colitis
E
Colonic lamina propria lymphocytes
Vehicle
+ AS605240
TNBS
4.59%
0.672%
94.4%
0.301%
0.00%
5.61%
60.3%
34.1%
0.781%
72.0%
17.9%
9.30%
FoxP3
CD25
F
Vehicle
CTL (Control TNBS)
AS605240 (30 mg kg−1, p.o.)
CD25+ FoxP3+ cells
TNBS-induced colitis
G
Control
Control IgG (100 μg kg−1, i.p.)
TNBS
+ anti-IL-10R (100 μg kg−1, i.p.)
+ AS605240 (30 mg kg−1, p.o.)
+ AS605240 (30 mg kg−1, p.o.) + anti-IL-10R (100 μg kg−1, i.p.)
Macroscopic score
H
Colon length (cm)
I
Body weight change (%)
TNBS
Days
J
1
2
3
4
5
6

Figure 8

AS605240 treatment increases regulatory T cells producing interleukin-10 (IL-10) after TNBS-induced acute colitis. Mice were given 100 μL of TNBS (in 35% ethanol) and after 24 h, treated with AS605240 (30 mg·kg^{-1}, p.o.) or with vehicle or dexamethasone (1 mg·kg^{-1}, s.c.). (A) IL-10 protein level was evaluated in colonic tissue at 72 h following TNBS administration. The cytokine levels were assayed by ELISA. Real-time PCR data shows (B) IL-10 (C) CD25 and (D) FoxP3 mRNA levels in colonic tissue 72 h after TNBS administration. Real-time PCR assays were performed in duplicate and with three mice per group. GAPDH mRNA was used to normalize the relative amount of mRNA. (E) Flow cytometry analysed for the percentage of $CD25^+FoxP3^+$ cells within the $CD4^+$ population and (F) absolute number of $CD4^+CD25^+FoxP3^+$ cells in colonic lamina propria lymphocytes 72 h after TNBS administration. Clinical parameters of acute TNBS colitis following *in vivo* block of IL-10 were evaluated in mice treated i.p. with the anti-IL-10R mAb (100 μg·kg^{-1}) alone, AS605240 (30 mg·kg^{-1}, p.o.) or the combination of AS605240 (30 mg·kg^{-1}) and the anti-IL-10R mAb (100 μg·kg^{-1}). Control mice received normal IgG at the same dose. (G) Macroscopic score, (H) colon length and (I) time-course of body weight change on day 3 after TNBS-induced colitis. (J) Representative photograph of colons from 1, vehicle-treated control; 2, vehicle plus control IgG (100 μg·kg^{-1}, i.p.); 3, TNBS-treated; 4, TNBS plus anti-IL10R mAb (100 μg·kg^{-1}, i.p.); 5, AS605240 (30 mg·kg^{-1}, p.o.); 6, AS605240 (30 mg·kg^{-1}, p.o.) plus anti-IL-10R mAb (100 μg·kg^{-1}, i.p.) mice. Each column represents the mean ± SEM of 8 to 10 mice per group. ##$P < 0.01$ versus vehicle-treated control group; *$P < 0.05$; **$P < 0.01$ versus TNBS-treated group. TNBS, trinitrobenzene sulphonic acid.

mechanisms of the anti-inflammatory action of AS605240 on TNBS-induced colitis, we assessed the protein expression of the angiogenic cytokine VEGF and the apoptotic cell death rate in colonic tissue. Importantly, the systemic treatment with AS605240, similar to dexamethasone, significantly and completely reversed the high tissue content of VEGF and fewer apoptotic epithelial cells were present in the colonic tissues of AS605240-treated mice when compared with the TNBS control group. Furthermore, the PI3K pathway has been shown to be an important factor for survival in monocytes (Kelley *et al.*, 1999), neutrophils (Cowburn *et al.*, 2002) and eosinophils (Pinho *et al.*, 2005; Sousa *et al.*, 2009) and a previous study demonstrated that treatment with wortmannin at the peak of eosinophilic inflammation decreased Akt phosphorylation and promoted eosinophil apoptosis (Pinho *et al.*, 2005), suggesting that the activation of the PI3K–Akt–mTOR pathway is extremely important for providing survival signals (Song *et al.*, 2005). However, whether apoptosis of epithelial cells in colonic tissues is inhibited directly by AS605240 or indirectly by AS605240-mediated iNOS suppression in the mucosa requires further investigation.

The MMPs have been demonstrated to exert an important regulatory role in the pathogenesis of inflammatory diseases and cancer (Hu *et al.*, 2007). In particular, activation of MMPs is thought to be involved in the pathogenesis of human IBDs (Kirkegaard *et al.*, 2004), as well as in the disease progression of experimental colitis (Ohkawara *et al.*, 2002; Medina and Radomski, 2006). Up-regulated expression of MMP-2, MMP-3, MMP-7 and MMP-9 has been observed in mice with DSS- or TNBS-induced colitis (Naito and Yoshikawa, 2005; Medina and Radomski, 2006) and elevated MMP-13 expression was detected in the colonic tissues of mice with DSS-induced colitis (Ohkawara *et al.*, 2002). Here, we demonstrated that AS605240 treatment suppressed the up-regulated expression of MMP-9 in acute colitis induced by TNBS in mice. Thus, blocking intestinal inflammation and pathoangiogenesis through the inhibition of MMP expression during IBD progression may, at least in part, explain how AS605240 attenuates experimental colonic inflammation.

It is now well accepted that excessive leucocyte recruitment triggered by chemokines into the injured colonic tissue plays a key role in the pathogenesis of IBD (Danese *et al.*, 2006; MacDermott *et al.*, 2008). Characteristically, innate immune responses are activated during the progression of IBD and up-regulate the expression of most pro-inflammatory cytokines and chemokines such as IL-1β, IL-6, TNF-α, KC and MIP-2 (Berndt *et al.*, 2007). In our TNBS model of acute colitis, oral treatment with AS605240 (30 mg·kg^{-1}) not only suppressed colonic infiltration of neutrophils but also suppressed intestinal levels of pro-inflammatory mediators, namely IL-1β, KC, MIP-2 and TNF-α. Previous studies have shown that PI3Kγ is able to regulate some crucial inflammatory functions including the chemotaxis, proliferation and cytokine production in leucocytes (Ohkawara *et al.*, 2002). In accordance with our data, it was recently demonstrated that T cells from PI3Kγ-null mice showed impaired proliferation and cytokine production of INF-γ and IL-2 when stimulated with Con-A (Sasaki *et al.*, 2000).

The NF-κB signalling cascade is a critical event in the regulation of immune and inflammatory responses and has been linked to the pathogenesis of colitis and other IBDs (Mattson and Camandola, 2001). NF-κB activation induces the expression of many cytokines, chemokines and adhesion molecules, which in turn mediate the recruitment and activation of immune cells (Karin *et al.*, 2002). Our results, using the phospho-p65 NF-κB antibody in the TNBS-induced colitis model, showed that AS605240 is able to inhibit the translocation of p65 into the nucleus, thus strongly suggesting that inhibition of NF-κB activation is a key mechanism through which this relatively selective PI3Kγ inhibitor modulates intestinal inflammation. These results are in agreement with a previous study that demonstrated that in lung neutrophils, LPS-induced activation of NF-κB and the production of IL-1β and TNF-α are inhibited by PI3K inhibition (Yum *et al.*, 2001). In addition, the inhibition of NF-κB by wortmannin, an inhibitor of PI3K-related protein kinase, has been reported in U937 (granulocytes), jurkat (T cells), HeLa and H4 glioma cells (Manna and Aggarwal, 2000). In contrast, most studies, especially those dealing with macrophages, indicate that PI3K inhibition augments the toll-like receptor (TLR)–induced activation of NF-κB. These conflicting results suggest that the effect of PI3K on NF-κB activation may depend on cell type. Thus, in macrophages, the inhibition of PI3K may augment the activation of NF-κB through GSK-3 activation. On the other hand, a considerable number of studies have reported that PI3K inhibition leads to the down-regulation of NF-κB in T cells (Lee *et al.*, 2005), B cells (Suzuki *et al.*, 2003) and many other cell types. One possible explanation for this discrepancy is that PI3K has multi-functional roles that may regulate various signalling pathways leading to NF-κB activation.

Here, our results suggest that after the blocking of PI3K by AS605240, there is an increase in the functional activity of $CD4^+CD25^+FoxP3^+$ Treg in the colon tissue and consequently a decrease in both inflammation and NF-κB activation.

As an important new aspect of the anti-inflammatory mechanism of AS605240, our results indicate that AS605240 strongly affects the activity of Treg *in vivo*. In the first set of experiments, we demonstrated that AS605240 led to a significant induction of IL-10, CD25 and FoxP3 expression in the colonic tissue after TNBS-induced colitis in mice. Recent work has confirmed that FoxP3 can be regarded as the most reliable Treg marker, being predominantly expressed in $CD4^+CD25^+$ Treg (Ziegler, 2006). The induction of $CD4^+CD25^+$ $FoxP3^+$ Treg was paralleled by an increase in IL-10 levels in the colonic tissue. Different subpopulations of Treg are responsible for immunological tolerance in the gut, thereby preventing mucosal inflammation, and their regulatory capacity has been linked to the expression of IL-10 and TGF-β (Coombes *et al.*, 2005).

To support the concept regarding the increase of functional activity of $CD4^+CD25^+$ Treg following treatment with AS605240, we performed an analysis of the lymphocytes present in the colon tissue. LPLs $CD4^+$ T cells isolated from AS605240-treated mice showed a significant increase in CD25 and FoxP3 expression compared with those from control mice. The importance of $CD4^+CD25^+$ Treg in mediating the inhibition of colitis was also reported in another recent study (Read *et al.*, 2000). These results support the concept that the $CD4^+CD25^+$ $FoxP3^+$ T cells function as the major players in mediating the regulatory potential in experimental colitis. In addition, recent data have suggested that signalling through the PI3K–Akt–mTOR pathway has a negative effect on Treg cell thymic selection, as constitutively active Akt impairs the thymic differentiation of Treg as well as their conversion by TGF-β (Haxhinasto *et al.*, 2008; Sauer *et al.*, 2008), consistent with a positive effect of the kinase mTOR inhibitor rapamycin on Treg cell selection and population expansion (Haxhinasto *et al.*, 2008; Merkenschlager and von Boehmer, 2010; Ouyang *et al.*, 2010). These effects are probably related to the enhanced induction of FoxP3 and the corresponding dearth of effector cytokines that occurs after TCR stimulation of mature T cells lacking mTOR, which seems attributable to the TORC2 complex (Delgoffe *et al.*, 2009). In the final set of experiments, we blocked the IL-10 axis using an ultra-purified IL-10R mAb and found that the therapeutic effect of AS605240 was significantly inhibited in mice administered the anti-IL-10R mAb. In accordance with these observations, the findings in our present study underline the potential of AS605240 to ameliorate TNBS-induced colitis in mice by increasing the functional activity of IL-10-producing $CD4^+CD25^+$ $FoxP3^+$ T cells.

In summary, the present results show, for the first time, that pharmacological inhibition of PI3Kγ can effectively ameliorate TNBS-induced colitis in mice. Our findings also suggest PI3Kγ as a new target in colitis injury with the potential to modulate multiple stages of colonic damage, including both acute and chronic colitis. Although the PI3Kγ inhibitor is still far from being used clinically, its immunomodulatory properties offer us a novel therapeutic strategy in the prevention and treatment of human IBDs, notably Crohn's disease.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

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