JPET Fast Forward. Published on October 13, 2015 as DOI: 10.1124/jpet.115.228205
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JPET #228205

Encenicline, an α7 nicotinic acetylcholine receptor partial agonist, reduces immune cell infiltration in the colon and improves experimental colitis in mice

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Running title: Encenicline in colitis.

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Number of pages: 36

Number of tables: 0

Number of figures: 10

Number of references: 42

Number of words in the Abstract: 242

Number of words in the Introduction: 495

Number of words in the Discussion: 2050

Recommended section: Gastrointestinal, Hepatic, Pulmonary and Renal

List of non-standard abbreviations:

5-HT_3Rs - type 3 serotonin receptors

CNS - central nervous system

DMSO - dimethyl sulfoxide

DSS - dextran sulfate sodium

GALT - gut-associated lymphoid tissue

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HEX - hexamethonium

HPRT - hypoxanthine-guanine phosphoribosyltransferase

HTAB - hexadecyltrimethylammonium bromide

i.c. - intracolonic

i.p. - intraperitonealf

IHC - immunohistochemistry

IL - interleukin

LPS - lipopolysaccharide

MLA - methyllycaconitine

MPO - myeloperoxidase

nAChRs - nicotinic acetylcholine receptors

NF-κB - nuclear factor κB

PBS - phosphate buffered saline

RORγt - RAR-related orphan receptor

TNBS - 2,4,6-trinitrobenzene sulfonic acid

Abstract

The α7 pentamer nicotinic acetylcholine receptors (nAChRs) are a target in transduction of anti-inflammatory signals from the central nervous system (CNS) to the gastrointestinal (GI) tract. The aim of this study was to investigate the anti-inflammatory action of the novel α7 nAChR partial agonist, encenicline and to determine the mechanism underlying its activity. Anti-inflammatory activity of encenicline was evaluated using trinitrobenzenesulfonic acid (TNBS)- and dextran sulfate sodium (DSS)-induced models of colitis. Macroscopic score, ulcer score, colon length and thickness, as well as myeloperoxidase (MPO) activity were recorded. Immunohistochemistry (IHC) was used to measure the infiltration of immune cells in the colon. Furthermore, we employed flow cytometry to determine the effect of encenicline on frequencies of $FoxP3^+$ and IL-$17A^+$ T cells in the mouse colon. Encenicline attenuated TNBS- and DSS-induced colitis in mice via α7 nAChRs, as indicated by significantly reduced macroscopic parameters and MPO activity. Treatment with encenicline significantly reduced the infiltration of macrophages, neutrophils and B cells in the colon of TNBS-treated animals, as indicated by the IHC. In the TNBS model encenicline reduced the frequency of $FoxP3^+$ IL-$17A^+$ T cells in the colon. In the DSS model treatment with encenicline increased the frequency of $FoxP3^+$ T cells and reduced IL-$17A^+$ T cells. Stimulation of α7 nAChR with partial agonist encenicline alleviates colitis via alteration of the number and/or activation status of the immune cells in the gut, emphasizing a potential role of α7 nAChRs as a target for anti-colitic drugs.

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Introduction

Inflammatory bowel diseases (IBD), comprising of ulcerative colitis (UC) and Crohn's disease (CD) are a group of chronic, relapsing gastrointestinal (GI) disorders manifested mainly by imbalanced immunological response leading to an inflammatory state in the gut, disrupted motility and abdominal pain (Salaga *et al.*, 2013;Sobczak *et al.*, 2014). Although several pharmacological strategies have been employed to treat IBD, none of them entirely succeeded. Recent studies point towards a role of α7 homopentamer nicotinic acetylocholine receptors (nAChRs) in the cholinergic modulation of immune cell activity (de Jonge and Ulolla, 2007;Snoek *et al.*, 2010). The α7 nAChRs are located in both central and the peripheral nervous systems (de Jonge and Ulolla, 2007;Snoek *et al.*, 2010). In the periphery, α7 nAChRs participate in the cholinergic transmission and are involved in control of the heart rate, hormone secretion, GI motility and immunomodulation (de Jonge and Ulolla, 2007).

The main neurotransmitter of the vagal nerve is acetylcholine, which is thought to control the functions of immune cells via nAChRs. Borovikova *et al.*, (2000a) reported that acetylcholine significantly attenuated the release of pro-inflammatory, such as tumor necrosis factor-α (TNF-α), but not the anti-inflammatory cytokines, such as interleukin-10 (IL-10), in human macrophages in vitro. From a pharmacological point of view, nAChRs agonists are more efficient than acetylcholine in inhibition of inflammatory signaling and production of pro-inflammatory cytokines. For example, it has been demonstrated that activation of nAChRs by nicotine reduces, in a dose-dependent way, the release of TNF-α and IL-6 from isolated mice peritoneal macrophages stimulated with lipopolysaccharide (LPS) (de Jonge *et al.*, 2005). This observation is further supported by studies in animal models of GI tract disorders indicating that activation of vagal α7 nAChRs may effectively attenuate colitis by reduction of cytokine secretion from immune cells (de Jonge and Ulolla, 2007;Ji *et al.*, 2014) as well as clinical reports indicating that smoking and administration of nicotine (i.e. via

patches) may provide beneficial effect on colonic inflammation in UC patients (Srivastava *et al*., 1991;Pullan *et al*., 1994).

To explore the therapeutic potential of α7 nAChRs in the treatment of IBD, we tested the anti-inflammatory properties and mechanisms of action of a novel partial α7 nAChR agonist, encenicline which exhibits high efficacy at α7 nAChRs with the K_i value of 4.33 nM vs. [^{125}I]-α-bungarotoxin in rat brain homogenate (Prickaetrs *et al*., 2012) and is currently in the phase 3 clinical trial (ClinicalTrials.gov Identifier: NCT01969136). To assess the anti-inflammatory activity of encenicline, we used trinitrobenezenesulfonic acid (TNBS)- and dextran sulfate sodium (DSS)-induced models of colitis. To investigate the mechanism of action of encenicline, we used α7 nAChRs antagonist, methyllycaconitine (MLA). Moreover, we applied the ganglionic blocker hexamethonium (HEX) to investigate whether the blockade of peripherally-located nAChRs may abolish the potential ant-inflammatory action of encenicline. Using quantitative immunohistochemistry (IHC) and flow cytometry, we examined whether treatment with encenicline affects the number of immunocytes (macrophages, neutrophils, T cells and B cells) and changes the ration of pro- and anti-inflammatory T cell subtypes in the colonic tissue.

Materials and methods

Animals

Male balbC mice obtained from Animal Facility of the University of Lodz, Poland, weighing 22–26 g were used for all experiments. Mice were housed at a constant temperature (22-24ºC) and maintained in sawdust-lined plastic cages under a 12-hour light/dark cycle with free access to laboratory chow and tap water ad libitum. The study was carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and with the institutional recommendations. The experimental protocol was approved by the Local Ethical Committee for Animal Experiments in Lodz (# 589/2011).

Induction of colitis and assessment of colonic damage

TNBS model

Colitis was induced by intracolonic (i.c.) instillation of 2,4,6-trinitrobenzene sulfonic acid (TNBS), as described before (Fichna *et al.*, 2012). Briefly, mice were lightly anesthetized with 1 % isoflurane (Baxter Healthcare Corp., IL, USA) and TNBS (4 mg in 0.1 ml of 30% ethanol in saline) was instilled into the colon through a catheter inserted into the anus (3 cm proximally). Control animals received vehicle alone (0.1 ml of 30% ethanol in saline). Preliminary experiments demonstrated that the dose of TNBS used in this study induced reproducible colitis.

DSS model

Colitis was induced by the addition of DSS (4% w/v; molecular weigh 40,000; MP Biomedicals, Aurora, OH, Lot No. 5237K) to drinking water from day 0 to day 5. On days 6 and 7 animals received tap water (without DSS). Control animals received tap water throughout entire experiment. Animal body weight was monitored daily.

Pharmacological treatments

We used two different therapeutic regimens in the TNBS model. In the acute TNBS model therapeutic effect of encenicline was evaluated as follows: colitis was induced on day 0 and encenicline (3 mg/kg, i.p. twice daily) was administered from day 0 to day 2 with the first treatment 30 min before the induction of colitis. Animals were sacrificed on day 3 and the evaluation of colonic tissue damage was performed (Fig. 1A). The α7 nAChRs antagonist MLA (3 mg/kg i.p.) was administered 60 min before encenicline (n = 8).
The ganglionic blocker HEX was administered at the dose of 10 mg/kg i.p., 30 min prior to encenicline. In the semi-chronic TNBS model a curative treatment mode was applied: inflammation was induced on day 0 and animals received encenicline treatment (3 mg/kg, i.p. twice daily) between day 3 and day 6 (Fig. 1B). On day 7 mice were sacrificed and the evaluation of colonic damage was performed.

In DSS model animals were treated with encenicline (3 mg/kg, i.p. twice daily) either from day 0 to day 6 (Fig. 1C) with the first treatment 30 min before the addition of DSS to the drinking water, or from day 3 to day 6 (Fig. 1D). On day 7 mice were sacrificed and the evaluation of colonic damage was performed. In all experiments control animals received vehicle (i.p.) alone.

For the in vitro assay, a stock solution of encenicline in DMSO (10^{-2} M) was prepared and diluted accordingly.

Evaluation of colonic damage

TNBS model

Animals were sacrificed by cervical dislocation. The colon was removed, opened longitudinally, rinsed with phosphate buffered saline (PBS), and immediately examined. Macroscopic colonic damage was assessed by an established semiquantitative scoring system

by adding individual scores for ulcer, colonic shortening, wall thickness, and presence of hemorrhage, fecal blood, and diarrhea, as described before (Fichna *et al.*, 2012). For scoring ulcer and colonic shortening the following scale was used: ulcer - 0.5 points for each 0.5 cm; shortening of the colon: 1 point for >15%, 2 points for >25% (based on a mean length of the colon in untreated mice of 8.01 ± 0.15 cm, n=6). The wall thickness was measured in mm: a thickness of n mm corresponds to n scoring points. The presence of hemorrhage, fecal blood, or diarrhea increased the score by 1 point for each additional feature. All colon samples for further experiments were collected during macroscopic evaluation process and were stored at -80 ºC until further processing.

DSS model

Mice were sacrificed by cervical dislocation 7 days after addition of DSS to the drinking water. The colon was rapidly isolated and weighed with fecal content. Colon was then opened along the mesenteric border and fecal material was removed. A total macroscopic damage score was calculated for each animal including stool consistence (where 0 means normal well-formed faecal pellets and 3 means diarrhea), colon epithelial damage considered as number of ulcers (0 - 3), colon length and weight scores, considered as a percentage loss of either parameter in the proportion to the control group (0 = <5% weight/length loss; 1 = 5-14% weight/length loss; 2 = 15-24% weight/length loss; 3 = 25-35% weight/length loss and 4 = >35% weight/length loss), where score = 0 means no inflammation (Fichna *et al.*, 2012). The presence (score = 1) or absence (score = 0) of fecal blood was also recorded.

Determination of tissue myeloperoxidase activity

The method described by Salaga *et al.*, (2014) was used to quantify the myeloperoxidase (MPO) activity, which is an indicator of granulocyte infiltration (Pulli *et al.*, 2013). Briefly, 1-cm segments of colon were weighed and homogenized in

hexadecyltrimethylammonium bromide (HTAB) buffer (0.5% HTAB in 50 mM potassium phosphate buffer, pH 6.0; 1:20 w/v), afterwards the homogenate was centrifuged (15 min, 13,200 g, 4 ºC). On a 96-well plate, 200 µl of 50 mM potassium phosphate buffer (pH 6.0), containing 0.167 mg/ml of O-dianisidine hydrochloride and 0.05 µl of 1% hydrogen peroxide was added to 7 µl of supernatant. Absorbance was measured at 450 nm (iMARK Microplate Reader, Biorad, United Kingdom). All measurements were performed in triplicate. MPO was expressed in milliunits per gram of wet tissue, 1 unit being the quantity of enzyme able to convert 1 µmol of hydrogen peroxide to water in 1 min at room temperature. Units of MPO activity per 1 min were calculated from a standard curve using purified peroxidase enzyme.

Immunostaining of immune cells in mouse colon tissue

Tissue processing

After the macroscopic scoring, segments of distal colon were stapled flat, mucosal side up, onto cardboard and fixed in 10% neutral-buffered formalin and stored in 4 ºC. Following fixation the tissue was washed extensively in 1x PBS, pH 7.4 and cryoprotected via subsequent overnight incubations in PBS containing 10% and 30% sucrose, respectively. The colon samples were frozen cryosectioned on the same day. Tissue sections (12 µm) were cut on a Microm HM 500 OM cryostat (Thermo Scientific, Germany) and collected on Superfrost Plus slides (Menzel-Gläser, Germany). Slides were air dried and stored at -20 ºC until further processing.

Immunohistochemistry

For IHC staining the method described by Blomster *et al.*, (2011) was used. Briefly, slides were washed for 3 x 5min in PBS followed by quenching of endogenous peroxidase activity with PBS containing 0.6% (v/v) H_2O_2 and 10% (v/v) methanol for 30 min at room

temperature. After another round of washing, slides were immersed in blocking solution/antibody diluent containing 2% (w/v) bovine serum albumin (BSA, Sigma), 5% (v/v) goat serum (Dako) and 0.2% (v/v) Triton X-100 in PBS for 1h to reduce nonspecific antibody binding. The sections were incubated overnight in a humidified chamber at 4 ºC with primary antibodies to visualize either macrophages (rabbit anti-Iba1, 1:1000; Wako Pure Chemical Industries), neutrophils and macrophages (rat anti-Ly6B.2; 1:100; Serotec), T cells (rat anti-CD3; 1:100; Serotec) or B cells (rat anti-CD19; 1:50; Serotec).

The following day, slides were washed extensively for 3 x 10 min in PBS and further incubated with the appropriate secondary antibody, i.e. biotinylated goat anti-rabbit or donkey anti-rat antibody (1:1000; Jackson ImmunoResearch Laboratories) for 1.5 h at room temperature. After another round of washing to remove unbound antibody, slides were incubated with an avidin–biotin complex reagent (1:200; Vector Laboratories) for 1 h at room temperature as per manufacturer's instructions. The staining was developed after a final round of washing by incubating the slides with 0.1% (w/v) 3,3-diaminobenzidine and 0.03% (v/v) H_2O_2 in Tris-HCl, pH 7.6. The reaction was stopped after approximately 4-5 min via immersion of the slides in distilled H_2O, after which they were dehydrated through a graded series of ethanol, cleared in Xylene and coverslipped in Pertex mounting medium. Evaluation of the staining was performed in the computerized Axo Imager Carl Zeiss light microscope.

Flow cytometry

Frozen tissue samples were placed in 2 ml of Hanks' Balanced Salt Solution (HBSS) buffer with collagenase type II (200 U/ml; Life Technologies) and incubated 4 hours at 37°C. Next, tissues were homogenized using tissue raptor (IKE) at rpm 20,000/min for 30 seconds and 0.5 ml of trypsin solution (0.05% in HBSS) was added. Samples were incubated for 20 min at +37°C and filter through 70 µm nylon mesh (BD) to separate the dispersed cells and

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tissue fragments from the larger pieces. Cells were washed twice in PBS (without Ca^{2+}/Mg^{2+}) and centrifuged at 500 x g, 5 min, RT. Cell pellet was resuspended in 1 ml of PBS and cells were counted using Turk's staining solution. Cells were stained using the mouse Th17/Treg phenotyping kit (BD Bioscience) following the manufacturer's protocol. Briefly, 1 mln cells/ml was fixed for 30 min at 4°C at dark, permeabilized for 30 min at +37°C at dark and stained with Th17/Treg phenotyping cocktail. The following antibodies were used: CD4 PerCP-Cy5.5 Clone: RM4-5, IL-17A PE Clone: TC11-18H10.1, Foxp3 Alexa Fluor® 647 Clone: MF23. After each step cells were washed twice and centrifuged at 300 x g, 7 min, RT. Finally cells were resuspended in 200 µl of PBS and analysed on flow cytometer (LSR II Fortessa, BD). Lymphocytes were gated using forward (FSC) and side scatter (SSC) plot, and $CD4^+$ cells were identified as $SSC^{low}CD4^+$ cells. The change in a frequency of FoxP3-, IL-17A- and FoxP3 IL-17A-positive cells was analysed.

Determination of α7 nAChRs mRNA level in colonic tissue

RNA was isolated according to manufacturer's protocol using PureLink RNA Mini kit (Life Technologies, Carlsbad, CA, USA). Briefly, tissue samples were homogenized in lysis buffer (600 µl), complemented with 1% 2-mercaptoethanol (St. Louis, MO, USA). Subsequently, the homogenates were centrifuged to remove the debris. Next, supernatants were placed onto ion-exchange columns and finally purified total RNA was eluted using diethyl pyrocarbonate (DEPC) treated water (50 µl). To assess the purity and quantity of isolated RNA, dedicated spectrophotometer (BioPhotometer; Eppendorf, Germany) was used. Total isolated RNA (1 µg) was transcribed onto cDNA with First Strand cDNA synthesis kit (Fermentas, Burlington, Canada). Subsequently quantitative analysis was performed using fluorescently labeled TaqMan probe Mm01312230_m1 for mouse α7 nAChR and Mm01545399_m1 for mouse hypoxanthine-guanine phosphoribosyltransferase (HPRT),

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which was used as the endogenous control (Life Technologies, Carlsbad, CA, USA) on Mastercycler S realplex 4 apparatus (Eppendorf, Hamburg, Germany) and TaqMan Gene Expression Master Mix (Life Technologies, Carlsbad, CA, USA) in accordance with manufacturer's protocol. All experiments were performed in triplicate.

The Ct (threshold cycle) values for examined genes were normalized to Ct values obtained for the housekeeping gene HPRT. Relative amount of mRNA copies was calculated using following equation: $2^{-\Delta Ct} \times 1000$.

Drugs

All drugs and reagents, unless otherwise stated, were purchased from Sigma-Aldrich (Poznan, Poland). Encenicline ((R)-7-chloro-N-quinuclidin-3-yl)benzo[b]thiophene-2-carboxamide 13) was provided by NeuroSearch A/S (Ballerup, Denmark). DSS (MW 40,000) was purchased from MP Biomedicals (Solon, OH, USA). [1α,4(S),6β,14α,16β]-20-Ethyl-1,6,14,16-tetramethoxy-4-[[[2-(3-methyl-2,5-dioxo-1-pyrrolidinyl)benzoyl]oxy]methyl]aconitane-7,8-diol citrate (MLA) was purchased from Tocris Bioscience (Ellisville, MO, USA). In the in vivo tests, drugs were dissolved in 5% DMSO in saline, which was used as vehicle in control experiments. The vehicles in the used concentrations had no effects on the observed parameters.

Statistics

Statistical analysis was performed using Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). The data are expressed as means ± SEM and calculated per mm^2 area of tissue (for IHC experiments). Macrophages, neutrophils, B cells and T cells were quantified from 10 randomly assigned high-powered fields (object magnification × 40) of view per animal (n = 4 mice per group) and P values were assessed by the use of the Mann–Whitney U test. Student

t-test or one-way ANOVA followed by Newman-Keuls post-hoc test were used for all other analysis. P values < 0.05 were considered statistically significant.

Results

Encenicline protects against TNBS-induced colitis in mice by stimulation of α7 nAChRs

To evaluate the anti-inflammatory activity of encenicline in the mouse GI tract, we used a well-established mouse model of acute colitis induced by TNBS. The i.c. injection of TNBS resulted in reproducible increase in macroscopic damage scores and elevated MPO activity (Fig 2). Encenicline (3 mg/kg, i.p. twice daily) significantly improved colitis as shown by lowered macroscopic score, ulcer score, bowel thickness, and MPO activity (Fig. 2 A -D), and significantly increased colon length (Fig. 2 E). The anti-inflammatory activity of encenicline was significantly inhibited after pre-administration of MLA (3 mg/kg, i.p., twice daily) as shown by changes in ulcer score, bowel thickness, MPO activity and colon length (Fig. 2 B - E).

To investigate any effect of encenicline on established colitis, the compound was injected twice daily from day 3 following administration of TNBS. The selection of the day from which the treatment started was based on literature data (Monteleone *et al.*, 2012;Sans *et al.*, 2001) and on our previous observations indicating that clinical symptoms of colitis (e.g. changes in body weight), which represent severity of the disease reach the maximum at day 3 after TNBS injury. Treatment with encenicline resulted in a significant attenuation of intestinal inflammation as shown by significant decrease of total macroscopic score, ulcer score, bowel thickness and MPO activity (Fig. 3 A - D). There was no difference in colon length between vehicle- and encenicline-treated animals (Fig. 3 E).

The ganglionic blocker hexamethonium blocks the effect of encenicline on colon length and thickness but not on other macroscopic and biochemical parameters

In order to investigate whether central or peripheral nicotinic receptors are involved in the anti-inflammatory activity of encenicline, we used a ganglionic blocker HEX (10 mg/kg

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i.p., 30 min prior to encenicline). We observed that treatment with HEX did not block the effect of encenicline on macroscopic score, ulcer score and MPO activity (Fig. 4 A, B, D). However, HEX inhibited the beneficial effect of encenicline on colon thickness and length (Fig. 4 C, E).

Encenicline prevents development of DSS-induced colitis in mice but has no healing effect on established DSS-induced colonic inflammation

Additionally we characterized the anti-inflammatory activity of encenicline in a mouse model of colitis induced by DSS. We used two different dosing regimens to distinguish between potential prophylactic and healing effects of encenicline. We observed that encenicline administered at the dose of 3 mg/kg i.p., twice daily for 6 constitutive days during the DSS-treatment significantly attenuated colitis as shown by significantly reduced macroscopic score and MPO activity, and increased colon weight and length (Fig. 5 A - D). On the other hand, administration of encenicline (3 mg/kg, i.p., twice daily) starting from day 3 to day 6 of the experiment did not improve any of the measured parameters indicating that the effect cannot be achieved when inflammation is established (Fig. 6 A - D).

Stimulation of α7 nAChRs reduces the number of immune cells in the inflamed colonic tissue

To investigate the infiltration of specific immune cells into the colon, we used semi-quantitative IHC staining of mouse colon tissue. Injection of TNBS resulted in a significant elevation of the number of macrophages, neutrophils, T cells and B cells both in mucosal and submusocal layers of the colonic tissue (Fig. 7). Administration of encenicline resulted in a significant reduction of number of macrophages in both colonic mucosa and submucosa (Fig. 7 A). This effect was completely reversed by pre-administration of α7 nAChRs antagonist

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MLA (Fig 7A). Moreover, treatment with encenicline reduced the number of neutrophils in the mucosa and submucosa (Fig. 7 B). In case of mucosal layer this effect was significantly reversed by MLA, but in submucosal layer the difference did not reach statistical significance (Fig. 7B). The number of T cells was not affected by administration of encenicline (Fig. 7 C). Quantification of B cells showed that administration of encenicline significantly reduced their number only in the submucosal layer of the colon and this effect was not affected by pre-treatment with MLA (Fig. 7 D). Representative photographs of neutrophil presence in the TNBS treated mucosa (Fig. $7E_i$) and submucosa (Fig. $7E_{ii}$). Treatment with encenicline reduced the number of neutrophils markedly (Fig. $7E_{iii}$ and E_{iv}).

Administration of MLA alone did not affect the number of any type of quantified immune cells except for neutrophils in the submucosal layer where the number of cells were increased following MLA treatment alone (data not shown).

Stimulation of α7 nAChRs alters the frequency of FoxP3$^+$ and IL-17A$^+$ T cells in the colon

Since we observed that administration of encenicline does not affect the total number of T cells in the inflamed colon, we used flow cytometry to investigate whether it alters the frequencies of different T cell subtypes, such as FoxP3$^+$ T-reg cells and pro-inflammatory IL-17A T-cells among all CD4$^+$ T cells in the colonic tissue. We observed that the frequencies of all subtypes of T cells, namely FoxP3$^+$ (Fig. 8 A), IL-17A$^+$ (Fig. 8 B) and FoxP3$^+$ IL-17A$^+$ (Fig. 8 C) T cells were elevated in the TNBS-treated groups. Treatment with encenicline significantly decreased the number of FoxP3$^+$ IL-17A$^+$ T cells (Fig. 8 C) in the colonic tissue, but it did not affect the frequencies of FoxP3$^+$ T cells and IL-17A$^+$ T cells.

The frequency of FoxP3$^+$ (Fig. 9 A) T cells was significantly elevated and the frequency of IL-17A$^+$ (Fig. 9 B) T cells was reduced in the groups treated with encenicline

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compared to DSS-treated group. There was also a significant elevation of $FoxP3^{+}$ $IL\text{-}17A^{+}$ (Fig. 9 C) T cells in DSS-treated animals, which was only partially reduced by treatment with encenicline.

α7 nAChRs mRNA level is increased in TNBS-induced colitis

In order to examine the changes in α7 nAChRs expression in the course of intestinal inflammation we determined the α7 nAChRs mRNA level in specimens obtained from control, TNBS-treated and encenicline-treated animals. A significant up-regulation of α7 nAChRs mRNA level was detected in TNBS-treated animals. Administration of encenicline partially reversed this effect but the difference did not reach statistical significance. (Fig. 10).

Discussion

The existence of so called "cholinergic anti-inflammatory pathway" has been proposed more than a decade ago (for review please see Marteli *et al.*, 2014). Early experiments showed that the vagal nerve is responsible for the transmission of anti-inflammatory stimuli from the CNS to the periphery (Borovikowa *et al.*, 2000a; Borovikova *et al.*, 2000b). Subsequently, it has been shown that secondary lymphoid organs, such as spleen are the primary target for vagal anti-inflammatory signals and α7 nAChRs have been implicated in their transduction (Wang *et al.*, 2003). There are several lines of evidences showing that nicotinic receptors, and α7 nAChRs in particular, may serve as a pharmacological target for anti-IBD drugs (de Jonge and Ulolla, 2007). However, the exact mechanism of this phenomenon is not fully understood. Current hypotheses concern the interplay between centrally located receptors (both nicotinic and muscarinic) and α7 nAChRs located on peripheral immune cells, such as macrophages, neutrophils, T cells, B cells and dendritic cells.

In this study we clearly evidenced that the stimulation of α7 nAChRs with a novel selective partial agonist encenicline alleviates symptoms of experimental colitis in mice and reduces the number of immune cells infiltrating the colon. The selectivity of encenicline for the target has been validated by inhibition of some of the effects with the selective α7 nAChRs antagonist MLA. Moreover, by employing the ganglionic blocker HEX we demonstrated that central, rather than peripheral sites are responsible for encenicline activity; this was stated based on the fact that HEX did not block overall macroscopic effects of the α7 nAChR agonist on intestinal inflammation and did not block the effect on biochemical parameters. Noteworthy, even though some of the macroscopic parameters (e.g. colon length and thickness) were worsened by HEX (compared to encenicline-treated group), the inflammatory state at the cellular level was alleviated as indicated by low MPO activity. Our

observation remains in line with recent findings reported by Munyaka *et al.,* (2014) who showed that central cholinergic activation improves DNBS-induced colitis and reduces secretion of colonic and splenic cytokines.

Previous studies showed that immune cells, such as macrophages, neutrophils, T cells and B cells express α7 nAChRs mRNA, thus their function may be modulated by cholinergic signaling (Munyaka *et al.*, 2014;Kawashima *et al.*, 2007;Koval *et al.*, 2009;Razani-Boroujerdi *et al.*, 2007;Su *et al.*, 2010;Yoshikawa *et al.*, 2006). Moreover, it has been demonstrated that α7 nAChRs are involved in the modulation of immune cell activity in the spleen (Munyaka *et al.*, 2014). In our study, TNBS infusion caused a massive infiltration of immune cells namely, macrophages, neutrophils, T cells and B cells in the mucosal and submucosal layers of the colon and stimulation of α7 nAChRs with encenicline reversed this process. Moreover encenicline inhibited the viability of spleen-derived monocytes stimulated with LPS, which may explain at least partially reduced number of macrophages infiltrating the colon of encenicline-treated animals (data not shown). The detailed mechanism of this process is not resolved, but may involve the Jak2-STAT3 signaling and/or reduced NF-κB activation as demonstrated for isolated peritoneal macrophages (de Jonge and Ulolla, 2007; de Jonge *et al.*, 2005).

Additionally, we observed reduced neutrophil infiltration indicated by decreased MPO activity in the colonic tissue of animals treated with encenicline. This result was confirmed by IHC staining showing significantly lower number of neutrophils both in the mucosal and submucosal layers of the colon. These two findings correspond to the effects observed at the macroscopic level, since neutrophils are mainly responsible for the damage of the colonic tissue in the course of colitis (Lampinen *et al.*, 2008; Knutson *et al.*, 2013). Again pre-treatment with MLA emphasized the specificity of α7 nAChRs are responsible for these effects, which is in line with findings reported by Gahring *et al.,* (2010) who showed an

increased number of neutrophils infiltrating the site of inflammation in α7 nAChRs KO animals. It has also been shown that nicotine affects various neutrophil functions e.g. superoxide anion production, chemokine secretion, chemotaxis and integrin expression (de Jonge and Ulolla, 2007). Disruption of these processes may contribute to the effects observed in this study.

Here we show that TNBS-induced colitis is associated with strong infiltration of B cells into the mucosa and submucosa of the colon. Changes in the number of B cells were more significant in the submucosal than mucosal layer, which is likely caused by the smaller number of residual B cells in the submucosa of control animals. Under physiological conditions the mucosa is constantly exposed to the external surrounding abundant in foreign antigens (e.g. bacterial); the reservoir of B cells in this layer is necessary for production of antibodies needed to protect against pathogens. TNBS insult results not only in the accumulation of antigen-like molecules in the colon, but also disrupts its mucosal barrier, thus the recruitment and/or proliferation of B cells is observed in deeper, submucosal layer of the colonic tissue (Lee *et al.*, 2007). Treatment with encenicline significantly reduced the number of B cells present in the colon. This effect may be attributed to the fact that α7 nAChRs negatively regulate B cells proliferation (Koval *et al.*, 2009).

Of note, the effect of encenicline on B cells was not blocked by MLA. This phenomenon could be explained by a possible non-selective action of encenicline on type 3 serotonin receptors (5-HT_3Rs); however, it is very unlikely since its other effects were successfully blocked by MLA, and the fact that 5-HT_3Rs are known to affect GI motility rather than immunological responses in the gut (Mawe and Hoffman, 2013). Hence, we suggest that the effect of encenicline on B cells may be indirect. It has been reported that B cell responses are controlled by neural signaling in the spleen (Mina-Osorio *et al.*, 2012) and that increased parasympathetic transmission decreases secretion of pro-inflammatory

cytokines that cause the recruitment of B cells (Ji *et al.*, 2014;Munyaka *et al.*, 2014;Ghia *et al.*, 2007). The reduction of B cell infiltration may thus be a result of increased parasympathetic signaling in secondary lymphoid organs, such as spleen or gut-associated lymphoid tissue (GALT), however the exact cellular target mediating this effect remains unknown. Moreover, it is possible that the effect of encenicline on B cells is caused by a lesser local tissue damage observed after the treatment, especially since infiltration of B cells to the site of inflammation is thought to be secondary to the inflammatory status of the tissue.

Interestingly, treatment with encenicline did not change the total number of T cells in the colonic tissue. T cells are crucially involved in the pathophysiology of intestinal inflammation and mediate various processes that lead to colonic tissue damage and maintenance of the inflammatory state, such as secretion of pro-inflammatory cytokines. Furthermore, inhibition of T cell migration to the gut is one of the strategies used in the treatment of IBD in humans (Raine 2014). On the other hand, T cells have been implicated in mediation of vagal cholinergic anti-inflammatory reflex. It has been shown that in the nude mice lacking T cells the stimulation of vagal nerve does not cause anti-inflammatory effect and that adoptive transfer of T cells into these animals partially restores the anti-inflammatory activity of vagal transmission (Martelli *et al.*, 2014). Furthermore, Rosas-Ballia *et al.*, (2011) characterized an acetylcholine-producing memory phenotype T cell population that is integral to the anti-inflammatory transmission and is necessary for inhibition of cytokine production by vagus nerve.

Here, given the dual nature of T cells in the context of intestinal inflammation we examined the frequency of three different subtypes of T cells that have a potentially strong impact on the inflammation in the colon, namely $FoxP3^+$ T, IL-$17A^+$ T and $FoxP3^+$ IL-$17A^+$ T cells. We found significant differences in the effect of encenicline on the frequency of these T cell subtypes between TNBS and DSS models of colitis. In the TNBS model, the frequency of

JPET Fast Forward. Published on October 13, 2015 as DOI: 10.1124/jpet.115.228205
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FoxP3$^+$ IL-17A$^+$ T cells was significantly reduced after treatment with encenicline, although there was no effect on other subtypes. The role of FoxP3$^+$ IL-17A$^+$ T cells in the inflammation is only partially understood. It has been shown that these cells are generated in the periphery, at mucosal sites during inflammation and their presence has been confirmed in the lamina propria of the gut (Zhou *et al.*, 2008;Voo *et al.*, 2009). Moreover, their differentiation is stimulated by IL-1β, IL-2, IL-21, and IL-23, i.e. cytokines involved in the pathophysiology of intestinal inflammation (Voo *et al.*, 2009). Although they resemble the immunosuppressive FoxP3$^+$ phenotype (Treg cells), the expression of FoxP3$^+$ in FoxP3$^+$ IL-17A$^+$ T cells is lower than that of FoxP3$^+$ IL-17A$^-$ T cells in CD4$^+$ population (Voo *et al.*, 2009). Therefore, the pro-inflammatory function has been attributed to these cells. In line, Ueno *et al.*, (2013) have recently found that prevalence of circulating FoxP3$^+$ IL-17A$^+$ T cells is increased in patients with IBD compared to healthy controls. Moreover, the conversion of Treg cells into FoxP3$^+$ IL-17A$^+$ T cells have been suggested as a mechanism of their generation since the ability of Treg cells to suppress autologous T cell proliferation was decreased in IBD patients (Ueno *et al.*, 2013). It has also been reported that FoxP3$^+$ IL-17A$^+$ T cells co-express RAR-related orphan receptor (RORγt), which promotes the differentiation of thymocytes into pro-inflammatory T helper 17 cells (Dong 2008;Sun *et al.*, 2000). In the DSS model, we observed an increased frequency of Treg and decreased frequency of pro-inflammatory IL-17A$^+$ T cells after treatment with encenicline, which most likely contribute to the anti-inflammatory effect observed at the macroscopic level. On the other hand, it should be noted that a differential effect of encenicline on T cell subpopulations in both models could be seen due to the different time point of the analysis, which correlates with the kinetics of the disease severity. Moreover, a less pronounced involvement of innate immune system in the acute TNBS- compared to DSS-induced colitis may be of importance. Taken together, our observations suggest that stimulation of α7 nAChRs with clinically effective partial agonist encenicline

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activates different anti-inflammatory mechanisms that in both models lead to the improvement of colitis. Our finding adds another layer of complexity to the knowledge of models of intestinal inflammation and differentiates these based on the role of $FoxP3^+$ IL-$17A^+$ T cells in their pathophysiology.

Additionally, we found that α7 nAChRs mRNA is increased in the colonic tissue of TNBS-treated animals. This phenomenon is most likely caused by a massive infiltration of α7 nAChRs-expressing immune cells to the colon. Moreover, it has been shown that expression of α7 nAChRs increases in immune cells upon their activation with concanavalin A (Nizri *et al.*, 2009), which could also account for the overall increase in a7 mRNA expression. Curiously, treatment with encenicline only partially reduced this effect. However, it has been reported that the expression of α7 nAChRs increases upon stimulation with nicotinic agonists, which may be the reason why the α7 nAChR mRNA did not go down to the control level (Van der Zanden *et al.*, 2012).

To summarize, our study extends the knowledge of the pathogenesis of colonic inflammation and provides novel information on the mechanisms underlying the anti-inflammatory activity of the α7 nAChR partial agonist encenicline. Although there have been some doubts concerning targeting α7 nAChRs in the treatment of colitis (e.g. it has been suggested that α7 nAChRs agonists worsen the disease (Snoek *et al.*, 2010)), we suggest that alteration of the frequencies of $FoxP3^+$ and/or IL-$17A^+$-expressing T cells may underlay the beneficial effects of encenicline on intestinal inflammation. Furthermore we highlight the superiority of partial over full α7 nAChR agonists in the treatment of colitis, which is supported by recent reports showing on one hand that in vitro partial α7 nAChR agonists exhibit anti-inflammatory properties (Thomsen and Mikkelsen, 2012) and on the other indicating that full agonists do not affect colitis in vivo (Snoek *et al.*, 2010).

Moreover, we show that the duration of treatment may be another factor that affects its outcomes. Our insights in the DSS model show that longer (7 day) stimulation of α7 nAChRs is necessary to obtain significant anti-inflammatory effect in the GI tract, which may be further supported by the fact that chronic nicotine intake prevents UC in humans. In line, Van der Zanden *et al.,* (2012) demonstrated that repeated exposure to nicotine upregulates α7 nAChRs expression in human monocytes and enhances the potency of α7 nAChRs agonists in reduction of TNF-α levels.

Conclusion

Given the results of our study, we postulate that cholinergic brain-immune system interactions, mediated mainly by vagal nerve regulate the abnormal immunological responses observed in the course of IBD. Hence we encourage further development of α7 nAChR-targeting compounds with anti-inflammatory activity.

Disclosures: The authors have nothing to disclose.

Authorship Contributions:

Participated in research design: Fichna, Mikkelsen, Salaga, Blomster

Conducted experiments: Salaga, Blomster, Piechota-Polanczyk, Zielinska, Jacenik, Cygankiewicz

Contributed new reagents or analytic tools: Mikkelsen

Performed data analysis: Salaga, Blomster, Piechota-Polanczyk, Zielinska, Jacenik, Cygankiewicz

Wrote or contributed to the writing of the manuscript: Salaga, Fichna, Mikkelsen, Blomster, Krajewska

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Footnotes

Supported by the Iuventus Plus program of the Polish Ministry of Science and Higher Education [#0107/IP1/2013/72 to JF] and the grants from the Medical University of Lodz [502-03/1-156-02/502-14-140 to MS and #503/1-156-04/503-01 to JF] and National Science Centre [#UMO-2013/11/N/NZ7/02354 to MS, #UMO-2013/11/B/NZ7/01301 and #UMO-2014/13/B/NZ4/01179 to JF]. LVB and JDM are supported by grants from the Danish Research Council for Strategic Research [COGNITO] and the NOVO Nordisk Foundation. Study sponsored by a Polpharma Scientific Foundation scholarship to MS.

Legends for figures

Figure 1. A scheme illustrating experimental protocols and treatment regimens used in TNBS (A and B) and DSS (C and D) models.

Figure 2. The i.p. administration of encenicline (3 mg/kg, twice daily) over 3 days attenuated TNBS-induced colitis in mice. Figure shows data for macroscopic score (A), ulcer score (B), bowel thickness (C), MPO activity (D) and colon length (E) after administration of encenicline alone or in the presence of MLA and for MLA alone. *P < 0.05, **P< 0.01 ***P < 0.001, as compared to control mice. #P<0.05, ##P < 0.01, ###P < 0.001, vs. TNBS-treated mice. &P<0.05, &&P<0.01, &&&P < 0.001, as compared to encenicline treated mice. Data represent mean ± SEM of 6–8 mice per group.

Figure 3. The i.p. administration of encenicline (3 mg/kg, twice daily) alleviates established TNBS-induced colitis in mice. Figure shows data for macroscopic score (A), ulcer score (B), bowel thickness (C), MPO activity (D) and colon length (E) after administration of encenicline. *P < 0.05, **P< 0.01 ***P < 0.001, as compared to control mice. #P<0.05, ##P < 0.01, ###P < 0.001, vs. TNBS-treated mice. Data represent mean ± SEM of 6–8 mice per group.

Figure 4. The i.p. administration of ganglionic blocker HEX (10 mg/kg, twice daily) does not block the anti-inflammatory effect of encenicline (3 mg/kg, twice daily) in model of TNBS-induced colitis. Figure shows data for macroscopic score (A), ulcer score (B), bowel thickness (C), MPO activity (D) and colon length (E) after administration of encenicline alone or in the presence of HEX. *P < 0.05, **P< 0.01 ***P < 0.001, as compared to control mice. #P<0.05, ###P < 0.001, vs. TNBS-treated mice. &P<0.05, &&P<0.01 as compared to encenicline treated mice. Data represent mean ± SEM of 6–8 mice per group.

Figure 5. Encenicline (3 mg/kg, i.p.) injected twice daily over 7 days attenuated DSS-induced colitis in mice. Figure shows data for macroscopic score (A), colon weight (B), MPO activity

(C), colon length (D) and body weight changes (E). *P < 0.05, **P < 0.01, ***P < 0.001, as compared to control mice. #P < 0.05, ##P < 0.01 vs. DSS-treated mice. Data represent mean ± SEM of 6–8 mice per group.

Figure 6. Encenicline (3 mg/kg, i.p.) injected twice daily from day 3 to day 6 does not exhibit healing effects on established DSS-induced colitis in mice. Figure shows data for macroscopic score (A), colon weight (B), MPO activity (C), colon length (D) and body weight changes (E). *P < 0.05, **P < 0.01, ***P < 0.001, as compared to control mice. Data represent mean ± SEM of 6–8 mice per group.

Figure 7. Encenicline (3 mg/kg, i.p.) injected twice daily over 3 days reduce the infiltration of macrophages, neutrophils and B-cells but not T-cells in the colonic tissue. Figure shows data for quantification of macrophages (A), neutrophils (B), T-cells (C) and B-cells (D) after administration of encenicline alone or in the presence of MLA. Panel (E) shows representative micrographs of mucosal (i) and submucosal (ii) layers of TNBS-treated mouse distal colon stained with neutrophil marker as well as mucosal (iii) and submucosal (iv) layers of TNBS+encenicline-treated mouse distal colon stained with neutrophil marker. Scale bar reefers to 100 μm. *P < 0.05, **P< 0.01 ***P < 0.001, as compared to control mice. #P<0.05, ##P < 0.01, vs. TNBS-treated mice. &P<0.05, &&P<0.01, as compared to encenicline treated mice. Data represent mean ± SEM of 4 mice per group.

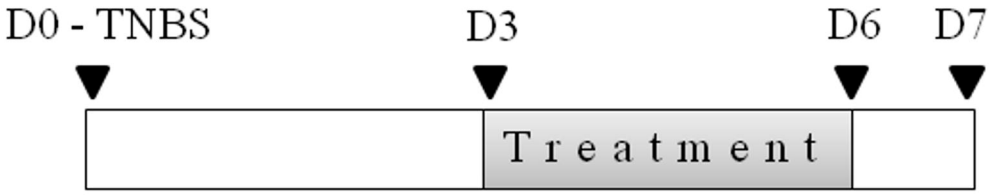
Figure 8. Effect of the i.p. administration of encenicline (3 mg/kg, twice daily) on the frequency of FoxP3 and IL-17A expressing $CD4^+$ T-cells in the colon of TNBS-treated mice. Figure shows data for the frequency of (A) $FoxP3^+$ T-cells, (B) $IL\text{-}17A^+$ T-cells and (C) $FoxP3^+$ $IL\text{-}17A^+$ T-cells. The frequencies were assessed by flow cytometry. Cells were gated on CD4 populations. Representative flow cytometric analysis is shown (D). *P < 0.05, as compared to control mice. #P<0.05, vs. TNBS-treated mice. Data represent mean ± SEM of 5-8 mice per group.

Figure 9. Effect of the i.p. administration of encenicline (3 mg/kg, twice daily) on the frequency of FoxP3 and IL-17A producing $CD4^+$ T-cells in the colon of DSS-treated mice. Figure shows data for the frequency of (A) $FoxP3^+$ T-cells, (B) IL-$17A^+$ T-cells and (C) $FoxP3^+$ IL-$17A^+$ T-cells. The frequencies were assessed by flow cytometry. Cells were gated on CD4 populations. Representative flow cytometric analysis is shown (D). *$P < 0.05$, as compared to control mice. Data represent mean ± SEM of 5-8 mice per group.

Figure 10. Determination of α7 nAChRs mRNA levels in mouse colon specimens. The level of α7 nAChRs mRNA is significantly incerased in TNBS-treated mice. There is an observable difference between TNBS- and encenicline-treated animals but it did not reach statistical significance. Data represent mean ± SEM of 6 mice per group.

A)

B)



C)

D)

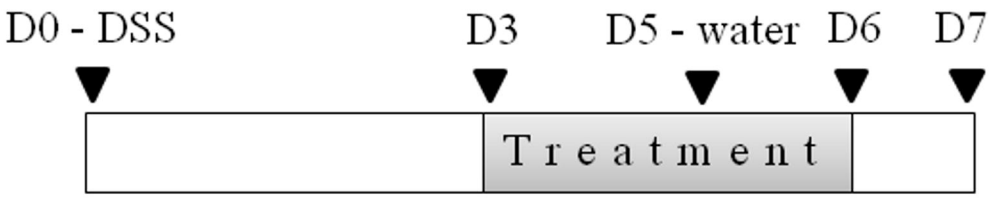


Figure 1

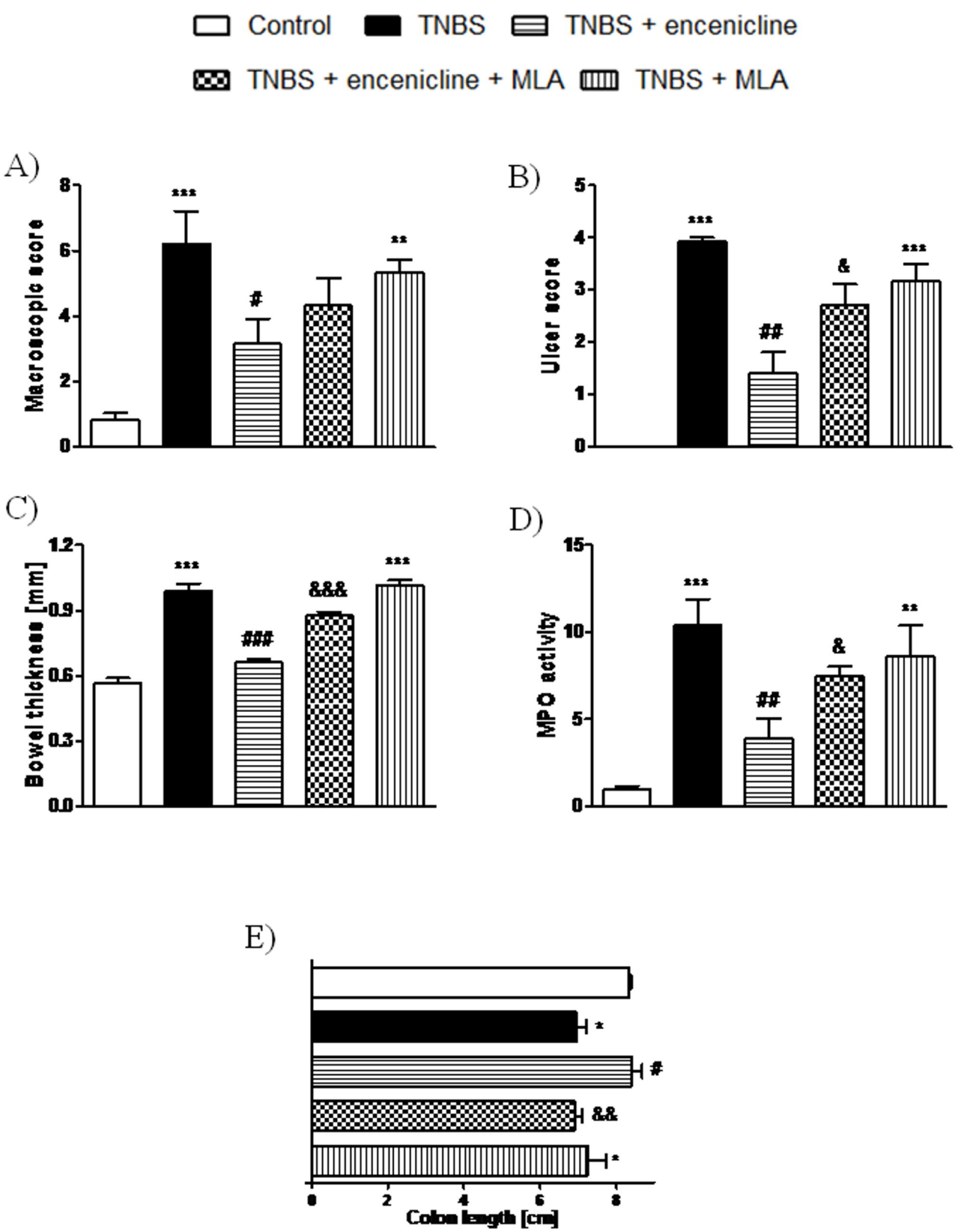

Control
TNBS
TNBS + encenicline
TNBS + encenicline + MLA
TNBS + MLA
A)
Macroscopic score
B)
Ulcer score
C)
Bowel thickness [mm]
D)
MPO activity
E)
Colon length [cm]

Figure 2

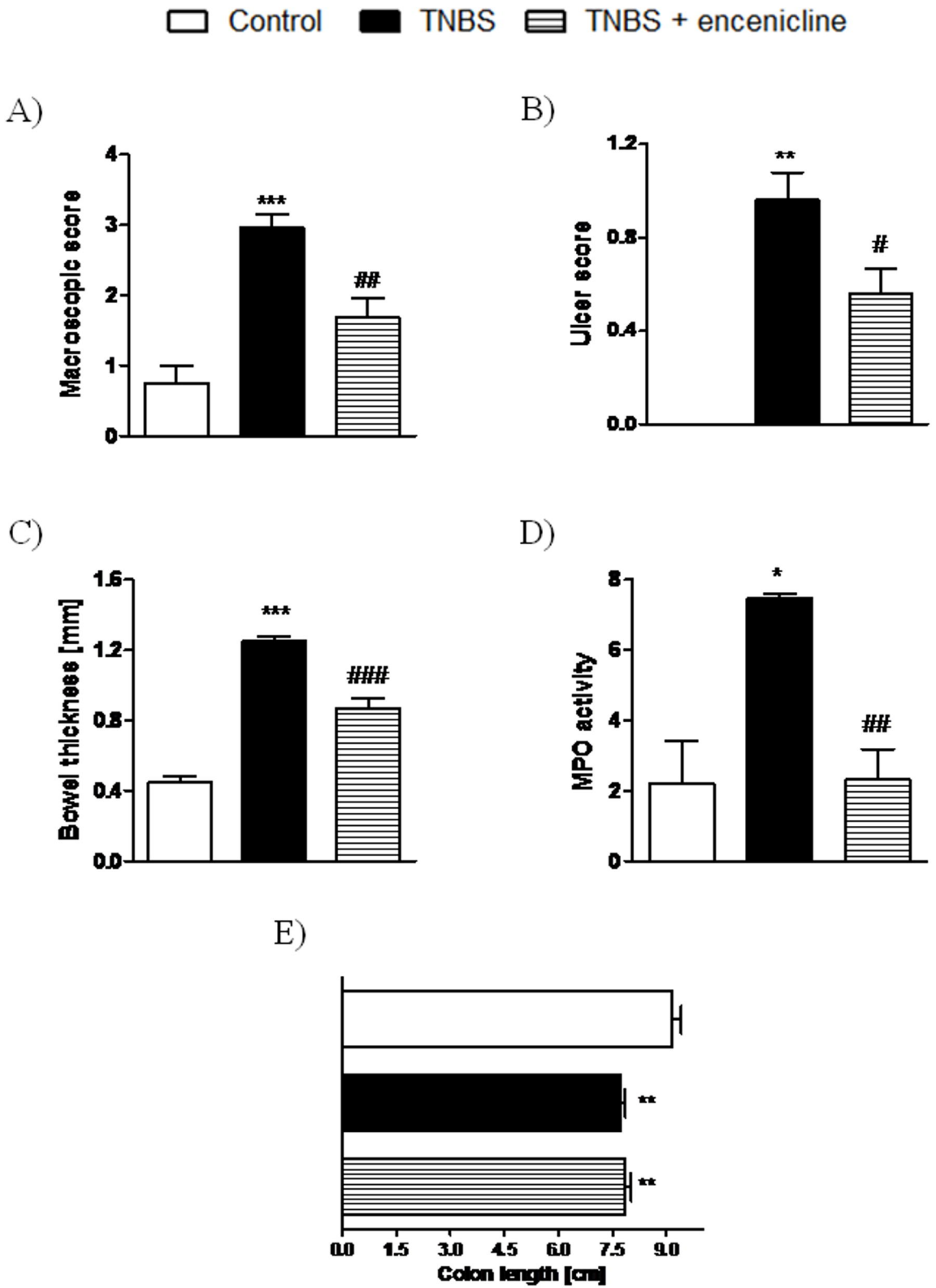

Control
TNBS
TNBS + encenicline
A)
Macroscopic score
0
1
2
3
4
***
##
B)
Ulcer score
0.0
0.4
0.8
1.2
**
#
C)
Bowel thickness [mm]
0.0
0.4
0.8
1.2
1.6
***
###
D)
MPO activity
0
2
4
6
8
*
##
E)
0.0
1.5
3.0
4.5
6.0
7.5
9.0
Colon length [cm]
**
**

Figure 3

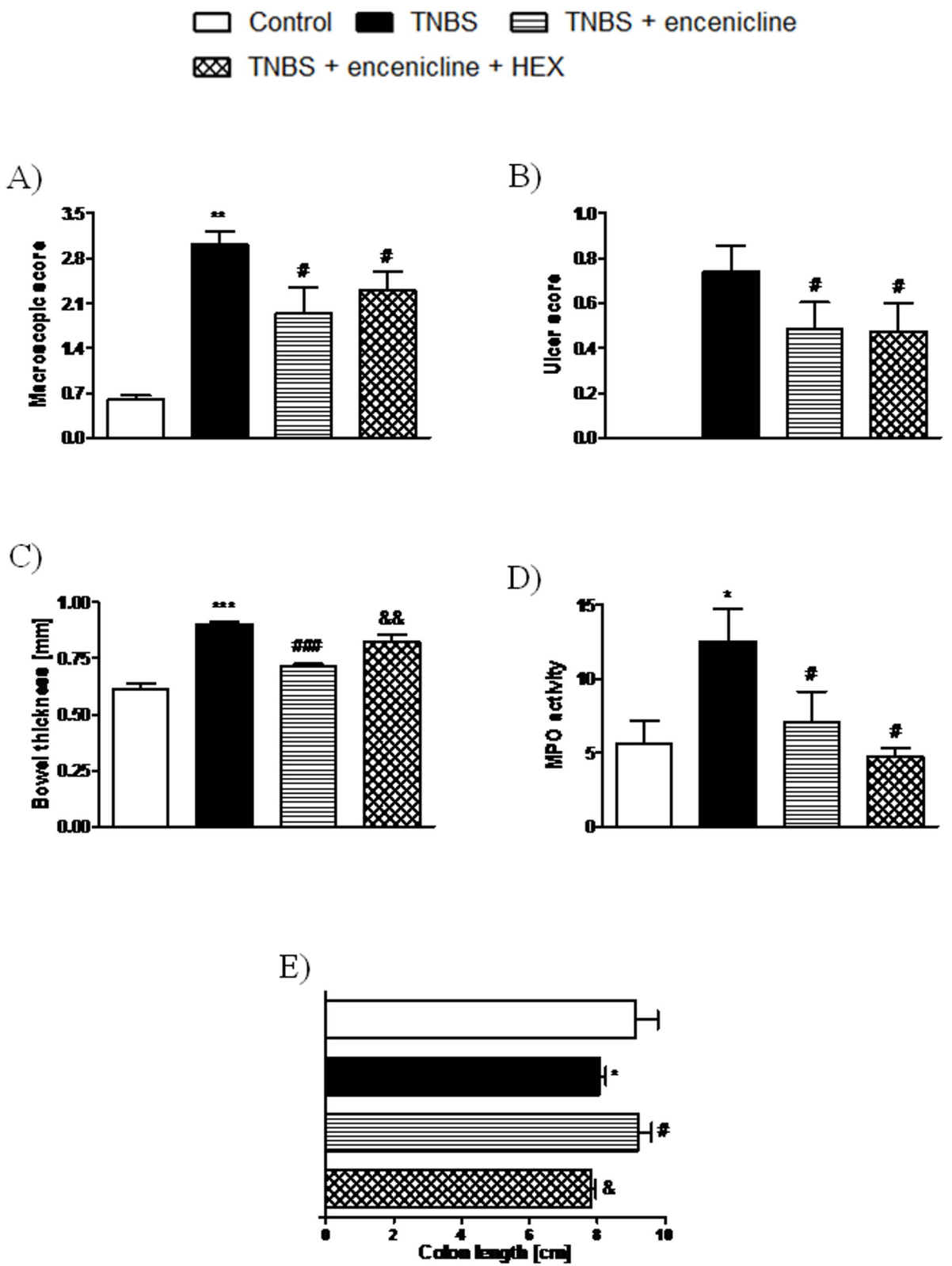

Control
TNBS
TNBS + encenicline
TNBS + encenicline + HEX
A)
Macroscopic score
3.5
2.8
2.1
1.4
0.7
0.0
**
#
#
B)
Ulcer score
1.0
0.8
0.6
0.4
0.2
0.0
#
#
C)
Bowel thickness [mm]
1.00
0.75
0.50
0.25
0.00
***
###
&&
D)
MPO activity
15
10
5
0
*
#
#
E)
0
2
4
6
8
10
*
#
&
Colon length [cm]

Figure 4

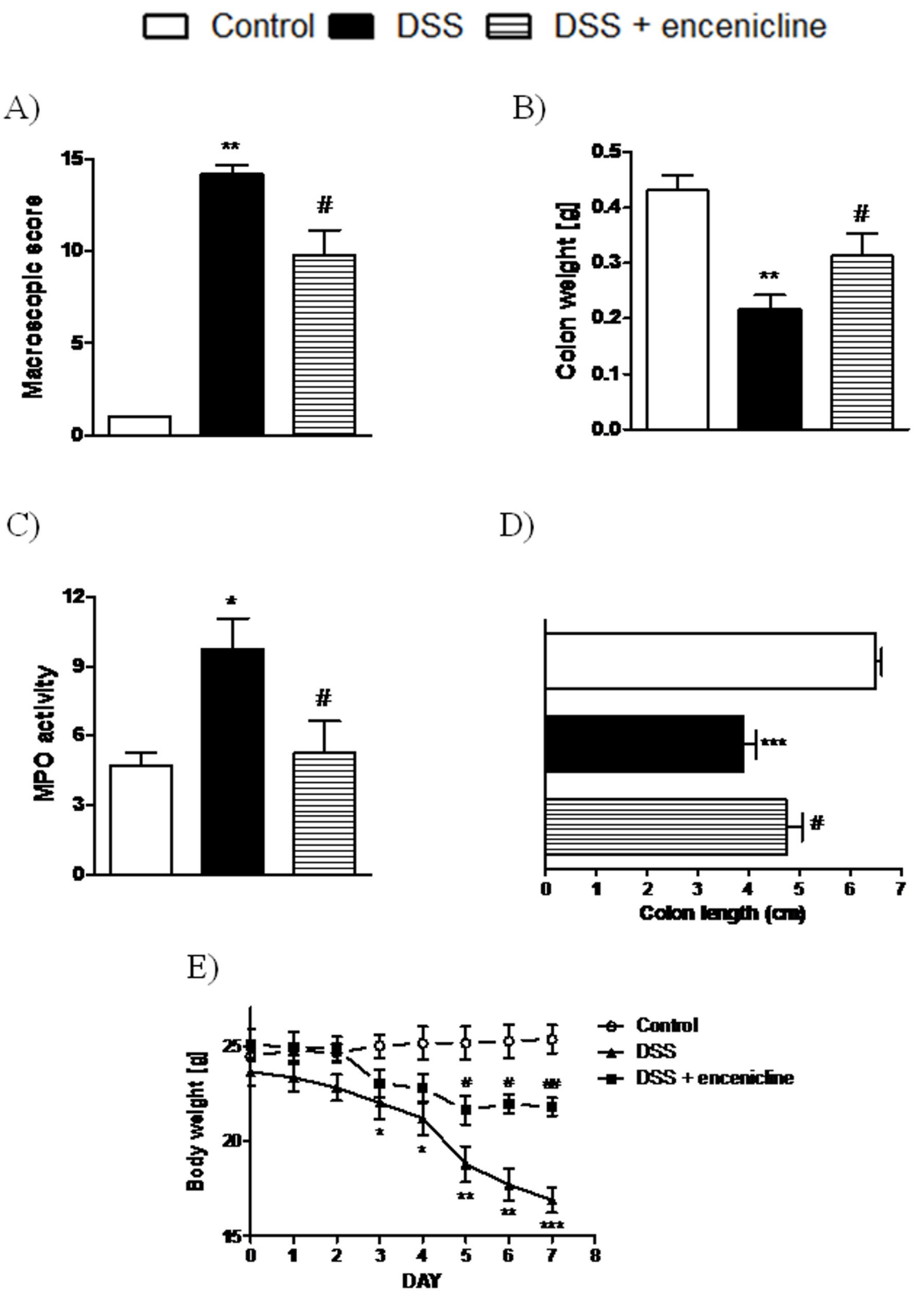

Control
DSS
DSS + encenicline
A)
Macroscopic score
B)
Colon weight [g]
C)
MPO activity
D)
Colon length (cm)
E)
Body weight [g]
DAY
Control
DSS
DSS + encenicline

Figure 5

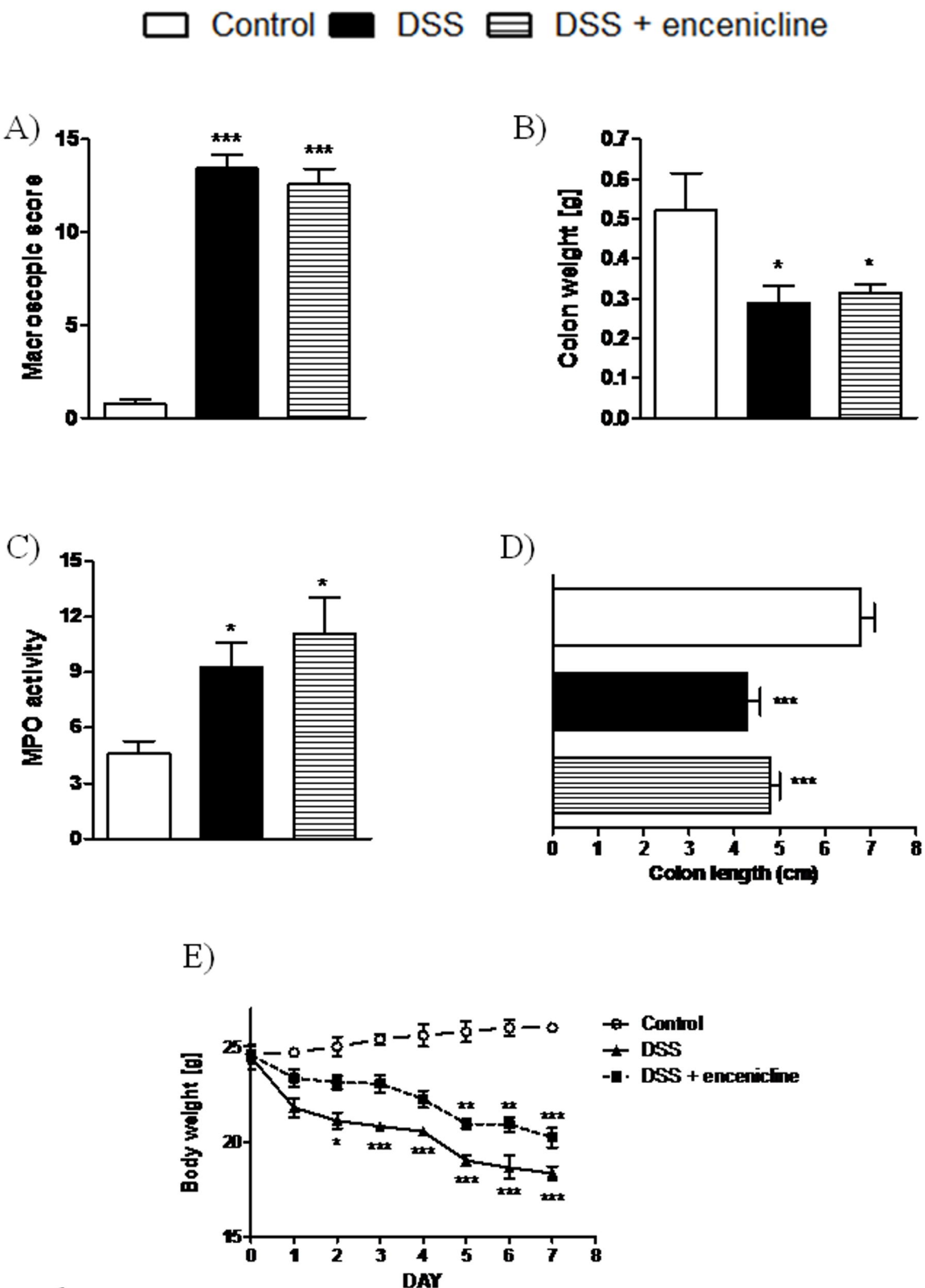

Control
DSS
DSS + encenicline
A)
Macroscopic score
15
10
5
0
***
***
B)
Colon weight [g]
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
C)
MPO activity
15
12
9
6
3
0
*
*
D)
***
***
0 1 2 3 4 5 6 7 8
Colon length (cm)
E)
Body weight [g]
25
20
15
0 1 2 3 4 5 6 7 8
DAY
Control
DSS
DSS + encenicline

Figure 6

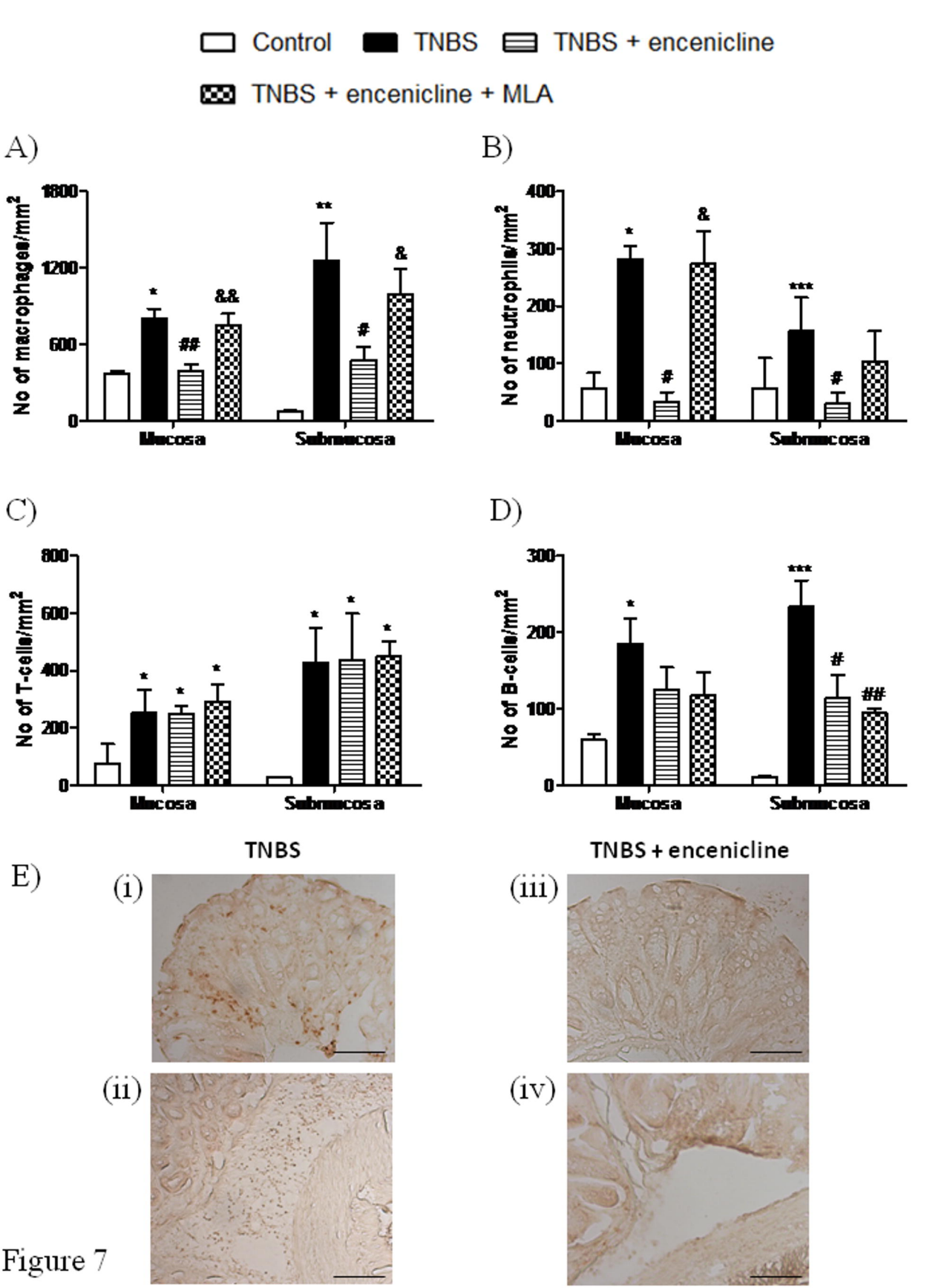

Control
TNBS
TNBS + encenicline
TNBS + encenicline + MLA
A)
No of macrophages/mm2
1800
1200
600
0
Mucosa
Submucosa
B)
No of neutrophils/mm2
400
300
200
100
0
Mucosa
Submucosa
C)
No of T-cells/mm2
800
600
400
200
0
Mucosa
Submucosa
D)
No of B-cells/mm2
300
200
100
0
Mucosa
Submucosa
E)
TNBS
TNBS + encenicline
(i)
(ii)
(iii)
(iv)

Figure 7

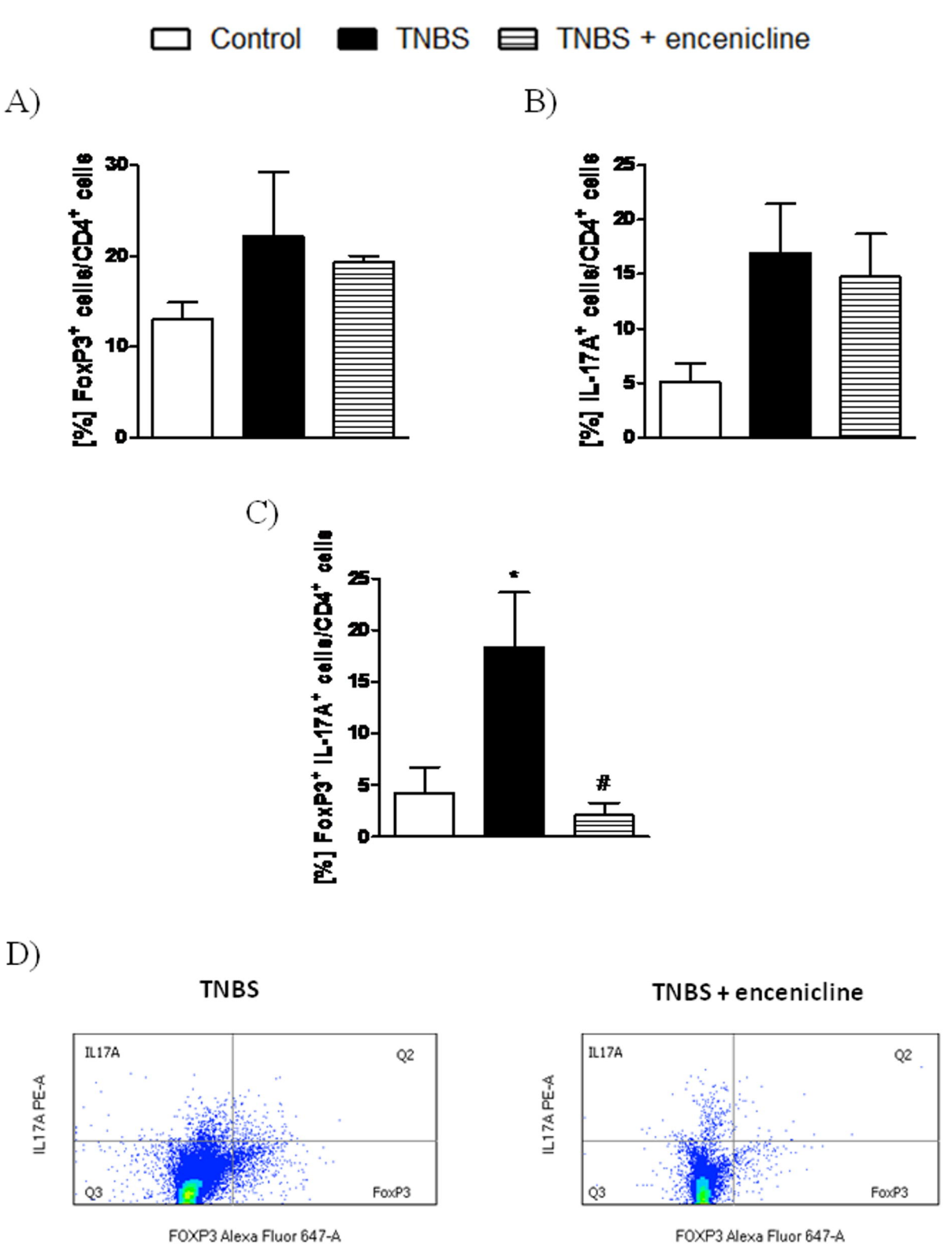

Control
TNBS
TNBS + encenicline
A)
[%] FoxP3+ cells/CD4+ cells
30
20
10
0
B)
[%] IL-17A+ cells/CD4+ cells
25
20
15
10
5
0
C)
[%] FoxP3+ IL-17A+ cells/CD4+ cells
25
20
15
10
5
0
*
#
D)
TNBS
TNBS + encenicline
IL17A
Q2
Q3
FoxP3
IL17A PE-A
FOXP3 Alexa Fluor 647-A

Figure 8

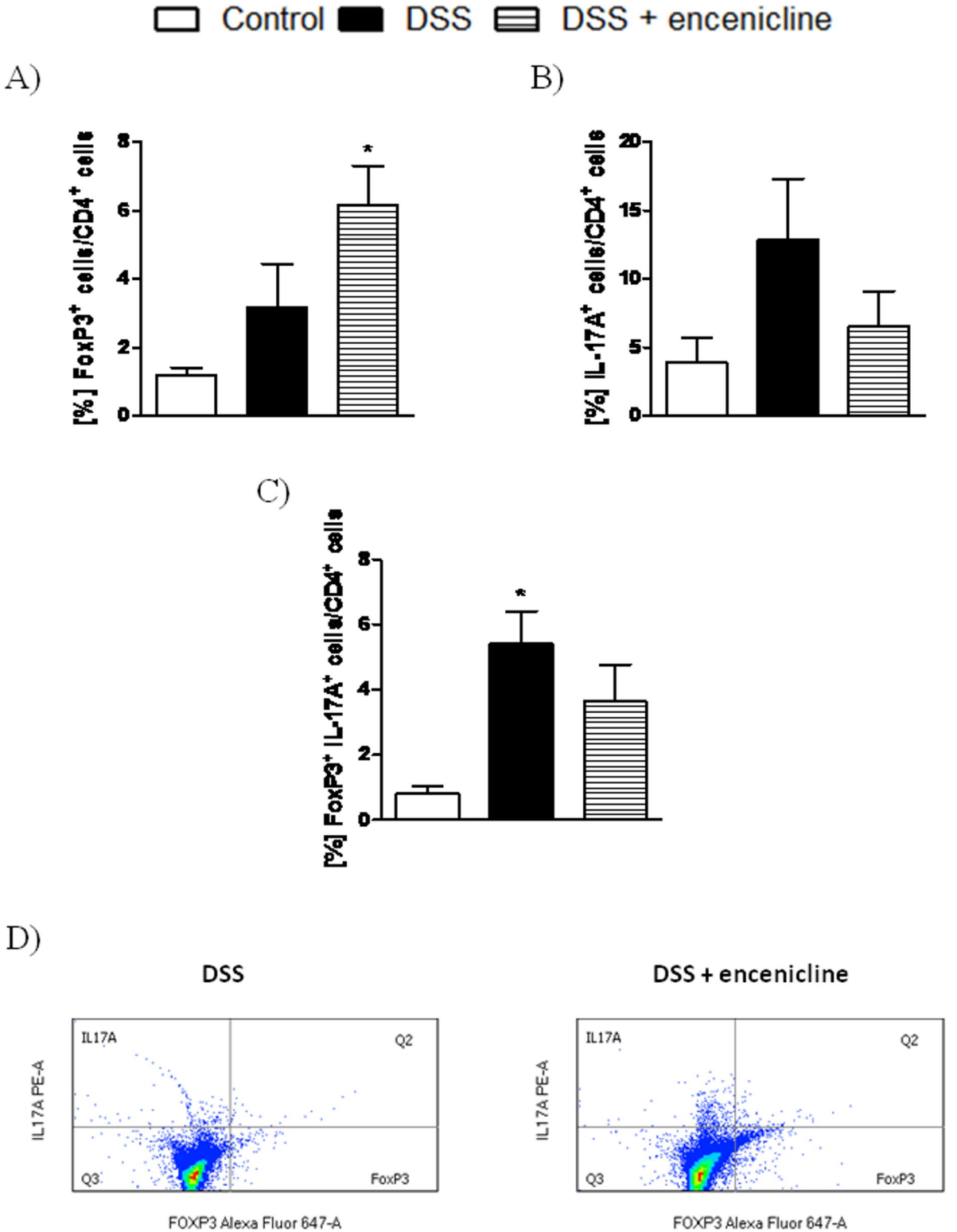

Control
DSS
DSS + encenicline
A)
[%] FoxP3+ cells/CD4+ cells
0
2
4
6
8
*
B)
[%] IL-17A+ cells/CD4+ cells
0
5
10
15
20
C)
[%] FoxP3+ IL-17A+ cells/CD4+ cells
0
2
4
6
8
*
D)
DSS
DSS + encenicline
IL17A
Q2
Q3
FoxP3
IL17A PE-A
FOXP3 Alexa Fluor 647-A

Figure 9

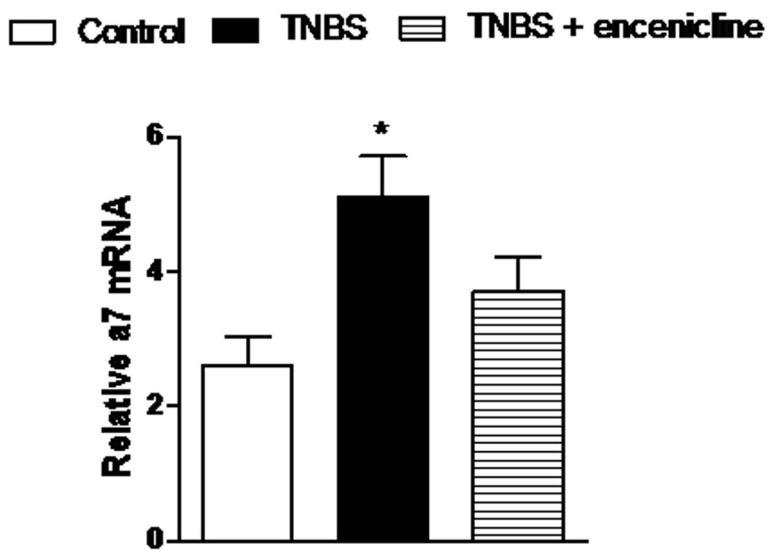


Figure 10