



Published in final edited form as:

Dig Dis Sci. 2017 August ; 62(8): 1977–1984. doi:10.1007/s10620-017-4631-x.

Estradiol Has Differential Effects on Acute Colonic Inflammation in the Presence and Absence of Estrogen Receptor β Expression

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Abstract

Background—Inflammatory bowel disease (IBD) increases the risk of developing colon cancer. This risk is higher in men compared to women, implicating a role for female hormones in the protection against this disease. Studies from our laboratory demonstrated that estradiol (E_2) protects against inflammation-associated colon tumor formation when administered following chemical carcinogen and induction of chronic colitis.

Aim—This study seeks to better understand the effect of E_2 on acute colitis in the presence and absence of estrogen receptor β ($ER\beta$).

Methods—Inflammation was induced by 2,4,6-trinitrobenzenesulfonic acid in wild-type (WT) and $ER\beta$ knockout ($ER\beta$ KO) mice implanted with a control or E_2 -containing pellet and killed 5 days later. Inflammation and injury were scored by a pathologist. Apoptosis and proliferation were assessed by immunohistochemistry. Cytokines were measured by multiplex analysis.

Results— E_2 treatment reduced inflammation in the middle colon in WT mice and the distal colon in $ER\beta$ KO mice compared to control mice. WT mice had reduced IL-6, IL-12, IL-17, GM-CSF, IFN- γ , MCP-1, MIP-1 α , and TNF- α , and $ER\beta$ KO had reduced IL-6 and IFN- γ expression in response to E_2 . Injury scores were lower in E_2 -treated $ER\beta$ KO mice compared to control $ER\beta$ KO mice. $ER\beta$ KO mice had increased proliferation in the basal third of crypts in the distal colon and decreased apoptosis in the proximal colon.

Conclusions—These data suggest that E_2 has differential protective effects against acute colitis in the presence or absence of $ER\beta$ and provide insight into how E_2 may protect against IBD.

Keywords

Colon cancer; Estradiol; Estrogen receptor beta; Crohn's disease; Inflammation

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Conflict of interest

None of the authors have any conflicts of interest to declare.

Introduction

Inflammatory bowel disease (IBD) affects over one million people in the USA. Typically, IBD occurs in persons in their twenties and thirties at which point symptoms lessen for a number of years followed by flare-ups of the disease later in life. The two primary forms of IBD, Crohn's disease and ulcerative colitis, both confer a greater lifetime risk of developing colon cancer [1, 2]. This increased risk is most likely due to an increased rate of growth due to the exposure to pro-inflammatory cytokines. Interestingly, women have been observed to be 60% less at risk than men to develop inflammation-associated colon cancer, suggesting that female hormones may play a role in the prevention of this disease [3].

A number of experimental models have demonstrated that estrogens have anti-inflammatory properties in tissues other than the intestine and one way in which estradiol (E_2), the most biologically active form of estrogen in the human body, may be protecting against inflammation-associated colon carcinogenesis is through the suppression of intestinal inflammation [4–7]. Epidemiological studies have observed that pre-menopausal women with Crohn's disease reported a worsening of their IBD-related symptoms during menses, the time during the estrous cycle that E_2 concentrations are at their lowest [8]. Not only does this suggest that E_2 could be protecting against intestinal inflammation, but the fact that the same worsening of symptoms was not observed in women with ulcerative colitis suggests that the role of E_2 in protecting against inflammation could be dependent on the subtype of IBD.

Experimental data to support the protective effects of E_2 on the development of inflammation-associated colon tumors, however, are conflicting. Data from our laboratory indicate that treatment with E_2 in mice following induction of inflammation-associated colon cancer using cotreatment with the colon-specific carcinogen azoxymethane (AOM) and dextran sulfate sodium (DSS) was effective at reducing both the number and size of colon tumors [9]. Other groups, however, have observed that in mice pre-treated with E_2 and then subjected to AOM/DSS, polyp number and size were increased [10]. In a separate study investigating the role of estrogen receptor (ER) β in AOM/DSS-induced polyp formation in the presence of endogenous E_2 levels, Saleiro et al. determined that mice lacking $ER\beta$ ($ER\beta$ KO) had more polyps at 9 weeks compared to wild-type (WT) littermates. This study further saw an induction of pro-inflammatory cytokines in the absence of $ER\beta$ at 16 weeks; however, at 9 weeks, no significant differences in cytokine expression were detected [11].

The disparity in the observed results is not limited to eventual tumor development. Studies investigating the effects of E_2 solely on inflammation have also reached varying data. Here though, the incongruent findings are likely the result of the models of inflammation used. Studies using DSS as the inflammatory agent found that E_2 worsened disease severity, whereas colitis induced by dinitrobenzene sulfonic acid was improved in the presence of E_2 [10, 12]. When 2,4,6-trinitrobenzenesulfonic acid (TNBS) was used to induce colonic inflammation in rats, estradiol benzoate and the phytoestrogen genistein improved pathological scores in models of acute and chronic inflammation, respectively [13, 14]. DSS and TNBS induce inflammation through distinct mechanisms resulting in colitis that resembles ulcerative colitis and Crohn's disease, respectively, and the differences in

response to E₂ observed between these two models may be reflective of distinct effects of E₂ on these two separate forms of IBD.

The focus of the present study is to investigate the physiological actions of E₂ during acute TNBS-induced colitis in WT and ERβKO mice. While previous studies suggest that estrogen is protective against TNBS colitis, the mechanism behind this protection is still poorly understood. Studies in our laboratory in both sporadic and inflammation-associated colon cancer models suggest that ERβ, the primary ER in the colon, mediates the protective effect of E₂ in this tissue [9, 15]. To date, however, the role of ERβ in TNBS-induced colitis is unknown. Understanding the function of E₂ and ERβ during acute inflammation is important, particularly when you take into consideration the fact that persons with active Crohn's disease have lower colonic expression of ERβ compared to when their disease is in remission [16].

Methods and Materials

Animals

C57Bl6/J mice heterozygous for ERβKO (±) were originally obtained from the Jackson Laboratory. The mice were housed at the Laboratory Animal Resources and Research Facility at Texas A&M University. Mice were bred to produce WT and ERβKO offspring, and genotype was confirmed using genomic tail DNA. All procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee at Texas A&M University.

Induction of Colitis

While it does not perfectly recapitulate human Crohn's disease, TNBS-induced colitis shares many characteristics with human Crohn's disease including the involvement of NOD2, the macroscopic pattern of inflammation, induction of mucosal edema, crypt distortion, and abscess formation [17].

For this study, female mice were weight and age matched (mean 27.8 g and 5.8 months) and divided into treatment groups ($n = 12-16$ mice per group). On day 1, mice were ovariectomized (OVX) and implanted with either a 20-mg cholesterol containing pellet or a 0.5-mg E₂ + 19.5-mg cholesterol (Sigma-Aldrich, St. Louis, MO) pellet subcutaneously and placed on a phytoestrogen-free diet. Corresponding plasma E₂ levels for the groups at the end of the study were 1.2 nM in the mice receiving E₂ and undetectable in the cholesterol mice with no significant differences between genotype and TNBS treatment group. Two weeks after surgery and pellet implant, mice were pretreated with 1% TNBS dermal absorption through the skin on their backs. One week following pre-treatment, a 2.5% TNBS solution was administered intrarectally 2–4 cm into the colon using a flexible plastic gavage tube (Instech Solomon) after a 12-h fast. Mice were killed 5 d postintrarectal TNBS. Colons were resected and longitudinally bisected. One half of the colon was rolled into a Swiss roll and fixed in PFA. The other half was snap-frozen in liquid nitrogen for cytokine analysis. Inflammation and injury in the colons were assessed by a board-certified pathologist on H&E-stained colon Swiss rolls on a scale of 0–3. In brief, a score of 0 would indicate no

inflammation or injury noted, while a score of 3 would indicate severe inflammation or injury.

Cytokine Multiplex Analysis

Snap-frozen colon tissues representative of the whole colon were homogenized in 1 mL Tissue Protein Extraction Reagent (Thermo Scientific). Total protein content was assessed using the *DC* Protein Assay (Bio-Rad) as per the manufacturer's instructions. The Magnetic Mouse Cytokine/Chemokine Milliplex Map Kit was used to measure tissue levels of IL-6, IL-10, IL-12(p40), IL-17, GM-CSF, IFN- γ , MCP-1, MIP-1 α , and TNF- α following the manufacturer's instructions. All samples were diluted to 10 M. In brief, 25 μ L diluted sample was added per well of a 96-well plate in addition to 25 μ L assay buffer and 25 μ L of the magnetic beads provided in the kit. The plate was sealed and agitated on a plate shaker overnight at 4 °C. The following day, the 96-well plate was placed on a handheld magnet, the contents of the wells removed, and then the plate was washed twice. Following washing, 25 μ L of detection antibodies was added to each well and the plate was incubated at room temperature for 1 h with agitation. Next, 25 μ L of streptavidin-phycoerythrin was added to each well and the plate was incubated at room temperature for half an hour with agitation. The plate was then washed twice, the magnetic beads resuspended in 150 μ L Luminex sheath fluid, and the plate was run on a Luminex 200.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling Assay

Paraffin-embedded, paraformaldehyde (PFA)-fixed colon Swiss rolls were rehydrated, and the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore; Billerica, MA) was used for the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays following the manufacturer's instructions with slight modifications. PFA-fixed tissues were rehydrated and treated with 10 μ g/mL proteinase K for 3 min at 37 °C. Endogenous peroxidase activity was blocked using 0.3% H₂O₂ in methanol for 30 min. Tissues were incubated at room temperature (RT) for 20 s in equilibration buffer and then incubated for 1 h at 37 °C in reaction buffer plus TDT enzyme. The TDT enzyme was omitted from an individual section as a negative control for the stain. Subsequently, slides were placed in stopwash solution for 10 min followed by 30 min at RT in anti-digoxigenin in a humidified chamber. A 0.5% DAB solution for 20 s was used as the chromogen, and nuclei were counterstained in 0.5% methyl green for 5 s. Lastly, slides were dehydrated and coverslipped.

Immunohistochemistry for BrdU

Four micrometer sections were taken from the PFA-fixed tissues. Sections were deparaffinized and rehydrated. Endogenous peroxidase was quenched using 3% H₂O₂ in methanol for 30 min, and antigen retrieval was achieved by microwaving in 10 mM citrate buffer for 20 min. Slides were then incubated in the primary antibody, anti-BrdU (Roche, Basel, Switzerland) diluted 1:20 at 4 °C overnight in a humidified chamber. During each stain, the primary antibody was left off of a single slide to serve as a negative control. The following morning, the slides were washed and then incubated in the secondary antibody, goat anti-mouse HRP (Abcam, Cambridge, MA) diluted 1:250. Meyer's hematoxylin was used as the counterstain. Lastly, slides were dehydrated and coverslipped.

Immunohistochemistry Analysis

The TUNEL and BrdU assays were analyzed in the same manner. For each, 20 well-oriented crypts per animal from both the distal and proximal colon were symmetrically bisected and the right halves of each were analyzed. The total number of positively stained cells was divided by the total number of cells in the crypt column to generate the percentage of apoptotic or proliferative cells for each crypt column.

Statistics

Analysis for all data was determined using one-way ANOVA or Student's *t* test using JMP Pro 10. Differences were considered significant if $P < 0.05$.

Results

E₂ Protects Against TNBS-Induced Weight Loss and Increases Colon Length

All four groups of mice had a significant weight loss from their pre-study weights. WT control mice lost significantly more weight than WT E₂-treated mice. There was no difference in weight loss between ERβKO mice in response to E₂ (Fig. 1a). Additionally, as it has been routinely observed in our laboratory, E₂ treatment increased the length of the colon in WT but not ERβKO mice (Fig. 1b).

E₂ Protects Against Inflammation and Injury in Regions of the Colon

After induction of acute colitis using TNBS, E₂-treated WT mice had reduced inflammation in the middle region of the colon. ERβKO mice had reduced inflammation in response to E₂ in the distal end of the colon (Fig. 2a). There were no changes in injury scoring in the WT mice for any region of the colon. In ERβKO E₂-treated mice, however, a reduction in injury was observed in the proximal, distal, and overall colon compared to ERβKO control-treated mice (Fig. 2b).

E₂ Reduces the Expression of Pro-inflammatory Cytokines

Cytokine expression profiles were determined using a multiplex magnetic bead assay. In WT mice, treatment with E₂ resulted in reduced expression of IL-6, IL-12(p40), IL-17, GM-CSF, IFN-γ, MCP-1, MIP-1α, and TNF-α. In ERβKO mice, IL-6 and TNF-α expression was significantly downregulated by E₂. Several other cytokines, IL-12(p40), GM-CSF, MCP-1, MIP-1α, and TNF-α, trended toward reduced expression in response to E₂ in ERβKO mice; however, the differences did not reach statistical significance (Fig. 3).

Proliferation Is Increased in ERβKO Mice

Proliferation was assessed by BrdU incorporation into actively dividing cells. Both the proximal and distal ends of the colon were analyzed. The crypts were further divided into bottom, middle, and top regions. No changes in proliferation were observed in either genotype in response to E₂ in the proximal colon for any region in the crypt (Fig. 4a). In the distal colon, E₂-treated ERβKO mice had increased proliferation compared to control-treated ERβKO mice in the bottom third of crypts. No other significant differences were observed in the distal colon (Fig. 4b).

Apoptosis Is Decreased in ER β KO Mice

Apoptosis was analyzed in the proximal and distal colon using a TUNEL assay. Again, the crypts were divided into bottom, middle, and top regions to observe changes in the different populations of colonocytes. In the proximal colon, E₂-treated ER β KO mice had reduced apoptosis both overall and in the top third of colon crypts compared to control ER β KO mice (Fig. 5a). There was a trend for increased apoptosis in E₂-treated ER β KO mice in the distal colon compared to ER β controls; however, no significant changes occurred in the distal colon in response to E₂ in any region of the crypt for either genotype (Fig. 5b).

Discussion

Epidemiological studies suggest that E₂ is protective against inflammation-associated colon tumor formation [3]. One mechanism by which this protection may occur is through the modulation of acute inflammatory episodes in the colon. Thus far, however, results from in vivo studies investigating the effects of E₂ on inflammation and subsequent tumor formation in colon cancer models have been inconclusive. The differences observed in response to the presence of E₂ may be due, in part, to the inherent differences in the pathology of each disease as well as the variety of models used to study them. This study utilized TNBS to mimic acute Crohn's disease inflammation.

In this model, E₂ was observed to protect against inflammation in WT and ER β KO mice in the middle and proximal regions of the colon, respectively. The likely mediator of the reduced inflammation in the middle and distal colon in WT and ER β KO E₂-treated mice, respectively, is the decreased expression of pro-inflammatory cytokines observed in the presence of E₂. Aberrant expression of cytokines is a hallmark for Crohn's disease, and several of the current therapies are aimed at blocking cytokine activity [18]. Highlighting the fact that E₂ has varying effects depending on ER β expression, cytokine production was reduced in both WT and ER β KO mice; however, only expression of two of the targets analyzed, IFN- γ and IL-6, was significantly downregulated in the ER β KO mice compared to the eight cytokines affected in the WT mice. Studies have shown that macrophages express ER β and that ER β -specific agonists can influence cytokine production [19, 20]. The lack of ER β expression in the ER β KO mice reduces the ability of these cells to respond to E₂ and might partially explain the reduced physiological effects observed in these mice in regard to cytokine production.

While the ER β KO mice lack ER β , they still express ER α and other, non-classical, ERs such as G protein-coupled estrogen receptor 1 (GPER or GPR30). Other studies have observed that ER α -specific agonists influence leukocyte rolling and adhesion to protect against sepsis in the colon, demonstrating that ER α has a protective role in the tissue [21]. GPER has also been implicated in mitigating inflammation; treatment of endothelial cells with a GPER-specific agonist has been demonstrated to reduce the expression of pro-inflammatory cytokines [22]. It is also important to note that the colonic inflammatory process is complex and does not rely solely on the colonic epithelia but is rather a systemic process with immune cells migrating into the tissue upon initiation of the inflammatory cascade. Unlike the colonic epithelia, ER β is not necessarily the primary ER in these immune cells. In human monocytes, Pelekanou et al. observed expression of GPER and ER α 36, a splice

variant of ER α , but they did not detect any expression of ER β or full-length ER α . Furthermore, they demonstrated that in these cells, E₂ treatment inhibited LPS-induced IL-6 inflammatory response and this could be inhibited by the addition of either the nuclear ER antagonist ICI or the GPER-specific antagonist G15 [23]. Another study observed GPER expression in mast cells in human colon biopsies. Contrary to the protective effects of E₂ in the colon observed in our study, this group correlated increased GPER expression in mast cells to enhanced abdominal pain severity in diarrhea-predominant irritable bowel syndrome patients [24]. Together, these studies suggest that ER α 36 and/or GPER has a role in inflammation and in the colon. Being as the ER β KO mice in our study retain ER α and GPER expression, it is possible that these estrogen receptors are responsible for the anti-inflammatory effects of E₂ observed in our model. While it goes beyond the scope of the presented study, ongoing experiments in our laboratory are investigating the contribution of immune cells as well as GPER and ER α and its variants in response to E₂ in both acute and chronic colitis.

Injury scores were also reduced by E₂ treatment. For all groups, injury scoring was highest in the distal colon, possibly a result of the intrarectal method of TNBS administration: Insertion of the gavage tube into the colon physically damages this portion of the tissue which is a limitation as to how data from this model may be compared to Crohn's disease in humans. However, due to the physical damage, the results observed in response to E₂ in the distal colon in regard to injury are partially reflective of wound healing and not solely inflammation. E₂ treatment was associated with a lower injury score in ER β KO mice in the proximal, distal, and overall colon. In the distal colon, in addition to the reduction in injury, an increase in proliferation was observed in ER β KO mice. This increase in proliferation could be indicative of damaged colonocytes being replaced and may be the underlying mechanism behind the increased healing in the distal colons of these mice resulting in reduced injury scores. Future studies will explore the timing of E₂ exposure with the goal to clearly define its potential role in wound healing in the colon.

The fact that the protection against inflammation was observed in different regions of the colon for WT and ER β KO animals and that injury was only reduced in the presence of E₂ in ER β KO mice suggests that during acute inflammation separate mechanisms of protection are being utilized for different regions of the colon in the presence and absence of ER β . The proximal and distal colon is distinct in developmental origin, morphology, and gene expression profiles [25–27]. Additionally, the microbial population also varies between regions of the colon, due in part to changes in the viscosity of the mucus allowing for different microbes to reach the mucosa [28]. These physiological variances between regions of the colon support the hypothesis that the mechanism of action of E₂ differs between the proximal, middle, and distal colon.

Overall, data from this study suggest that E₂ is partially protective against acute TNBS-induced colitis and reduces the expression of pro-inflammatory cytokines in the colon. This protective effect differs between WT and ER β KO mice in regard to location of protection against inflammation, injury, and the cytokine expression profiles. This suggests unique actions of E₂ in the presence and absence of ER β which has clinical relevance due to the reduced ER β expression in the colon of people with active Crohn's disease. Furthermore,

reduction of acute inflammation could be a contributing mechanism by which E₂ reduces the risk of inflammation-associated colon cancer.

Acknowledgments

Funding was provided by American Cancer Society (Grant No. RSG-11-179-01-TVE).

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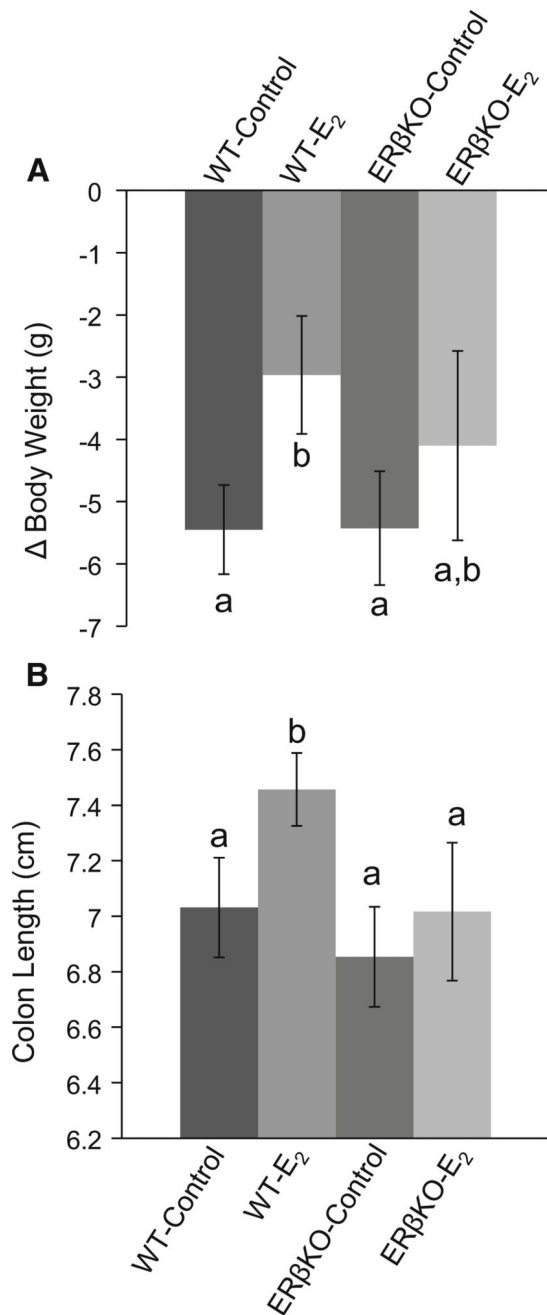


Fig. 1.
a Effect of E₂ on TNBS-induced weight loss. Values are mean weight loss per animal in grams \pm SEM and **b** effect of E₂ on colon length. Values are mean colon length \pm SEM. $n = 12-16$ mice per group. Bars without a common letter differ, $P < 0.05$

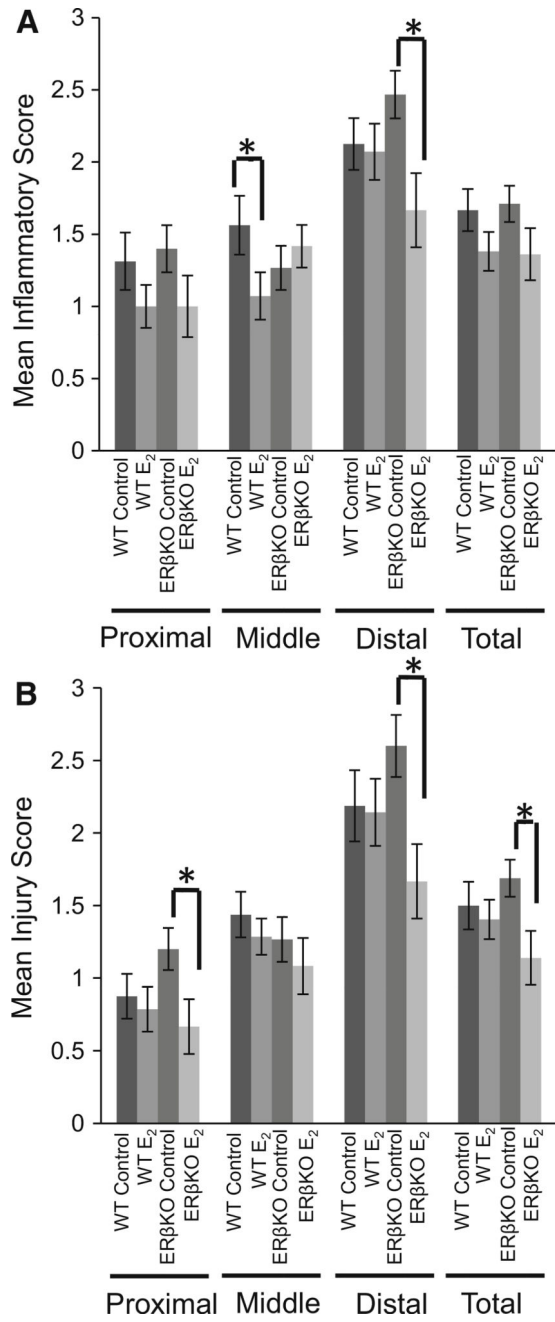


Fig. 2. Inflammation was induced in the colon of ovariectomized mice with TNBS. Sectioned tissues were H&E stained and analyzed by a board-certified pathologist. **a** Inflammation scores and **b** injury scores. Values are mean score ± SEM. *n* = 12–16 mice per group. “*” denotes significance between indicated groups, *P* < 0.05

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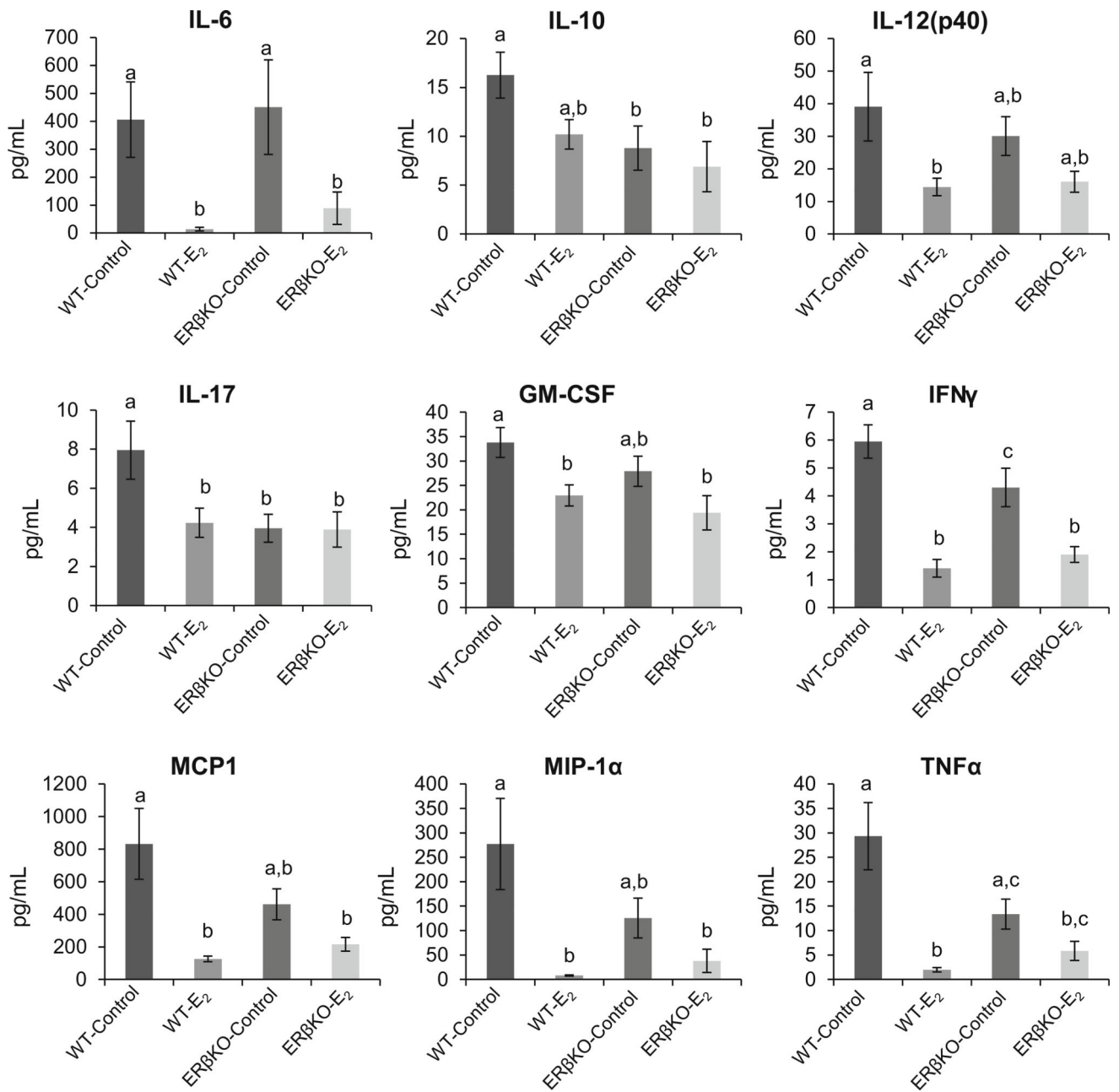


Fig. 3. Cytokine expression in colon tissue was assessed using a multiplex assay. Values are mean expression \pm SEM. Bars without a common letter differ, $P < 0.05$

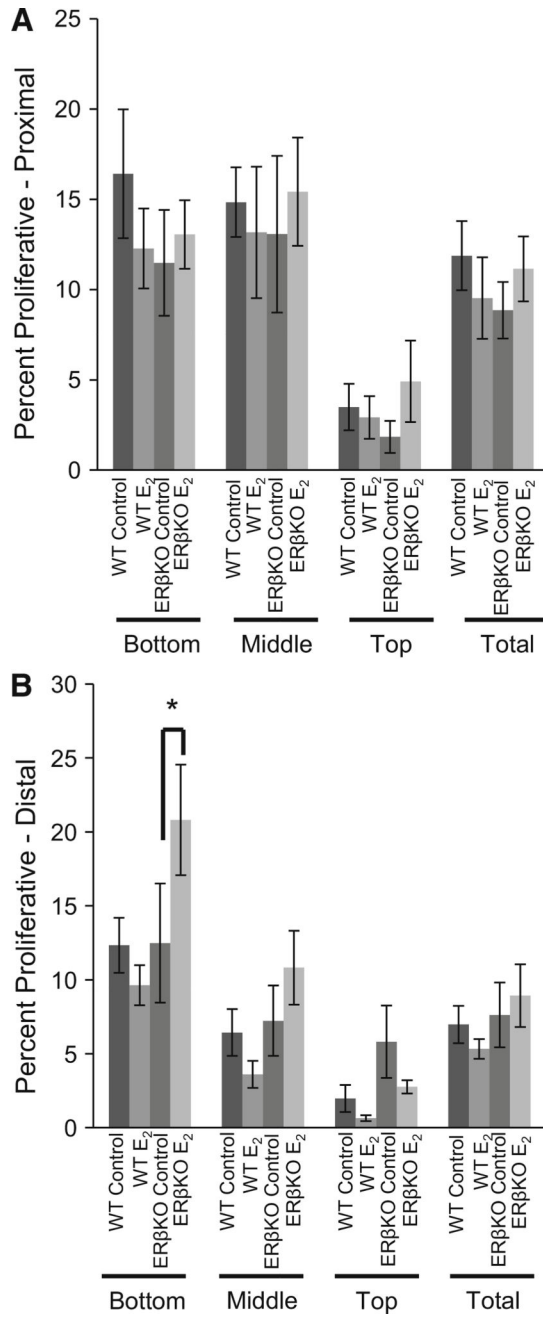


Fig. 4. Effect of E₂ on proliferation during acute inflammation. Two hours prior to kill, mice were injected with 5-bromo-2'-deoxyuridine (BrdU). Immunohistochemistry for BrdU was performed on sectioned tissues from the proximal and distal colon. Data are expressed as the percentage of proliferative cells compared to the total number of cells for either the full crypt column or the indicated region of the crypt. Data are representative of 20 well-oriented crypts per animal per proximal or distal colon with $n = 12-16$ mice per group. **a** Proliferation in the proximal colon and **b** proliferation in the distal colon. “*” denotes significance between indicated groups, $P < 0.05$

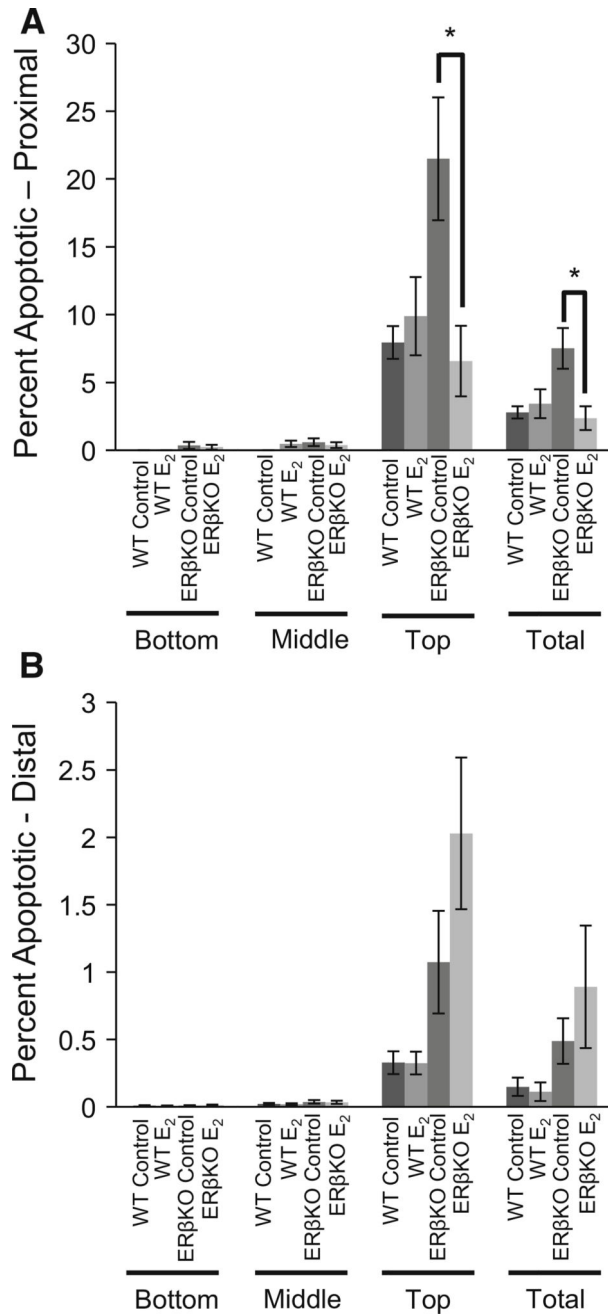


Fig. 5. A TUNEL assay was performed on sectioned tissues from the proximal and distal colon. **a** Apoptosis in the proximal colon and **b** apoptosis in the distal colon. Data are expressed as the mean percentage of apoptotic cells compared to the total number of cells for either the full crypt column or the indicated region of the crypt ± SEM. Data are representative of 20 well-oriented crypts per animal per proximal or distal colon with $n = 12-16$ mice per group. “*” denotes significance between indicated groups, $P < 0.05$