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Two rat strains develop different clinical signs of experimental colitis: does inflammation make all the difference?

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30 **Abbreviations**

31 TNBS - trinitrobenzenesulfonic acid

32 IBD - Inflammatory bowel disease)

33 AO - Albino Oxford

34 DA - Dark Agouti

35 MHC - Major Histocompatibility Complex

36 HLA - Human Leukocyte Antigen

37 PMSF - phenylmethyl sulfonyl fluoride

38 ISPF - α -isonitrosopropiophenone

39 OPD - *o*-phenylenediamine

40 FCS - Fetal calf serum

41 MLN - mesenteric lymph nodes

42 FSC - forwards scatter

43 SSC - low side scatter

44 LPS - Lipopolysaccharide

45 ConA - concanavaline A

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Abstract

Inflammatory bowel disease is a chronic intestinal inflammation resulting from host-microbial interactions in a genetically susceptible individual. Two rat strains, Albino Oxford and Dark Agouti, differ in gut microbiota composition and the overall intensity of inflammatory responses. We investigated whether trinitrobenzenesulfonic acid-induced colitis in these two rat strains may be useful for studying therapies for colitis.

Male Albino Oxford and Dark Agouti rats were intrarectally injected with trinitrobenzenesulfonic acid and clinically evaluated for 7 days. Colon tissue, peritoneal cavity and mesenteric lymph node cells were analyzed for nitric oxide, urea and cytokine production. The phenotype of peritoneal cavity and lymph node cells was determined by flow cytometry.

Dark Agouti rats exhibited higher mortality following colitis induction compared to Albino Oxford rats, accompanied by the extended segments of colon necrosis/inflammation and superior relative balance of IL-17A/IFN- γ in colon tissue and lymph node cells. An increased proportion of neutrophils and diminished proportion of macrophages in peritoneal cavity, and vigorous peritoneal cavity cell IL-6 response was also observed in Dark Agouti rats. Conversely, Albino Oxford rats displayed a higher proportion of peritoneal cavity resident macrophages and lymphocytes from lymph nodes, and dramatically increased production of peritoneal cavity cell IL-10 and urea.

Genetically determined inflammatory phenotype of Dark Agouti rats, in contrast to less inflammatory character of Albino Oxford rats, may serve as a useful model for investigating therapies targeting inflammatory processes in colitis. The use of rats with distinctive inflammatory characteristics may point to specific molecular targets in non-responsive individuals.

Clinical trial number: not applicable.

Key words: Anti-*E. coli* antibodies; rat strains; Trinitrobenzenesulfonic acid (TNBS)-induced colitis;

Introduction

Inflammatory bowel disease (IBD), represented by ulcerative colitis and Crohn's disease, is a chronic relapsing disorder of the gastrointestinal tract that leads to debilitating physical and psychosocial symptoms. These symptoms affect society through loss of schooling, absenteeism, and health-care costs [1]. Due to the complex interactions between environmental, genetic, microbial and immune factors that contribute to multifactorial etiology of IBD [2], mechanisms that trigger IBD are not fully understood. To improve this understanding, several animal models have been developed, one of which is trinitrobenzenesulfonic acid (TNBS)-induced colitis. In addition to the direct toxic effect of TNBS on colonocytes [3], mediated by its conversion into highly toxic reactive oxidants *via* colonic enzymes [4], several other possibly synergistic modes of TNBS action have been proposed. One of them is its ability to complex with surface-active phospholipids, promoting the transition of the mucus gel layer from a hydrophobic to a hydrophilic state. This chemically induced reduction in colonic mucosal hydrophobicity in turn facilitates bacterial adhesion and inflow of water-soluble bacterial products and toxins present in the lumen [5]. Although small TNBS molecules do not elicit an immune response, its intrarectal administration haptenizes mucosal proteins - mostly of commensal microbiota origin - which renders them immunogenic and provokes Th1 inflammation [6,7].

It is suggested that key factors - such as impairment of the intestinal epithelial barrier, disturbed gut microbiota homeostasis, deregulated neural function and inflammatory response - contribute to the etiology of TNBS-induced colitis in rats, as well as IBD in humans [8]. IBD pathogenesis is a result of the interplay of genetic susceptibility and environmental impact on the microbiome that, through a weakened intestinal barrier, will lead to inappropriate intestinal immune activation [9]. This understanding applies to the development of inflammation in TNBS-induced colitis in rats and supports the use of this model in rodents as an appropriate tool for investigating IBD in humans [10].

Innate immune response during TNBS-induced colitis involves macrophage-mediated recognition and lysis of TNBS-modified autologous cells within the mucosa without genetic restrictions [11]. In contrast, cell-mediated cytotoxicity targeting TNBS-modified cell surface components is controlled by genes within distinct regions of the MHC I and II [12,13]. In humans, multiple alleles of conventional antigen-presenting HLA molecules within MHC regions have been linked to IBD pathogenesis [14]. Therefore, immune response to TNBS-altered proteins may vary between MHC II non-compatible animals, such as Albino Oxford (AO) and Dark Agouti (DA) rats. Previous studies have shown that DA rats exhibit higher proliferative activity in draining lymph node cells and a more pronounced inflammatory cytokine response during the challenge and sensitization phases of contact

hypersensitivity reaction to another hapten, dinitrochlorobenzene, compared to AO rats[15]. These rat strains are at the opposite ends of the sensitivity spectrum to the induction of autoimmune response toward neural tissue or cartilage[16,17], and the differences in the composition of gut microbiota have been partially attributed to this phenomenon[18,19]. It was reported that the distinct types of microbial communities may directly affect the severity of intestinal inflammation *via* both innate and adaptive immune cells[20]. Therefore, the aim of the present study was to investigate whether AO and DA rats differ in the rate of the development and clinical signs of TNBS-induced colitis and, if so, to determine whether differences in inflammatory responses between these rat strains may serve as suitable model for researching therapies that target inflammatory processes in colitis in humans. Given that colitis development results in the breakdown of the intestinal epithelial barrier, and that the numbers of fecal Gram-negative *Escherichia coli* (*E. coli*) increases in bowel content[21], we also investigated the profile of the systemic immune response to autologous *E. coli* during TNBS-induced colitis, in addition to differences in the overall inflammatory response of mucosal and lymphoid cells.

Materials and Methods

Animals

Young 3-month-old rats of AO and DA strains were obtained from the local breeding colony (Immunology Research Centre “Branislav Janković”, Belgrade, Serbia). Considering the sex differences in the colonic inflammatory response to bacterial endotoxin[22], and the well-documented sex dimorphism of IBD[23,24], only male rats were used in the study. A total of 99 rats were used (57 AO and 42 DA) across 4 independent experiments, with 8–16 rats per group (Fig. 1).

The animals were housed in standard cages (3–4 rats/cage) in a controlled environment (22 ± 2 °C, humidity ranging 40% to 70% and under 12:12-hour light:dark cycle) with free access to conventional food pellets (Veterinary Institute of Subotica, Serbia) and tap water. Animals were handled daily for seven days prior to experiment, then placed in separate cages and deprived of food for 24h before colitis induction. Seven days after colitis induction animals were euthanized by increasing dose of CO₂. The experimental protocols and animal procedures including their care were approved by the Ministry of Agriculture and Environmental Protection (license number 323-07-12928/2022-05/2, issued on 12-05-2022), and adhered to the principles outlined in Directive 2010/63/EU of the European Parliament and of the Council from 22 September 2010 on the protection of animals used for scientific purposes (revising Directive 86/609/EEC). Animal experimentation and reporting were conducted in accordance with the principles recommended by ARRIVE guidelines 2.0 (originally published in PLOS Biology: [doi:10.1371/journal.pbio.3000411](https://doi.org/10.1371/journal.pbio.3000411) and available at <https://arriveguidelines.org/arrive-guidelines>)

Chemicals and immunoconjugates

Trinitrobenzenesulfonic acid (TNBS), phenylmethyl sulfonyl fluoride (PMSF), α -isonitrosopropiophenone (ISPF), *o*-phenylenediamine (OPD), and RPMI-1640 medium (RPMI) were purchased from Sigma (St. Louis, MO, USA). Fetal calf serum (FCS) was obtained from Gibco (Grand Island, NY, USA).

Monoclonal PE-conjugated mouse anti-rat CD163 antibody (clone HIS 36, 554901), biotin-conjugated mouse anti-rat CD11b (clone WT.5 554981) antibody, PE-Cy5-conjugated anti-rat CD45RA (557015), FITC-conjugated goat anti-mouse IgG (554020), mouse anti-rat MHCII (554929), and mouse anti-rat CD8a (554856), PerCP-conjugated streptavidin (Sav-PerCP, 554064) and appropriate IgG isotype controls were purchased from BD Biosciences Pharmingen (Mountain View, CA, USA). Biotin-conjugated mouse anti-rat granulocyte (clone HIS48, 13-0570-82) and PE-conjugated mouse anti-rat CD43 (clone W3/13, 202812) antibodies were purchased from BioLegend

(San Diego, CA, USA). APC-conjugated anti-rat CD4 (MR5105) and PerCP-eFluor 710-conjugated anti-rat CD25 (46-0390-80) antibodies were purchased from Invitrogen (Carlsbad, CA, USA).

Induction of colitis and clinical signs monitoring

Colitis was induced according to the procedure described previously[25,26]. Rats were first anaesthetized with a cocktail containing ketamine and xylazine (60/7.5 mg/kg). Upon reaching deep anesthesia, rats were intrarectally injected with approx. 300 µl of 50% ethanol (EtOH), or different doses of TNBS solution (10 mg/kg and 40 mg/kg) in 50% ethanol *via* flexible tubing ($\varnothing=0.58\text{mm}$), approximately 8 cm deep into the colon. The control rats received 300 µl of sterile saline applied in the same manner. Rats were then held in a head-down position for about 5 minutes to minimize passive rectal outflow. Our study used an acute model of TNBS-induced colitis, as it offered an insight into the short-lasting barrier alterations and innate immune effects without complications that accompany chronic diseases model, such as neoplasia and tissue fibrosis[7]. Over the next six days, food and water consumption were monitored daily, as well as the behavior and outward appearance of the rats.

Tissue and cell sampling

All specimens were removed aseptically seven days following the colitis induction. The abdomen of each rat was soaked in 70% alcohol, the abdominal skin with fur retracted to expose the intact peritoneal wall and a small incision was made along the midline with sterile scissors. Ten milliliters of sterile ice-cold phosphate buffer (PBS) pH 7.4 supplemented with 2% fetal calf serum (FCS) was injected, the entire body was gently shaken for 10 seconds and the fluid was gently aspirated. Individual peritoneal cell suspensions were washed twice in PBS/2%FCS (250 x g) at 4°C and counted.

Then, the incision was widened to expose the heart below the cage ribs and blood was obtained by cardiac puncture. The blood (approx. 1 ml) was allowed to curd for 1 h at room temperature and for another hour at 4°C, centrifuged for 15 minutes at 100 x g, and sera were removed and stored at -20°C until antibody levels were determined.

The mesenteric lymph nodes (MLN) were carefully extirpated from excess mesenteric tissue, weighed, mechanically disrupted using a plunger and cells were released through sterile cell strainer (Corning®, pore size 70 µm) with RPMI/5%FCS, washed twice in RPMI/5%FCS and counted.

The colon was excised from the cecal folds to the pelvic region and photographed. It was then opened longitudinally and gently rinsed in sterile physiological saline to remove fecal residues. Colon segments affected by hyperemia/oedema and ulceration/necrosis were measured. Segments of inflamed colon tissue were either rolled up, fixed in buffered 4% paraformaldehyde, and embedded in paraffin, or immediately homogenized with potassium-phosphate buffer supplemented with 1% protease inhibitor cocktail (Thermo Scientific, Rockford, IL,

USA) and centrifuged at 10,000 x g for 10 minutes. Supernatants were used to estimate urea and cytokine levels. Protein concentration in colon tissue homogenates, determined by Bradford method[27] was used to normalize all the results. Optical densities (OD) were determined using a Multiscan Ascent plate reader (Labsystems, Helsinki, Finland).

Flow cytometric analysis of peritoneal and MLN cells

Flow cytometric analysis was performed immediately following peritoneal and MLN cell isolation. Individual cell aliquots (50 μ l, 1×10^7 cells/ml) were immunolabelled and then analyzed on a FACSVerse™ flow cytometer using BD FACSuite™ software (Becton Dickinson, Mountain View, CA, USA). Debris and dead cells were gated out based on low forwards scatter (FSC) and low side scatter (SSC). Cell doublets were excluded by displaying the FSC height values over the FSC area values. A total of 25,000 events were recorded within the live cell gate. Surface marker assessment for peritoneal cells was performed by immunolabeling with biotin-conjugated anti-CD11b and HIS48, followed by PerCP-conjugated streptavidin, and with directly labelled PE-conjugated anti-CD163, anti-CD43, and anti-HIS48, or FITC-conjugated anti-CD4 and anti-MHCII antibodies. MLN cells were immunolabeled with PE-Cy5-conjugated anti-CD45RA, APC-conjugated anti-CD4, FITC -conjugated anti-CD8 and PCP eFluor 710-conjugated anti-CD25 antibodies.

Peritoneal and MLN cell cultures

Peritoneal and MLN cells were adjusted to 1×10^6 cells/ml in RPMI/2%FCS. Peritoneal cells were plated to tissue culture plates for 2 h at 37°C in a humidified atmosphere containing 5% CO₂ in the air and non-adherent cells were removed by washing the plates twice with warm PBS. Adherent peritoneal cells were incubated for an additional 24 h with 1 μ g/ml LPS in RPMI/5%FCS (for cytokine determination), or for 48 h with RPMI/5%FCS (for nitric oxide and urea determination), Since MLN cells are loosely adherent, they were directly stimulated with 2.5 μ g/ml of concanavalin A (Con A) in RPMI/5%FCS for 24 h (for cytokine content determination).

Cytokine level determination

Cytokine levels (TNF, IL-6, IL-10, IL-17A and IFN- γ) were determined in supernatants from peritoneal cavity and in MLN cell cultures after 24 hours of incubation, and in supernatants of freshly prepared colon tissue homogenates using commercially available ELISA kits (R&DSystems, Minneapolis, MN, USA).

Nitric oxide (NO) and urea determination

Supernatants of peritoneal cavity cell cultures following 48 hours of incubation were analyzed for nitrite concentration by a method based on the Griess reaction[28]. The concentration of nitrite in the samples was

determined according to optical densities measured at 545 nm and calculated using a NaNO_2 (1-40 μM) as a standard.

Arginase activity in peritoneal cells and in supernatants of colon tissue homogenates was indirectly determined by measuring the metabolite urea[29,30]. Adherent peritoneal cells on plates were first lysed by the addition of 0.1% Triton X-100 containing 2 mM PMSF and shaken for 30 minutes (200 cycles/minute). Then, arginase was activated in peritoneal cell lysates and in supernatants of freshly prepared colon tissue homogenates by 10 mM MnCl_2 /25 mM Tris-HCl, pH 7.5, for 10 minutes at 56°C and hydrolyzed by the addition of 0.5 M arginine, pH 9.7 at 37°C for 22 h. The reaction was stopped by adding an acid mixture containing H_2SO_4 , H_3PO_4 and H_2O (1:3:7). Urea concentration was measured at 545 nm after adding ISPF dissolved in absolute ethanol, followed by heating at 95°C for 45 minutes. Urea concentration was calculated according to the standard curve obtained with known concentrations of a urea (25-2500 μM).

Histological examination of colon tissue segments

Multiple sections (5 μm thick) of colon tissue segments were stained with hematoxylin and eosin. An Olympus® BX53 microscope (Tokyo, Japan) equipped with a digital camera (Olympus) was used to capture images.

Enzyme linked immunosorbent assay (ELISA) for determination of IgG1, IgG2a, IgG2b and IgA classes of specific anti-E. coli antibodies in sera

Fifty microliters of 1×10^8 /ml formalin-killed *E. coli* suspended in PBS were coated onto 96 well flat-bottomed tissue culture plates (Nunc Maxisorb). Fecal commensal *E. coli* was isolated from stool specimens collected from healthy AO and DA rats and prepared as previously described[31]. Plates were centrifuged for 15 min at 350 g to ensure bacterial adherence to the plastic, then incubated at 4° C overnight, washed four times with PBS/0.05% (v/v) Tween 20 (PBS/Tween), and blocked by using 2% bovine serum albumin (BSA) solution in PBS for one hour at 37° C. Plates were emptied and sera in 1% BSA/PBS/Tween were added at a 1:400 dilution for the IgG1, IgG2a and IgG2b ELISAs, and at a 1:50 dilution for the IgA ELISA. Sera were incubated for two hours at room temperature and washed two times with PBS/Tween and two times with PBS, followed by the addition of biotin conjugated anti-rat IgG1, IgG2a, IgG2b or IgA antibodies diluted 1:1000 in 1% BSA/PBS/Tween. Plates were left for one hour at room temperature, washed as before, and extravidin-peroxidase (1:4000) was added to the wells. After additional incubation for 30 minutes at room temperature, plates were washed with washing buffer, H_2O_2 -activated OPD (1 mg/ml) was added and color was allowed to develop for 15 minutes in the dark. Finally, the reaction was terminated with 1M H_2SO_4 . Optical densities (OD) were determined at 492/620 and results were expressed as OD x 1000.

236 ***Statistical analysis***

237 Survival analysis was performed by Kaplan-Meier's method with TIBCO Statistica® v13.3 package (Palo Alto,
 238 CA, USA), while all other calculations and statistical analysis were performed by using GraphPad Prism 5
 239 (GraphPad Software, San Diego, CA, USA). The effects of TNBS treatment on cytokine and NO/urea production
 240 in cells and tissue, as well as antibody levels in sera were analyzed by two-way ANOVA with factors Rat strain
 241 (AO vs. DA) and Treatment (saline, EtOH, 10 mg/kg, or 40 mg/kg TNBS). Bonferroni's multiple comparison was
 242 used as a *post hoc* test. Relative changes in body weight and changes in the proportion of cells within peritoneal
 243 cavity and MLN during colitis were analyzed by non-parametric Kruskal-Wallis's test. When the null hypothesis
 244 was rejected and (if necessary) corrected for type I error, Dunn's post-test was performed. Results show mean +
 245 SEM, or mean $\pm$ SEM. Data were collected from 4 independent experiments and the number of rats was 8–16 per
 246 group. Total number of rats used in the study was 99 (57 AO and 42 DA). Differences were regarded as statistically
 247 significant if $p < 0.05$.

248 ***Data Availability Statement***

249 Research data are deposited to InTOR repository. During the revision process research data will be available upon
 250 request from Irena Nježić, repository administrator, *via* mail injezic@torlak.rs. After manuscript acceptance,
 251 research data will be available online at https://hdl.handle.net/21.15107/rcub_intor_974 under CC BY license.

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Results

Survival analysis and clinical course of colitis in AO and DA rats

Mortality following colitis induction was considerably higher in DA rats relative to AO rats. Ethanol, as well as both TNBS doses, contributed to the decreased survival rate of DA rats during colitis development with mortality ranging from 25-40% in ethanol, 10 mg/kg and 40 mg/kg TNBS groups, respectively. Instillation of 40 mg/kg TNBS resulted in the death of DA rats as early as day after the intrarectal injection (Fig. 1A). In contrast, only 1 out of 16 AO rats instilled with 40 mg/kg TNBS died, resulting in a mortality rate of 6.25% in this group, whereas all AO rats instilled with ethanol or 10 mg/kg TNBS survived till the end of the experiment (Fig. 1A). The survival analysis was shown in Fig. 1B. Both rat strains developed colitis following ethanol or TNBS instillation, as assessed by the colon necrosis and hyperemia observed macroscopically (Fig. 2A). Diarrhea, sometimes with the traces of blood, was observed in the first few days following ethanol and TNBS instillation in both rat strains, followed by impacted stool observable within a severely dilated colon at the end of experiment. Colon necrosis induced by 40 mg/kg of TNBS was more prominent than that induced by ethanol in rats of AO strain. However, in DA rats, necrosis and hyperemia were similar between ethanol- and TNBS (10 mg/kg and 40 mg/kg)- injected groups (Fig. 2B). The portion of colon affected by necrosis was greater in DA than in AO rats treated with 10 mg/kg TNBS, but the segment of edematous/hyperemic colon was larger in all treated rats of DA compared to AO strain. (Fig. 2B). Despite evident pathological damage in all injected rats, the portions of the colon affected were substantially larger in DA compared to AO rats.

Food intake was reduced in both AO and DA rats injected with ethanol or TNBS compared to saline- injected controls from their respective strains (Fig. 3A). Food consumption in AO rats decreased similarly regardless of the treatment (ethanol or TNBS) or TNBS dose (10 mg/kg or 40 mg/kg), whereas it was less expressed in ethanol-injected than in TNBS-injected rats of DA rat strain (Fig. 3A). Water intake in AO rats during colitis was either comparable or decreased relative to saline-injected AO controls. Quite the opposite, water intake was significantly increased in DA rats instilled with 10 or 40 mg/kg TNBS on days 3-5 following colitis induction (Fig. 3B). Nevertheless, rat weight alteration was similar in ethanol- and TNBS (10 or 40 mg/kg)-injected rats of both rat strains relative to their respective saline-injected controls which represented 100% (Fig. 3C).

Paraffin-embedded colon sections of the saline-treated AO and DA rats exhibited unmarred mucosal architecture, with preserved crypts and no visible thickening or fibrosis of any tissue layer (Fig.4).

Ethanol injection in the colon of AO rats caused mucosal congestion with concomitant demarcation of lamina propria. Hemorrhage in mucosa was accompanied by overt eosinophilic (Fig. 4, insert I, arrows) and siderophage

infiltration (Fig. 4, insert II, asterisks) in submucosa, along with transmural inflammation along the attenuated muscularis propria layer. Similar ethanol-initiated mucosal alterations were observable in DA rats, including diffuse necrosis and crypt resorption with hemorrhage and demarcation of lamina propria, as well as a large erosive defect spanning the mucosa and submucosa layers (Fig. 4, triangle). Related, but more severe alterations were observed in colon segments of AO and DA rats treated with 10 mg/kg of b.w. of TNBS. These segments displayed severe congestion in lamina propria, dysplastic and aberrant crypts resorption, necrosis, and demarcation of the submucosa with transmural infiltration along the lamina muscularis propria. In addition to these similar alterations in the lamina propria, colon segments from AO and DA rats treated with 40 mg/kg of b.w. of TNBS exhibited edematous lamina muscularis mucosae and vacuolar degeneration of lamina muscularis interna myocytes (Fig. 4, insert III, arrows). The serosa beneath the damaged mucosal layer in DA rats was visibly thickened.

The level of urea and cytokines in colon tissue homogenates following colitis induction in AO and DA rats

In colon tissue homogenates from controls (saline-injected rats), the concentration of urea was higher in DA compared to AO rats, while the levels of IFN- γ , IL-17, IL-6 and TNF were comparable between these rat strains (Fig. 5A and 5B). Instillation of ethanol or TNBS significantly increased urea concentrations to a greater extent in AO rats than in DA rats, and increased the concentration of IL-6 in colonic tissue of all treated rats (Fig. 5A and 5B). Intrarectal injection of ethanol increased TNF levels in AO rats, whereas treatment with a lower dose of TNBS (10 mg/kg) augmented the concentration of IL-17 and TNF in colonic tissue of DA rats and the concentration of IFN- γ in both rat strains. The higher TNBS dose (40 mg/kg) reduced IFN- γ and IL-17 levels in DA rats (Fig. 5B). However, the relative IL-17/IFN- γ ratio remained consistently higher in colon tissue homogenates of all DA groups compared to to AO rat strain (Fig. 5C).

Phenotype and functional properties of peritoneal cavity cells following colitis induction in AO and DA rats

Instillation of ethanol or TNBS significantly increased the proportion of CD11b^{int}CD4^{low} cells in both rat strains (Fig. 6B, left), but had no effect on the peritoneal cavity CD11b^{hi}CD4^{hi} macrophages, except for 40 mg/kg TNBS, which increased macrophage proportion only in DA rats (Fig. 6B, right). The proportion of HIS48⁺CD43⁺ peritoneal cavity neutrophils within the CD11b^{int}CD4^{low} cell pool also significantly rose in both rat strains upon treatment (Fig. 6C, left). However, the percentages of resident CD163⁺MHCII^{lo} macrophages within the CD11b^{hi}CD4^{hi} macrophage cell pool, which were higher in control AO rats than DA rats, significantly decreased following ethanol or TNBS in both AO and DA rats (Fig. 6C, right). At the same time, the proportion of CD163⁻MHCII^{hi} cells (“inflammatory” peritoneal cavity macrophage/dendritic cells within CD11b^{hi}CD4^{hi} macrophage cell pool), which was higher in control DA rats than in AO rats, significantly increased following ethanol and

TNBS in AO rats but remained unchanged in the peritoneal cavity of DA rats (Fig. 6C, right). Cells were gated according to FSC and SSC (Fig. 6A)

Adherent peritoneal cavity cells from control (saline-injected) AO rats produced comparable amounts of NO, urea, TNF and IL-10, but a lower amount of IL-6 than cells from control DA rats (Fig. 7A, 7B). Ethanol injection increased cytokine production only in adherent cells from AO rats (Fig. 7B), while none of the treatments affected NO release in adherent cells from either rat strains (Fig. 7A). In both rat strains, instillation of 10 mg/kg TNBS or 40 mg/kg TNBS increased adherent peritoneal cavity cell TNF, IL-6, IL-10 and urea release, except in DA rats where 40 mg/kg TNBS had no effect on urea production (Fig. 7A, 7B).

Phenotype and functional properties of mesenteric lymph node (MLN) cells following colitis induction in AO and DA rats

In AO rats, ethanol and 10 mg/kg and 40 mg/kg TNBS reduced the proportion of CD8⁺ MLN cells and increased the proportion of CD45RA⁺ MLN cells. None of the treatments affected CD4⁺ and CD4⁺CD25⁺ MLN cell proportions (Fig. 8A). In DA rats intrarectal injection of 40 mg/kg TNBS reduced the proportion of CD4⁺ cells and increased the proportion of CD45RA⁺ cells in MLNs, but none of the treatments altered the percentages of CD4⁺CD25⁺ and CD8⁺ cells in MLNs (Fig. 8A).

At the same time, intrarectal injection of 40 mg/kg TNBS increased Con A-stimulated MLN production of IL-17 in both AO and DA rats, while none of the treatments influenced the IFN- γ level in either rat strain (Fig. 8B). The relative IL-17/IFN- γ ratio increased following colitis induction in both rat strains, and was significantly higher in MLN cells of DA than in AO rats injected with 40 mg/kg TNBS (Fig. 8C).

The level of specific anti-*E. coli* antibodies in sera of AO and DA rats following colitis induction

The total serum proteins levels were comparable in AO and DA rats, irrespective of their intrarectal treatments (Fig. 9A). The levels of specific anti-*E. coli* antibodies of the IgG1, IgG2a, and IgG2b classes were also comparable between saline-treated AO and DA rats, whereas the level of anti-*E. coli* IgA antibodies were significantly higher in saline-treated DA compared to saline-treated AO rats (Fig. 9B). Further analysis revealed that intrarectal injection of 40 mg/kg TNBS significantly increased the levels of all classes of anti-*E. coli* antibodies in the sera of AO rats compared to saline-treated AO rats (Fig. 9C-F). However, intrarectal injection of TNBS increased the level of anti-*E. coli* IgG1 and IgG2b (40 mg/kg) or IgG2a (10 mg/kg and 40 mg/kg) antibodies in DA rats, but none of the treatments changed the level of specific anti-*E. coli* antibodies of IgA classes in sera of DA rats (Fig. 9C-F).

DISCUSSION

Our results revealed several interesting points. Intrarectal injection of ethanol induced colitis in both rat strains, with the segment of colon affected by ulceration being shorter than that induced by TNBS in AO rats, and comparable between ethanol and TNBS injections in DA rats. Almost all AO rats survived until the end of experiment whereas DA rats experienced a significant mortality rate. Finally, both rat strains developed systemic anti-*E. coli* antibodies of IgG classes, but only AO rats mounted an IgA response following colitis induction. Comparative degree of colonic changes induced by ethanol and TNBS in DA rats suggests that the loss of epithelial integrity induced by ethanol alone[26] was sufficient to cause colitis. However, induction with ethanol, as opposed to TNBS, did not change the proportion of peritoneal cavity macrophages and proportion of lymphocytes within MLN, nor did it affect cytokine levels detected in colon tissue, or synthesized by peritoneal cavity macrophages or MLN lymphocytes in DA rats. Moreover, the order of lethal outcome in these rats was 40 mg/kg TNBS > 10 mg/kg TNBS > ethanol. The fact that TNBS, and not ethanol injection alone, elevated the concentration of specific anti-*E. coli* IgG antibodies further support the idea that haptenization of microbiota is necessary for the immune-mediated development and perpetuation of TNBS-induced colitis in DA rats. These findings could point to the involvement of gut bacteria in the mechanism of TNBS-induced colitis in these rats. While colitis induction diminished the amount of food and water consumed over a 6-day period in AO rats, which was in line with wasting observed in animals with colitis[7], all treated DA rats began consuming more water than saline-injected controls from day 3 following disease induction. This phenomenon has been occasionally recorded in TNBS-treated rats[32], likely representing a compensation for reduced fluid absorption during colitis[10]. Both rat strains eventually developed compacted stools observable within a heavily dilated colon. This may have been caused by a reduction in the contractility of colonic muscles, possibly due to changes in the neural control of colonic motility[33]. Colonic urea levels increased in both rat strains following ethanol and TNBS treatments. Urea is freely diffusible from the blood, as well as produced by arginase activity in the gastrointestinal microvasculature, and spontaneously hydrolyzes to ammonia, which is later converted to ammonium hydroxide[34,35]. This caustic base causes tight junction protein depletion in the colonic epithelial cell[35,36]. Several studies have revealed an enhanced expression and activity of arginase in the intestinal mucosa of patients with IBD and in mice with colitis[37,38] which is linked to heightened vascular pathophysiology in inflamed gut blood vessels[35]. AO rats treated with ethanol or TNBS, and DA rats treated with TNBS, produced higher colonic levels of TNF, IL-6 and IFN- γ than the control group, which is consistent with the Th1 nature of IBD. The main sources of cytokines in the inflamed colons are infiltrating immune cells, intestinal epithelial cells, and subepithelial

myofibroblasts[39]. Elevated TNF levels contribute to epithelial cell shedding, their necroptosis and degradation of tight junction proteins[40]. Increased levels of IFN- γ , produced by innate lymphoid cells within mucosa, contribute to epithelial cell apoptosis[41,42] and IL-6 signaling is required for induction and maintenance of Th17 cells[43]. Interestingly, MLN lymphocytes of both rat strains produced increased levels of IL-17A during colitis, whereas in colon tissue, IL-17A production was augmented only in DA rats. MLNs are the primary site for priming of naive T lymphocytes, while their differentiation into gut-tropic Th17 cells requires the presence of commensal bacteria[44,45]. Since gut bacteria translocation was common in MLNs of mice with chemically-induced colitis and patients with IBDs[46–48], as we have previously observed in DA rats[49], the decrease in the proportion of CD4⁺ T lymphocytes within MLNs of DA rats treated with a higher TNBS dose may indicate their priming by enteric microbiota and migration to the colon, where they contribute to IL-17A production. This cytokine seems to be paramount in colitis[50], and it further perpetuates a series of downstream inflammatory mediators within the microenvironmental cytokine colon niche. Th17 cells display substantial plasticity, and some Th17 cell precursors retain a predominantly IL-17A⁺ phenotype, whereas others extinguish IL-17 expression by acquiring IFN- γ expression, giving rise to “Th1-like” cells[51]. The levels of IL-17A and IFN- γ depend on the inflammatory milieu[52]. It is worth noting that the relative balance of IL-17A / IFN- γ in MLNs of rats treated with the highest TNBS dose, and in colon tissue of virtually all experimental groups, was higher in DA rats compared to AO rats. High production of IL-17A in draining LN cells of DA rats was associated with susceptibility to experimental autoimmune encephalomyelitis, whereas impaired IL-17A production correlated with the resistance of AO rats to this disease[17].

Contrary to the detrimental role of urea in colon tissue, urea is advantageous for peritoneal macrophages during inflammation. Urea is a stable by-product of the macrophage arginase activity, which catalyzes the conversion of L-arginine to L-ornithine, involved in proline and polyamines synthesis and subsequent cell proliferation, collagen synthesis and wound repair[53]. Given that macrophage enzymes inducible nitric oxide synthase (iNOS)[54] and arginase compete for the common substrate, semi-essential amino acid L-arginine[55], and that macrophage urea production increases during colitis in both rat strains (except in DA rats treated with higher TNBS dose), a shift in favor of macrophage arginase over iNOS activity during colitis in AO and DA rats is suggested. Colitis development did not affect adherent cell NO release in either AO or DA rats in the present study, which was previously observed for DA rats[49]. Arginase I is induced in macrophages by Th2 cytokines[56], which could be locally produced in a paracrine manner by the abundant infiltrating eosinophils in the outer smooth muscle layer of the affected colon. Given that macrophage NO production did not change during colitis in either rat strain, and

AO rats treated with 40 mg/kg b.w. TNBS had a greater macrophage urea production compared to cells from DA rats, it can be surmised that these cells have a significant anti-inflammatory effect in AO rats.

The role of peritoneal immune cells in colon inflammation is suggested by data showing that intraperitoneal injection of mitomycin-C, which activates peritoneal macrophages, produces colitis[57,58], whereas intraperitoneally delivered inhibitor of nuclear factor NF- κ B ameliorates severe TNBS-induced colitis *via* migration of drug-bearing macrophages from the peritoneal cavity to inflammation site[59]. Breakdown of the intestinal epithelial barrier during colitis results in the inflow of commensal flora, and Gram-positive bacteria specifically induce local expression of C–C chemokine receptor type-2 (CCR2) ligands, which mobilize CCR2-expressing monocytes/macrophages into the colon[60]. Large peritoneal cavity macrophages expressing distinctive transcription factor GATA6[61], can detect luminal injury and rapidly invade the injured site *via* peritoneal route in a CCR2- and microbiota-independent manner[62].

Colitis induced secondary peritoneal inflammation in both rat strains, probably because of dissemination of fecal bacteria. This was characterized by the influx of CD11b^{int}CD4^{low} (HIS48⁺CD43⁺) neutrophils into the peritoneal cavity and a decrease in the percentage of resident peritoneal CD163⁺MHCII^{low} macrophages within the CD11b^{hi}CD4^{hi} pool, part of which may have been directed toward injured colon sites. Saline-treated DA rats had a higher proportion of CD163⁺MHCII^{high} “inflammatory” macrophages and a lower percentage of resident “anti-inflammatory M2”[63] CD163⁺MHCII^{ow} macrophages, compared to AO rats. In addition to that, DA rat macrophages displayed a higher LPS-stimulated secretion of IL-6 and lower secretion of IL-10 in DA compared to AO rats. These results point to more intense steady-state priming of cells in DA rats by local peritoneal cytokine milieu, as previously suggested[64]. Greater inflammatory polarization of peritoneal macrophages in DA rats may also contribute to the increased IL-17A / IFN- γ ratio in the colon tissue of these rats compared to AO rats[65]. The therapeutic effect of bone marrow-derived mesenchymal stromal cells on experimental colitis has been shown to be exerted through the induction of peritoneal macrophage IL-10 expression, which led to bystander IL-10 expression in intestine-resident B and T cells[66]. Likewise, *in vivo* polarization of M2 macrophages by *Trichinella spiralis* adult worms, chromofungin or the TLR2/1 agonist PAM3, which increase macrophage arginase expression and their IL-10 production, reduce clinical signs of colitis by migrating to the colon[67–69]. It is noteworthy that compared to DA rats, peritoneal cells of AO rats dramatically increased their IL-10 production in response to epithelial barrier breakdown during colitis in the present study. Of course, IL-10 is an important factor in the induction of macrophage arginase I[70]. Since the depletion of resident macrophages increases sepsis lethality[71], a greater number of resident CD163⁺MHCIII^{ow} macrophages in the control, and subsequently in the colitis group

(TNBS 40 mg/kg b.w.) of AO rats compared to DA rats, may be an additional factor in their protection against sepsis. Altogether, this suggests that a less inflammatory macrophage phenotype in AO rats may blunt colitis-induced inflammation in these rats, contributing to their higher survival rate. The proportion of CD163⁺MHCII^{high} “inflammatory” macrophages remained unchanged in the pool of peritoneal cells during colitis in DA rats, alongside a decrease in the percentage of “anti-inflammatory M2” MHCII^{low} macrophages, which was observable in both rat strains. Given the role of MHC molecules in mounting innate immune response[72], decreased availability of MHCII⁺ peritoneal cells in DA rats may lead to insufficient control over peritoneal bacterial burden during colitis and to disease worsening in this rat strain.

Our next avenue of the research was to investigate whether the levels of specific anti-*E.coli* antibodies differ in the sera of AO and DA rats with colitis, and to what extent it may correlate with strain differences in colitis development and survival. When the intestinal barrier is intact, indigenous bacteria translocate *via* an intracellular route through the epithelial cells lining the intestines and travel *via* the lymph to the MLNs. However, damage to the mucosal epithelium allows their intercellular translocation between the epithelial cells, providing direct access to the blood[73].

Increased anti-microbial antibody response was shown to be an early pre-disease event in the development of Crohn’s diseases[74] and increased levels of antibodies directed to outer membrane porin-C (Omp-C) of *E. coli* or to clostridial flagellin were detected in these patients[75]. Inability to mount vigorous IgG1 response to Omp-D of another Gram-negative gut microorganism, *Salmonella* typhimurium, has been shown to result in less efficient bacterial opsonization, reduced uptake by macrophages, and diminished protection from *Salmonella* typhimurium infection[76]. Moreover, anti-commensal IgA antibodies in serum have been shown to be protective against widespread and lethal bacterial invasion following intestinal barrier disruption[77]. In our study, a more robust rise in the levels of specific anti-*E. coli* antibodies of IgG1, IgG2a, IgA and, to lesser extent, IgG2b classes in AO rats point to a superior systemic antibacterial defense in this rat strain during colitis. A protective role of serum immunoglobulins of IgG1, IgM and IgA classes against mortality has been previously recognized in postsurgical patients with septic shock[78], and a combined systemic deficiency of IgG1, IgA and IgM antibodies has the greatest influence on survival in septic patients[79]. Hence, the relatively modest rise in the levels of anti-*E.coli* antibodies of IgG classes, along with the complete inability to increase anti-*E. coli* IgA antibody response following colitis induction, suggests that poor anti-*E.coli* humoral immunity may contribute to significant mortality of DA rats during colitis.

CONCLUSION

Considering that host genetics in an inflammation-dependent manner affect the expression of genes associated with the progression of colitis, some of which are potential drug targets[80], the use of rats with distinctive inflammatory characteristics may point to specific molecular targets in non-responsive individuals. Despite all limitations, animal models are indispensable for investigating mechanistic details that facilitate better preclinical drug/therapy design to target specific components involved in human disease pathogenesis.

Our results suggest that superior IL-17 / IFN- γ ratio in colon and draining lymph node cells, an augmented proportion of neutrophils, diminished proportions of both MHCII⁺ and MHCII⁻ macrophages in peritoneal cavity accompanied with vigorous IL-6 response of the peritoneal cells created an inflammatory setting that predisposed DA rats to aggravated clinical signs of colitis, such as polymicrobial sepsis following TNBS treatment. Even though AO rats also develop colitis following TNBS instillation, a greater number of resident peritoneal macrophages and a significant increase in IL-10 and urea production by peritoneal cavity cells blunt colitis-induced peritoneal inflammation sufficiently to prevent lethal outcomes from septic shock in AO rats. The absence of an increase in systemic levels of anti-*E. coli* IgA antibodies in DA rats, in contrast to strong humoral immune response to gut commensal antigens following epithelial barrier breakdown in AO rats, probably also contribute to the distinctions in clinical signs of colitis. The differences displayed between the two rat strains in this study could possibly mean the difference between survival and demise, which goes to highlight the importance of seemingly minute variances in gut microbiota and immunity-related parameters.

Genetically determined inflammatory phenotype of DA rats, in contrast to less inflammatory character of AO rats, may serve as a useful model for investigating therapies targeting inflammatory processes in colitis. Additionally, we hope these results contribute to the steadily growing body of evidence that the commonly mucosal tissue-associated class of antibodies, IgA, also play an indispensable role in serum during life-threatening conditions such as septic shock.

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507

508 **Conflict of interest statement:**

509 The authors declare that there are no financial, professional, or personal conflicts of interest regarding this
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Statements and Declarations

Ethics approval;

The experimental protocols and animal procedures including their care were approved by the Ministry of Agriculture and Environmental Protection (license number 323-07-12928/2022-05/2, issued on 12-05-2022), and adhered to the principles outlined in Directive 2010/63/EU of the European Parliament and of the Council from 22 September 2010 on the protection of animals used for scientific purposes (revising Directive 86/609/EEC). Animal experimentation and reporting were conducted in accordance with the principles recommended by ARRIVE guidelines 2.0 (originally published in PLOS Biology: [doi:10.1371/journal.pbio.3000411](https://doi.org/10.1371/journal.pbio.3000411) and available at <https://arriveguidelines.org/arrive-guidelines>)

Data Availability Statement:

Research data are deposited to InTOR repository. During the revision process research data will be available upon request from Irena Nježić, repository administrator, *via* mail injezic@torlak.rs. After manuscript acceptance, research data will be available online at https://hdl.handle.net/21.15107/rcub_intor_974 under CC BY license

Legends for figures

Fig. 1. The number (A) and the survival (B) of AO and DA rats in days following intrarectal instillation of 300 μ l of saline, ethanol or TNBS (10 and 40 mg/kg b.w.). Results are presented as number of rats on each day relative to day 0 (A), or as a proportion (%) of rats that died over 7 days (B).

Fig. 2. Macroscopic colon structure (A) and the length (cm) of colon segment affected by necrosis (grey) or hyperemia/edema (white) (B) of AO and DA rats 7 days following intrarectal instillation of 300 μ l of saline, ethanol and TNBS (10 and 40 mg/kg b.w.). Results are presented as mean + SEM. Statistically significant differences: * $p < 0.05$ vs. ethanol and a, $p < 0.05$ vs. respective group of AO rats.

Fig. 3. Consumption of food (g) (A) and water (ml) (B) in AO and DA rats in days following intrarectal instillation of 300 μ l of saline, ethanol or TNBS (10 and 40 mg/kg b.w.). Weight loss (%) (C) in AO and DA rats 7 days following intrarectal instillation of 300 μ l of ethanol and TNBS (10 and 40 mg/kg b.w.). Results are presented as mean $\pm$ SEM (A, B) or as individual values (C). Statistically significant differences: * $p < 0.05$ and ** $p < 0.01$ vs. saline.

Fig. 4. Microscopic colon structure of AO and DA rats 7 days following intrarectal instillation of 300 μ l of saline, ethanol and TNBS (10 and 40 mg/kg b.w.). Magnification 400x, bar = 100 μ m. Inserts indicate infiltration of eosinophils (I, arrows) and siderophages (II, asterisks), and vacuolar degeneration (III, arrows). Triangle = large erosive defect among mucosa and submucosa layers. Insert bar = 200 μ m.

Fig. 5. The level of urea (μ M/mg proteins) (A), cytokines (pg/mg proteins) (B) and relative balance of IL-17/IFN- γ production in colon tissue homogenates of AO and DA rats 7 days following intrarectal instillation of 300 μ l of saline, ethanol and TNBS (10 and 40 mg/kg b.w.). was shown in 5C. Results are presented as mean + SEM. Statistically significant differences: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. saline; a, $p < 0.05$, b, $p < 0.01$ and c, $p < 0.001$ vs. respective group of AO rats.

Fig. 6. The proportion (%) of cell populations in peritoneal washing of AO and DA rats 7 days following intrarectal instillation of 300 μ l of saline, ethanol and TNBS (10 and 40 mg/kg b.w.). Cells were gated according to FSC and SSC (A), and then further analyzed for the expression of CD4 and CD11b (B). The proportion of HIS48^{hi}CD43⁺ cells among CD11b^{int}CD4^{low} cells (left) and the proportion of CD163⁺MHCII^{lo} and CD163⁺MHCII^{hi} cells among resident CD11b^{hi}CD4^{hi} cell pool (right) is shown in (C). Results are presented as mean + SEM. Statistically significant differences: * $p < 0.05$ and ** $p < 0.01$ vs. saline; b, $p < 0.01$ vs. respective group of AO rats.

Fig. 7. The level of NO and Urea (μM) in unstimulated (A), and the level of TNF, IL-6 and IL-10 (pg/ml) in LPS-stimulated (B) adherent peritoneal cells of AO and DA rats 7 days following intrarectal instillation of 300 μl of saline, ethanol and TNBS (10 and 40 mg/kg b.w.). Results are presented as mean + SEM. Statistically significant differences: * $p < 0.05$ and ** $p < 0.01$ vs. saline; a, $p < 0.05$, b, $p < 0.01$ and c, $p < 0.001$ vs. respective group of AO rats.

Fig. 8. The proportion (%) of CD4+, CD4+CD25+, CD8+ and CD45RA+ cells in mesenteric lymph nodes (A) and the concentration (pg/ml) of IL-17 and IFN- γ (B) and the relative balance of IL-17/ IFN- γ production (C) in Con A-stimulated mesenteric lymph node cells of AO and DA rats 7 days following intrarectal instillation of 300 μl of saline, ethanol and TNBS (10 and 40 mg/kg b.w.). Results are presented as mean + SEM. Statistically significant differences: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. saline; a, $p < 0.05$, b, $p < 0.01$ and c, $p < 0.001$ vs. respective group of AO rats.

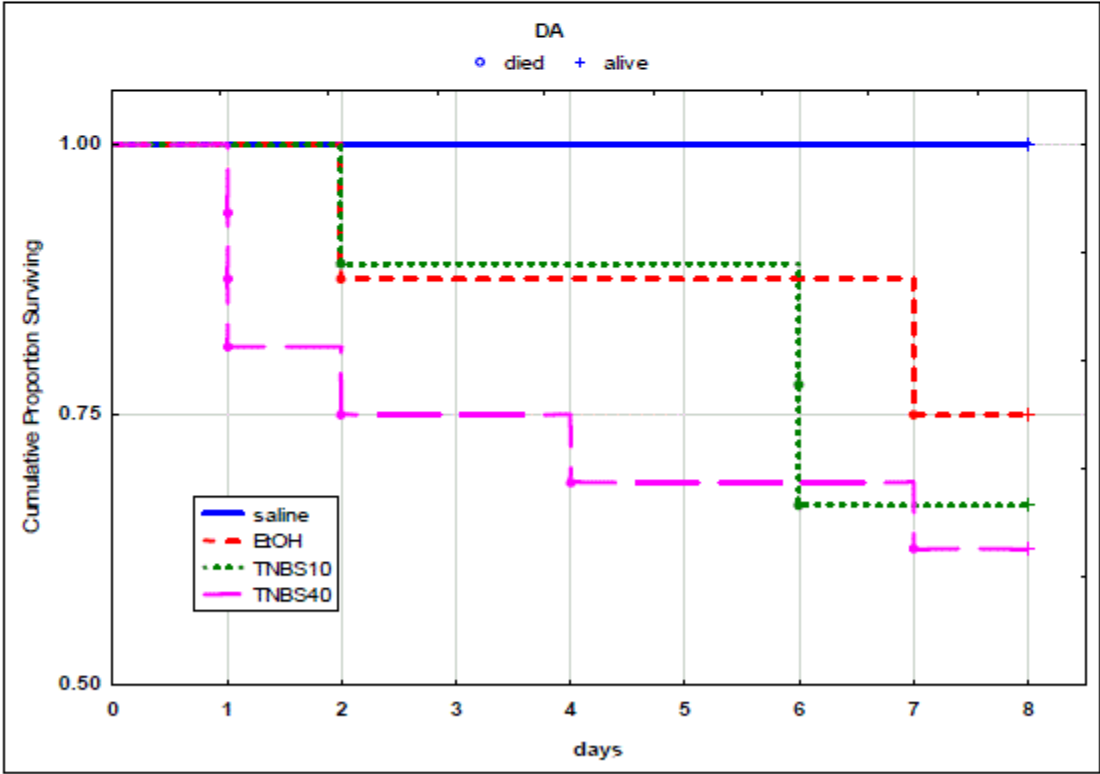
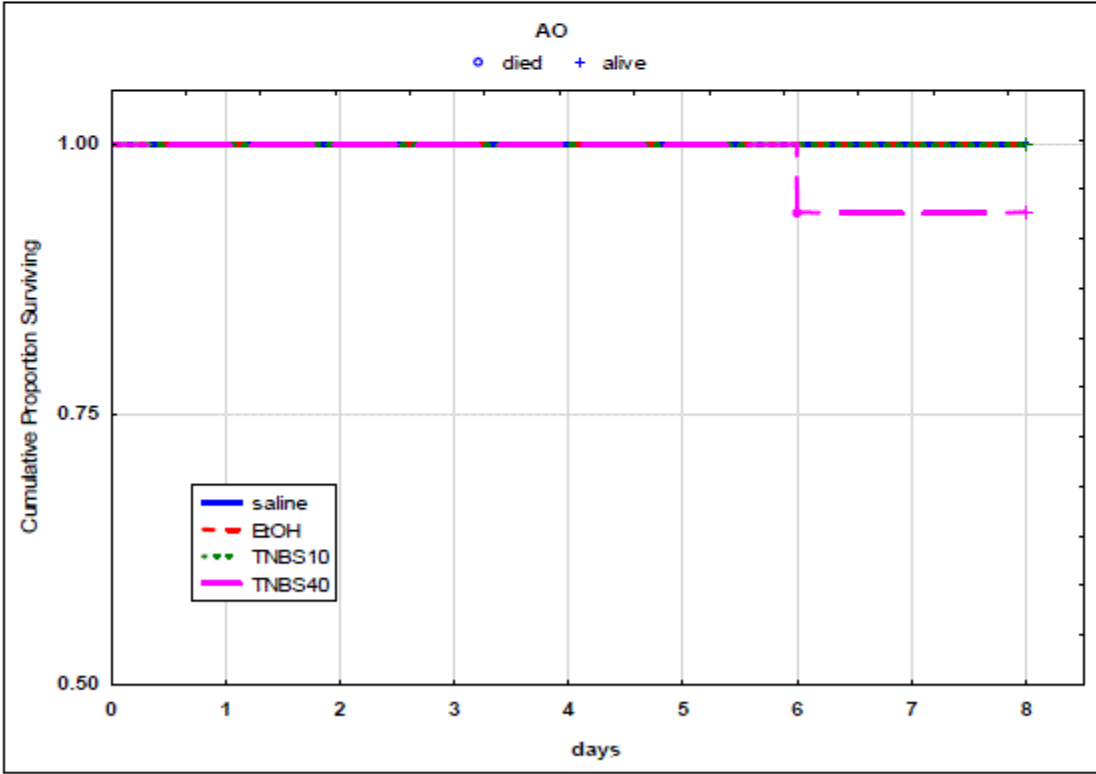
Fig. 9. Concentration of proteins (mg/ml) in sera of untreated AO and DA rats (A) and relative levels (OD) of anti-*E. coli* antibodies of IgG1, IgG2a, IgG2b and IgA classes in sera of saline-treated AO and DA rats (B). Relative levels (OD) of anti-*E. coli* antibodies of IgG1 (C), IgG2a (D), IgG2b (E) and IgA (F) classes in sera of AO and DA rats 7 days following intrarectal instillation of 300 μl of saline, ethanol and TNBS (10 and 40 mg/kg b.w.). Results are presented as mean + SEM. Statistically significant differences:

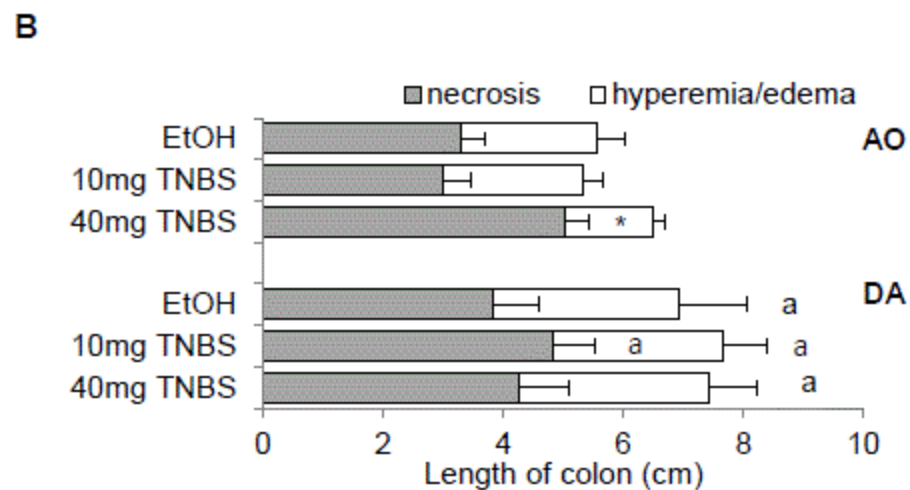
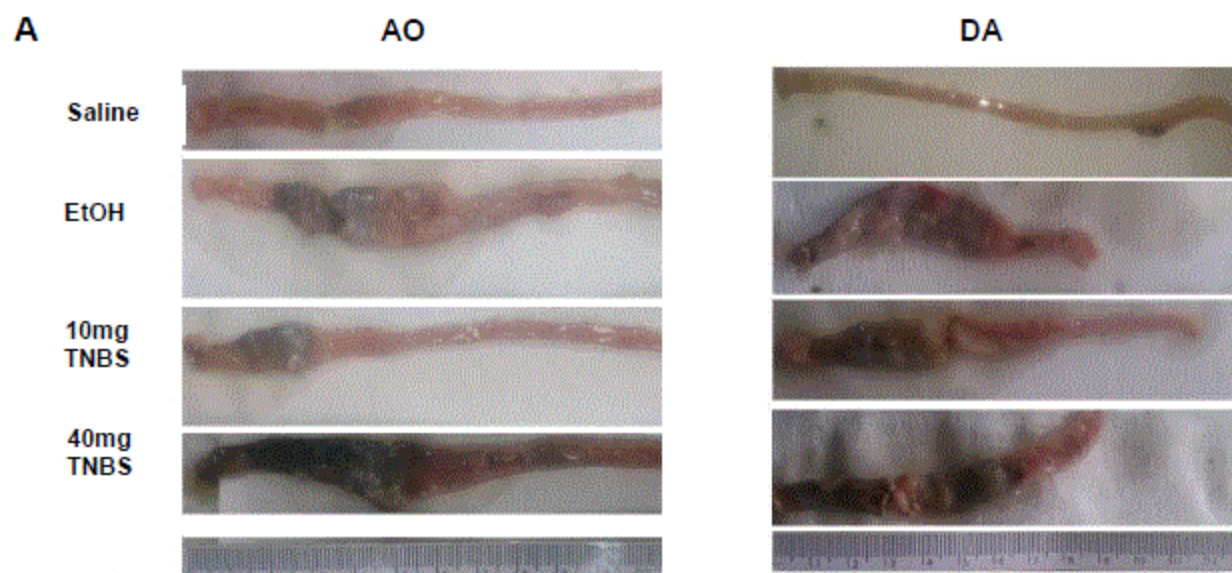
A

days	AO			
	saline	EtOH	10mg TNBS	40mg TNBS
Number of rats in exp. group relative to day 0				
day 0	15	14	12	16
day 1				
day 2				
day 3				
day 4				
day 5				
day 6				-1
day 7				
Mortality (%)	0,00	0,00	0,00	6,25

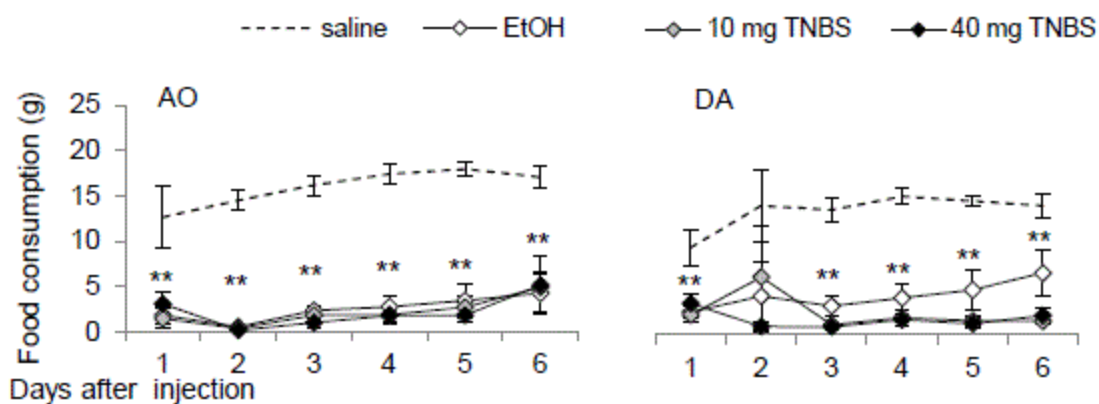
days	DA			
	saline	EtOH	10mg TNBS	40mg TNBS
Number of rats in exp. group relative to day 0				
day 0	10	8	9	15
day 1				-3
day 2		-1	-1	-1
day 3				
day 4				-1
day 5				
day 6			-2	
day 7		-1		-1
Mortality (%)	0,00	25,00	33,33	40,00

B

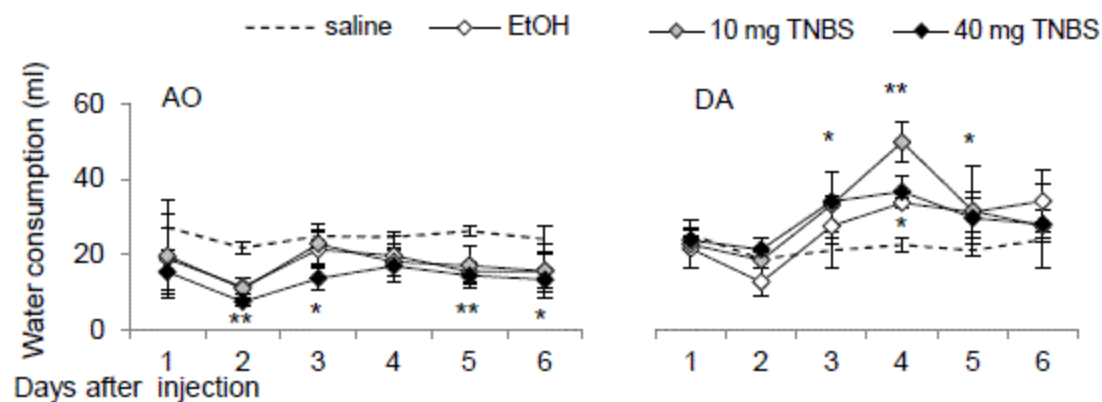




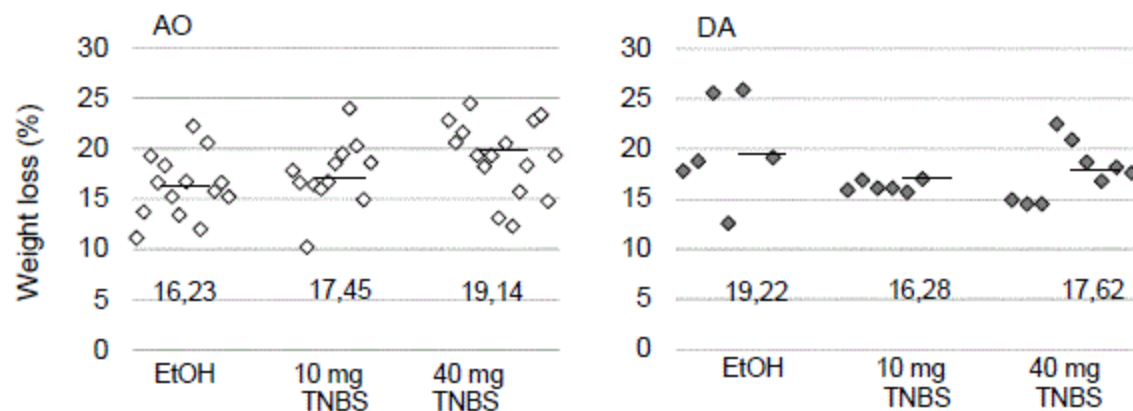
A



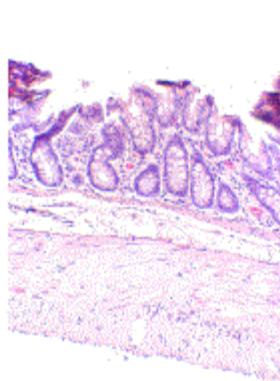
B



C



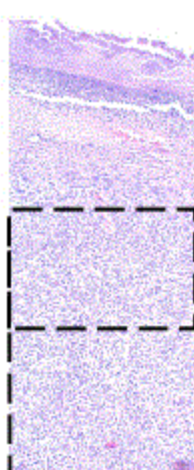
AO_{saline}



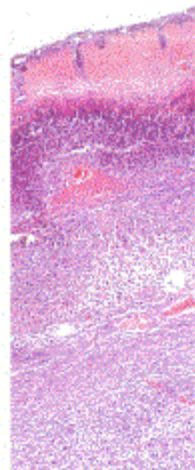
DA_{saline}



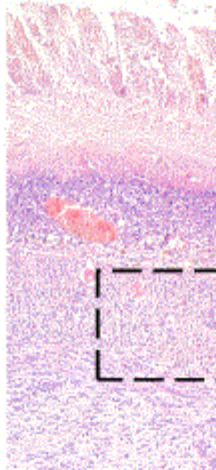
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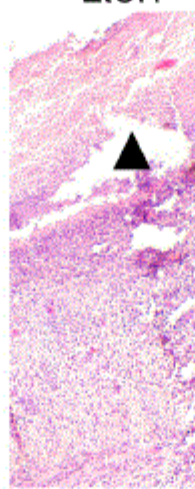
AO_{TBNS10}



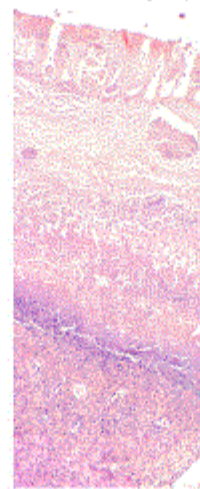
AO_{TBNS40}



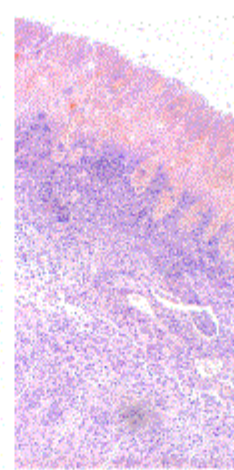
DA_{EtOH}



DA_{TBNS10}



DA_{TBNS40}



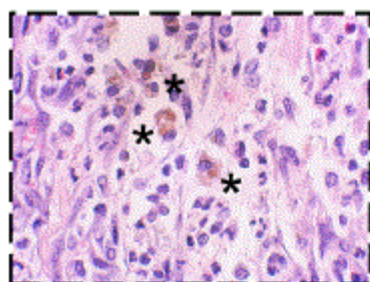
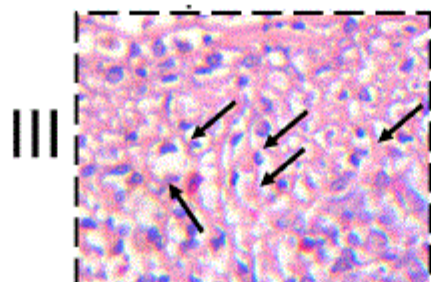
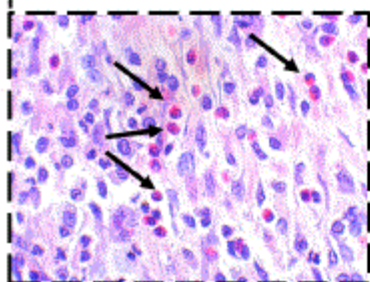
Lamina propria (mucosa)

Submucosa

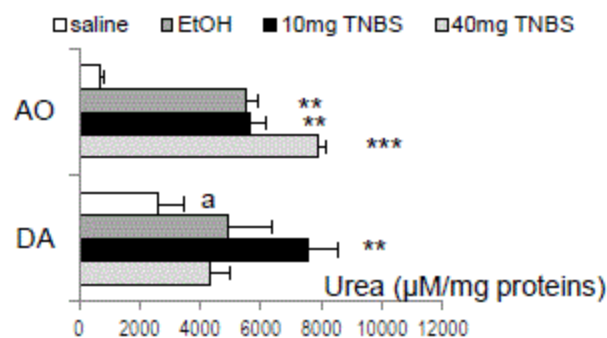
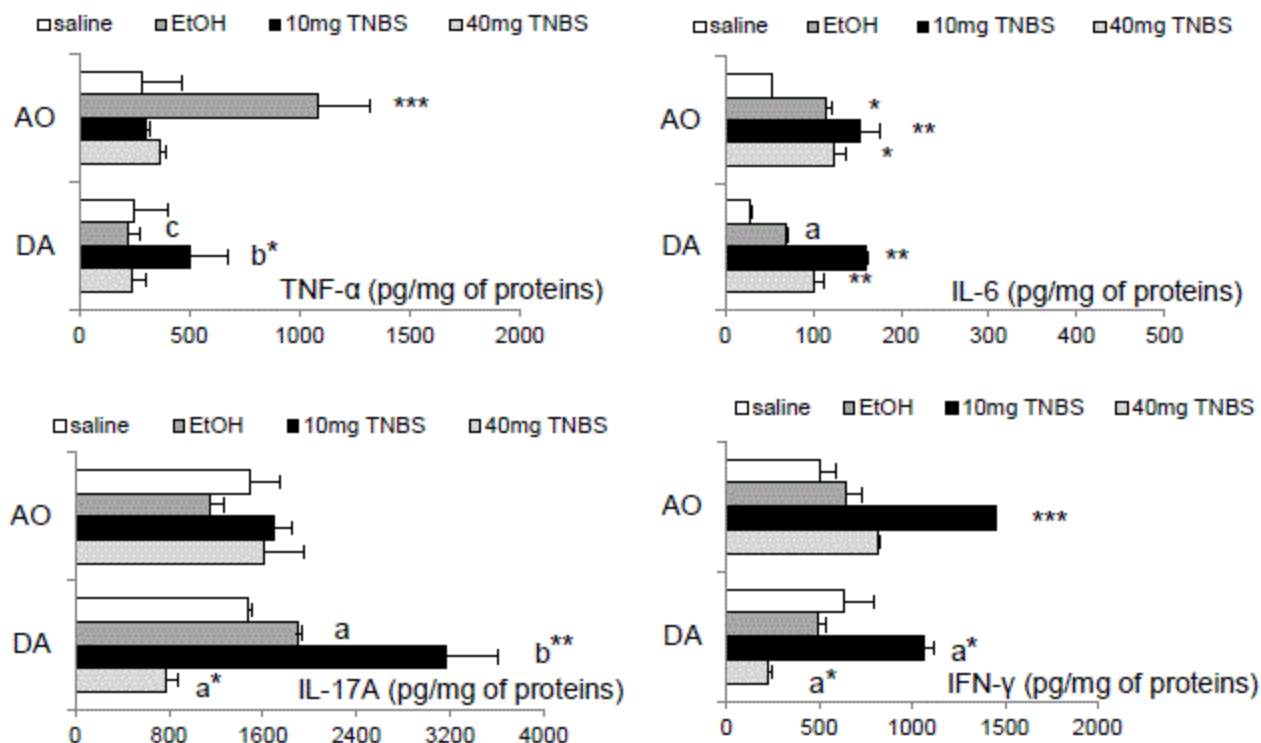
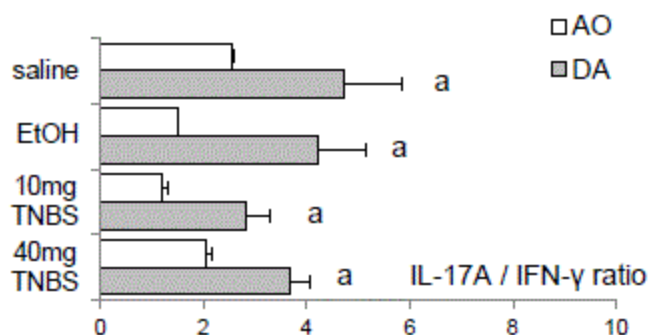
Lamina muscularis propria

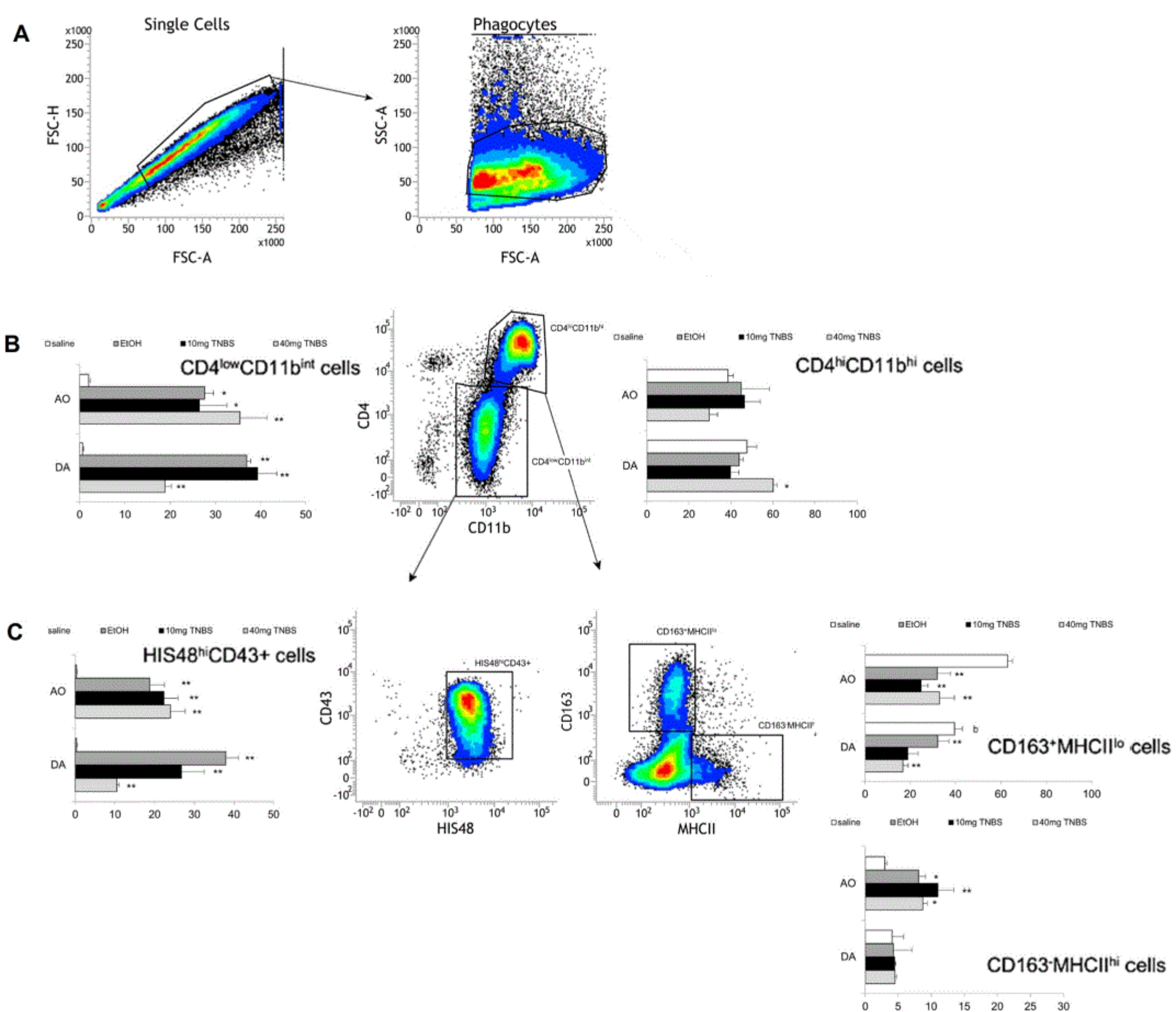
Serosa

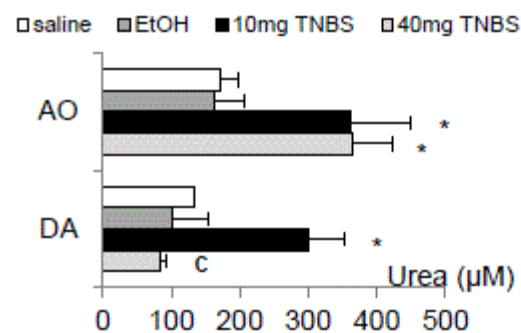
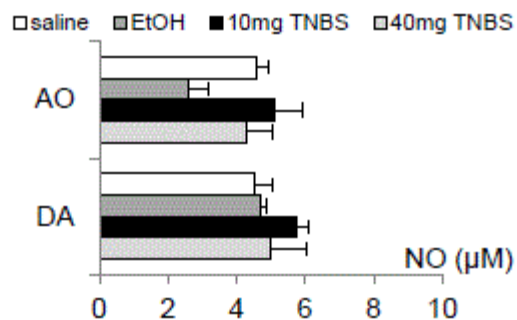
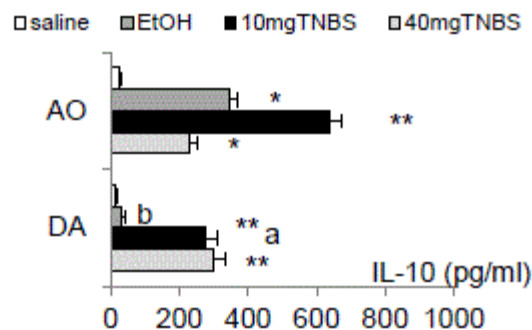
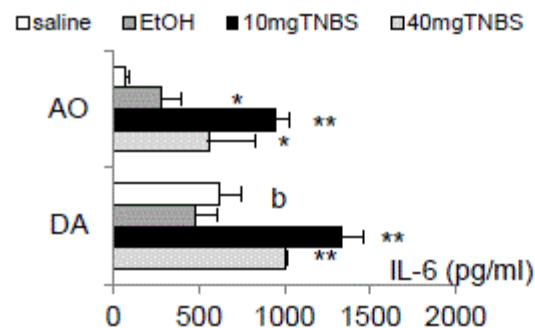
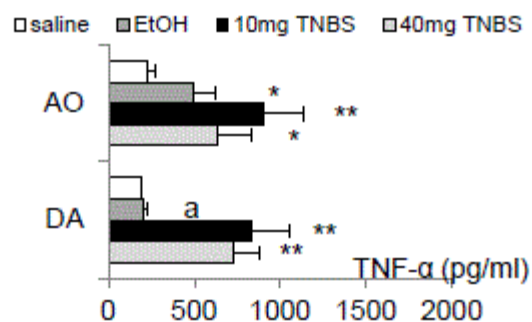
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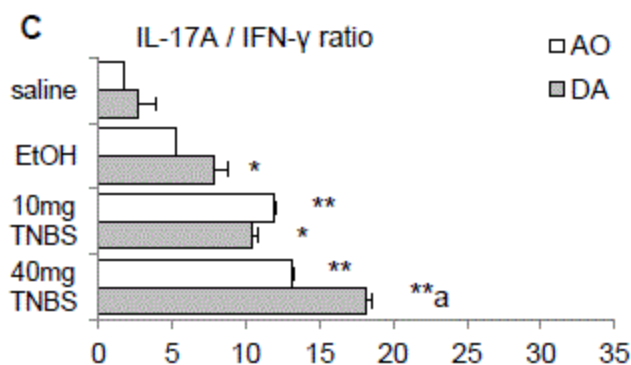
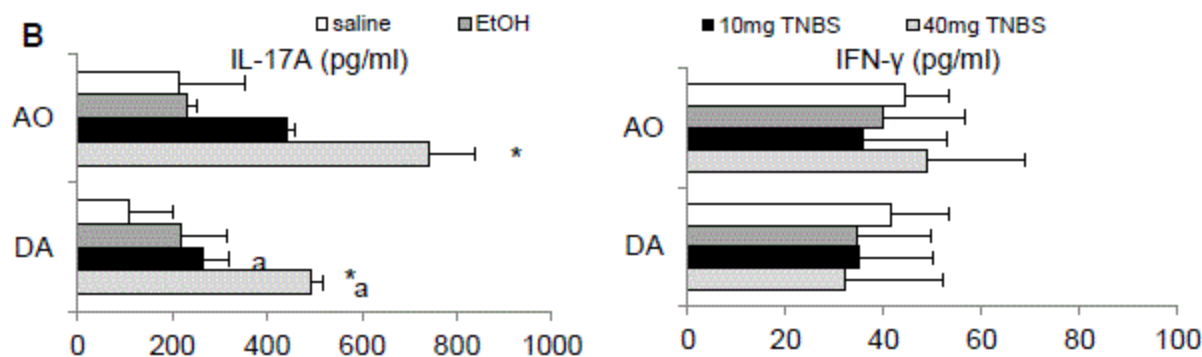
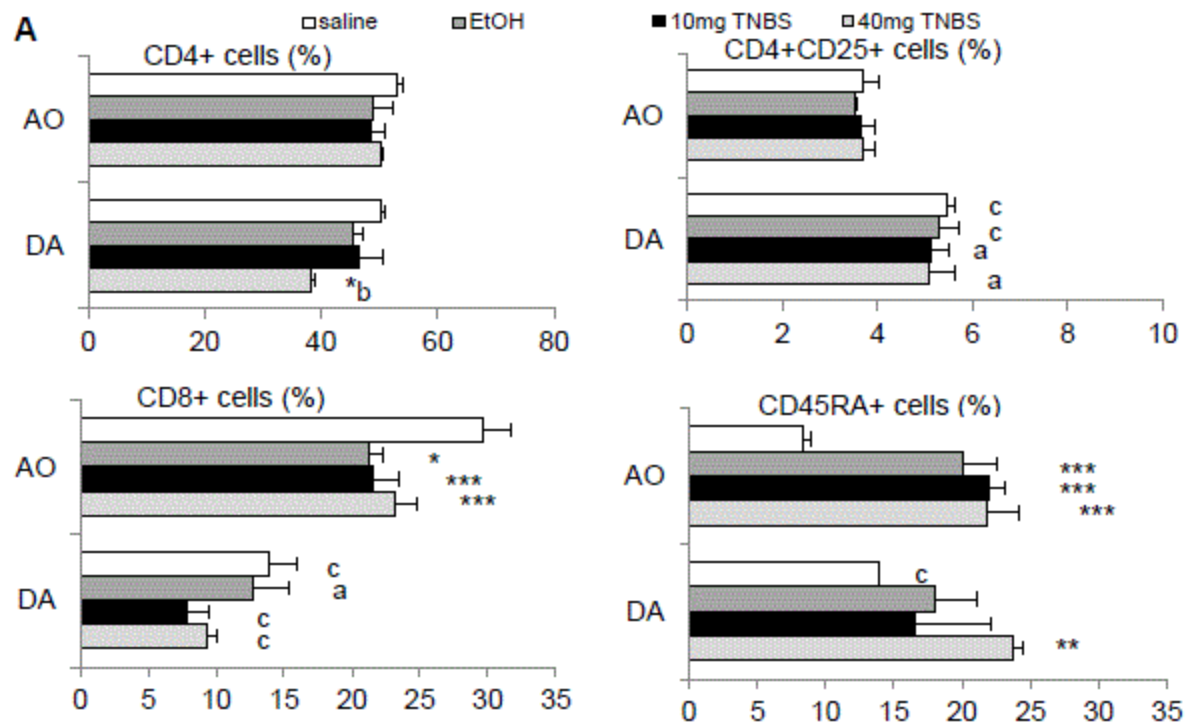


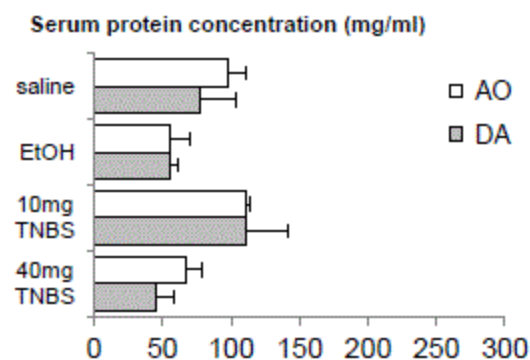
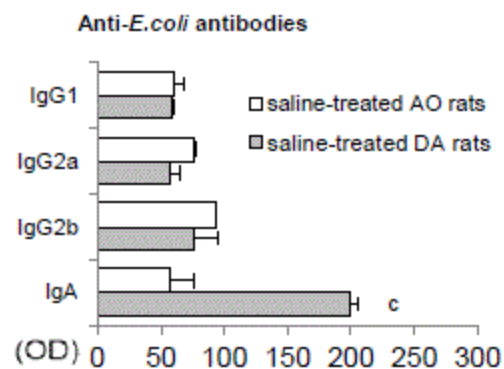
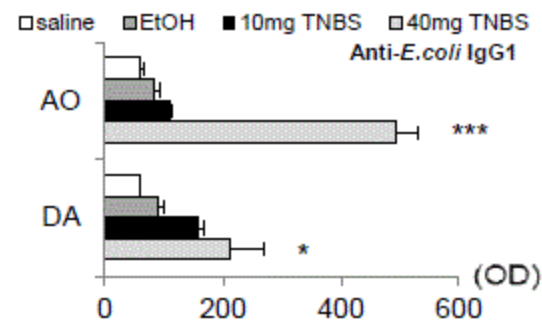
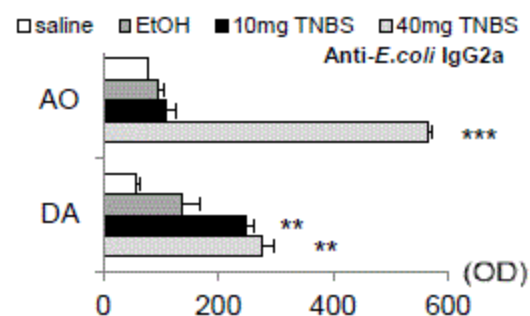
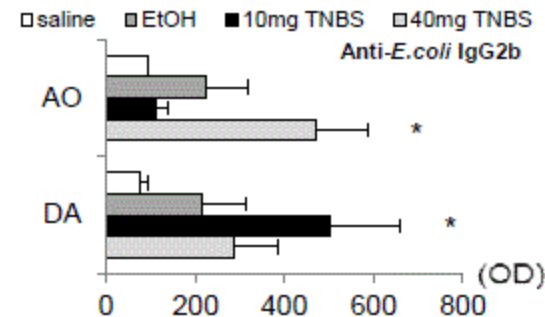
200 μm

A**B****C**



A**B**



A**B****C****D****E****F**