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# **Lack of TNFRI signaling enhances annexin A1 biological activity in intestinal inflammation**

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**Graphical abstract****Abstract**

We evaluated whether the lack of TNF- $\alpha$  signaling increases mucosal levels of Annexin A1 (AnxA1); the hypothesis stems from previous findings showing that TNF- $\alpha$  neutralization in Crohn's disease patients up-regulates systemic AnxA1 expression. Biopsies from healthy volunteers and patients under anti-TNF- $\alpha$  therapy with remittent ulcerative colitis (UC) showed higher AnxA1 expression than those with active disease. We also evaluated dextran sulfate sodium (DSS)-acute colitis in TNF- $\alpha$  receptor 1 KO (TNFR1<sup>-/-</sup>) strain with impaired TNF- $\alpha$  signaling and C57BL/6 (WT) mice. Although both strains developed colitis, TNFR1<sup>-/-</sup> mice showed early clinical recovery, lower myeloperoxidase (MPO) activity and milder histopathological alterations. Colonic epithelium from control and DSS-treated TNFR1<sup>-/-</sup> mice showed intense AnxA1 expression and AnxA1<sup>+</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells were more frequent in TNFR1<sup>-/-</sup> animals, suggesting an extra supply of AnxA1. The pan antagonist of AnxA1 receptors exacerbated the colitis outcome in TNFR1<sup>-/-</sup> mice, supporting the pivotal role of AnxA1 in the early recovery. Our findings demonstrate that the TNF- $\alpha$  signaling reduction favors the expression and biological activity of AnxA1 in inflamed intestinal mucosa.

**Key words:** therapy; DSS-induced colitis; inflammation; gut; epithelium.

## 1. Introduction

Annexin A1 (AnxA1) is a phospholipid binding protein able to inhibit leukocyte transmigration and activation. Originally described as a glucocorticoid (GC)-induced factor with anti-phospholipase activity, later on AnxA1 has been implicated in prevention of inflammation in several disease models, such as peritonitis, air-pouch edema, myocardium infarct, respiratory tract allergy, endotoxemia, kidney ischemia/reperfusion, uveitis, arthritis and others [1-9]. AnxA1 binds to formyl peptide receptors (FPR) [10] and supports the resolution of inflammation and the wound healing suggesting a protective role on the intestinal epithelium damage [11-14].

Inflammatory bowel diseases (IBD) are chronic gut illnesses. Between them, ulcerative colitis (UC) is a prevailing entity with continuous superficial colonic inflammation, abdominal pain and recurrent diarrhea [15]. The proposed mechanism for UC development is the breakdown of the interaction between environmental stimuli and gut microbiota that results in mucosal inflammation [16]. This change may include disruption of the barrier function and alteration in the regulation of acute and adaptive immune responses [17].

The pro-inflammatory cytokine TNF- $\alpha$  plays a pivotal role in the signaling cascade that causes chronic intestinal inflammation in IBD [18]. Biologic agents such as anti-TNF- $\alpha$  antibodies Infliximab (IFX) and Adalimumab (ADA) attenuate the inflammatory process and have been approved for the treatment of moderate-to-severe UC that is refractory to conventional therapy [19]. These antibodies are well tolerated, induce and maintain clinical remission and mucosal healing, and permit the tapering of corticosteroids while allow remission [19, 20]. Interestingly,

the anti-TNF- $\alpha$  therapy produces early changes in the gene expression profiles of intestinal epithelial cells that could be predictive of clinical responses [21, 22]. The availability of predictive correlates of clinical response is highly relevant as could enable to determine the benefits or risks of enduring biologic therapies on individual basis. In agreement, in a longitudinal assessment of peripheral blood samples from IBD patients recently we showed that systemic mononuclear cell transcripts as well as plasma levels of the anti-inflammatory biomarker AnxA1 are affected by initial and continuous IFX therapy [23]. Surprisingly, the response correlated with lower C-reactive protein and better quality of life of these patients.

We hypothesized that the absence or reduction of TNF- $\alpha$  signaling could amplify mucosal levels of the protein AnxA1. Herein, biopsies from patients under anti-TNF- $\alpha$  therapy during UC remission showed an increase in mucosal AnxA1 expression. We also used mice lacking TNFR1 (transmembrane and soluble receptor of TNF- $\alpha$ ) to mimic the activity of anti TNF- $\alpha$  antibody, and we examined the expression and anti-inflammatory activity of AnxA1 in the colon mucosa in the well characterized animal model of dextran sulfate sodium (DSS) induced colitis. We found that the blockade of the TNF- $\alpha$  pathway upregulated the AnxA1 expression in the epithelium and colon mucosa and simultaneously accelerated the resolution process of the colitis.

## **2. Methods**

### **2.1 Ethic statement**

This study was approved by the Ethics Committee of the Hospital Privado-Centro Medico de Córdoba (Approval Number HP 4-215). All patients medically assisted in the 2013 year gave their written informed consent prior to participation in this study. Animal experiments were approved by and conducted in accordance with guidelines of the Committee for Animal Care and Use of the Chemical Science Faculty, National University of Cordoba. (Approval Number HCD 15-09-69596) in strict accordance with the recommendation of the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care (OLAW Assurance number A5802-01). Our animal facility obtained NIH animal welfare assurance (assurance no. A5802-01, Office of Laboratory Animal Welfare, NIH, Bethesda, MD, USA).

### **2.2 Patients**

Colonic mucosal biopsies were obtained from healthy individuals without history of UC (healthy volunteers, n=3) undergoing colonoscopy for colorectal cancer screening and individuals with UC. Patients with UC (n=11) fell between four groups: with active disease without previous therapy (no therapy, n=6); with clinical response on maintenance therapy with either GC (n=2) or anti-TNF- $\alpha$  therapy (anti-TNF- $\alpha$  group, n=3). Patient characteristics such as gender, age and clinical data obtained from medical records are displayed in Table 1. Mucosal biopsies were taken from the left colon in close proximity to biopsies used for histological assessment and fixed in 10% neutral-buffered formalin.

### **2.3 Immunohistochemistry**

For AnxA1 expression analysis, 5  $\mu$ m sections were incubated in 3% H<sub>2</sub>O<sub>2</sub> for 25 min and then incubated for 30 min in 10 mM citrate buffer (pH 6.0), followed by 20 min in distilled water. Sections were blocked in 10% normal serum, and incubated with rabbit anti-human-AnxA1 (clone MRQ3, Cell Marque, California, USA) for 45 min at room temperature. Sections were washed in phosphate buffer saline (PBS), incubated with anti-rabbit biotinylated secondary antibody (Dako North America Inc. California, USA) and visualized by avidin-biotin-peroxidase detection. For the enzymatic reaction diaminobenzidine (DAB) (Vector Laboratories, California, USA) was used as the chromagen for 8 min, and sections counterstained with Mayer's hematoxylin (Sigma Aldrich, San Luis, MO, USA) for 5s. Images were taken with a Nikon optical microscope (Nikon Eclipse TE2000-U, Melville, USA). Densitometric analysis for AnxA1 staining was performed using the ImageJ software and displayed as an arbitrary scale ranging from 0 to 255 units.

### **2.4 Mice**

Male and female 6-8-wk-old mice C57BL/6 (WT, the Jackson Laboratory, California, USA ) and 6-8-wk-old TNF- $\alpha$  receptor 1 KO (TNFR1<sup>-/-</sup>, kindly provided by Dr. Silvia DiGenaro, National University of San Luis, Argentina) were used in these studies. For each group of experiments, mice were matched by age, sex, and body weight. Animals were maintained in specific pathogen-free conditions, housed in collective cages at 22  $\pm$  1°C under a 12-hour light/dark cycle (lights on at 7:00 a.m.), with free access to laboratory chow and drinking water. Experiments were performed during the light phase of the cycle.

## 2.5 Induction and assessment of DSS-induced colitis

Mice received a solution of filtered water containing 3% (w/v) DSS (MW 40 000 kDa, Dextran Prod Ltd, Ontario, Canada) ad libitum over a 5-day period. Every other day, the DSS solution was replenished and at the end of this period, DSS was replaced by normal drinking water for 2 days. Control mice received only normal drinking water. All animals were examined once a day and the disease activity index (DAI) was assessed during 18 days as described previously [24, 25] [25, 26]. Briefly, DAI was the combined score of weight loss, stool consistency, and bleeding. For weight loss, a value of 0 was assigned if body weight increased or remained within 1% of baseline, 1 for a 1% to 5% loss, 2 for a 5% to 10% loss, 3 for a 10% to 15% loss, and 4 for weight loss >15%. For stool consistency a value of 0 was for no diarrhea, 2 for loose stool that did not stick to the anus and 4 for liquid stool that did stick to the anus. For fecal blood, 0 was for none, 2 for moderate, and 4 for gross bleeding. Mice were humanely sacrificed on days 5, 7, 10 and 18 by cervical dislocation. Colons were removed and examined for weight, macroscopic appearance and length (which were measured from 1 cm above the anus to the top of the cecum).

## 2.6 Myeloperoxidase assay

Neutrophil infiltration was assessed indirectly by measuring myeloperoxidase (MPO) activity in colon samples. The MPO assay was performed as described previously (26). On day 7, animals were killed and colon tissue segments were homogenized in 5% EDTA/NaCl buffer (pH 4.7) and centrifuged at  $10\,000 \times g$  for 15 min at 4°C. Pellets were resuspended in 0.5% hexadecyl trimethyl ammonium bromide buffer (pH 5.4), and samples were frozen in liquid nitrogen and thawed three times. On final thawing, the samples were similarly centrifuged, and 25 µl of the



supernatant was used for MPO assay. The MPO enzymatic reaction was assessed by the addition of 1.6 mmol/L tetramethylbenzidine, 80 mmol/L NaPO<sub>4</sub> and 0.3 mmol/L H<sub>2</sub>O<sub>2</sub>. The absorbance was measured spectrophotometrically at 690 nm and MPO was calculated with a standard curve generated for each experiment. Results were expressed in optical density as ng/mg tissue protein.

## **2.7 Histopathological analysis**

Excised portions of the distal colon were fixed immediately in a 4% (w/v) formaldehyde solution and embedded in paraffin. Next, 5 µm sections were mounted on glass slides, and deparaffinized. For histological analysis, slices were stained using standard H&E techniques. For kinetic evaluation, we defined the histological score that represents the scoring sum of histoarchitecture alterations, mononuclear and polymorphonuclear infiltrate in epithelium, lamina propria (LP) and submucosa, crypt alterations, pseudopolyp formation, edema and ulcers, as described [26]. Images were taken with a Nikon optical microscope (Nikon Eclipse TE2000-U, USA).

## **2.8 Immunofluorescence**

Sections of the distal colon were embedded in Optimal Cutting Temperature Compound (Tissue-Teck O.C.T. compound, Sakura Finetek Europe B.V., The Netherlands). Sections (5 µm) were blocked with 3% w/v bovine serum albumin (BSA, Sigma Aldrich, San Luis, MO, USA) for 1 h and incubated with 1:400 rabbit anti-mouse AnxA1 antibody overnight at 4°C, washed, and incubated for 1 h with 1:100 fluorescein isothiocyanate (FITC) labeled goat anti rabbit secondary antibody (Sigma Aldrich, San Luis, MO, USA). All incubations were carried out in a humidity chamber. Nuclei were counterstained with DAPI and slides were mounted in Aqueous

Mounting Medium with fluorescence tracers (FluorSave, Calbiochem, USA). Images were taken with a Confocal Olympus Fluoview 1000 microscope (Olympus, USA).

## **2.9 Explant cultures**

Colons were flushed with RPMI and opened along the longitudinal axis. Then, 10 mm<sup>2</sup> punch biopsies were obtained from the medial colon and incubated for 24 h in RPMI supplemented with 10 % fetal bovine serum (FBS) and antibiotics (one punch biopsy per 1 ml medium). Supernatants were collected and kept in -20 °C to assess IL-6 production by ELISA (BD Biosciences, California, USA), as specified by the manufacturers or AnxA1 by Western blot with the rabbit anti-mouse AnxA1 antibody, as described [27]. The amount of 10 µl of total protein in each colon explant supernatant sample was employed for analysis [28, 29].

## **2.10 Isolation of lamina propria cells and flow cytometry analysis**

Isolation of colonic LP cells was performed as previously with slight modifications [30]. Briefly, extraintestinal fat tissue and blood vessels were carefully removed and colons were flushed with cold PBS, opened longitudinally and cut into small pieces. Epithelial cells and mucus were removed by 45 min incubation in RPMI containing 5% FCS and 2 mM EDTA and 1 mM dithiothreitol (DTT) (Sigma Aldrich, San Luis, MO, USA) at 37°C with 250 rpm shaking. Colon pieces were then digested in PBS containing 5% FBS, 1 mg/ml Collagenase IV (Sigma Aldrich, San Luis, MO, USA) for 90 min and 37°C shaking at 250 rpm. The digested cell suspension was washed with PBS and passed through 40 µm cell strainers. Isolated 1×10<sup>6</sup> LP cell aliquots were resuspended in FACS buffer (PBS, 5% FBS, 0.02% NaN<sub>2</sub>) containing FBS 10% on ice for 15 min to prevent non-specific antibody binding. Thereafter samples were resuspended in FACS buffer containing CD3 (PECy7), CD4 (PE), CD8 (PERCP) and CD11b (APC) antibodies at the

concentration suggested by the manufacturers. For intracellular staining, samples were resuspended in FACS buffer containing 1% paraformaldehyde for 15 min and thereafter in CytoFix/CytoPerm buffer (BD Biosciences, California, USA) for 1 h and washed in the PermWash buffer (BD Biosciences, California, USA). The anti-AnxA1 antibody was added at 1:100 dilution from stock solution of 1.0 mg/ml. The FITC-conjugated anti-mouse IgG (Sigma Aldrich, San Luis, MO, USA) was used at 1:400 dilution and incubated on ice for 30 min. On the basis of forward and side light scatter, debris and dead cells were gated out; 100 000 events were analyzed using a FACSAria flow cytometer (BD Bioscience, California, USA). Determination of positive and negative populations was performed based on the control staining with an irrelevant IgG isotype labeled with FITC, PE, PerCP-Cy5.5 or APC. Flow cytometry analysis was done with the FlowJo software.

### **2.11 In vivo BOC2 and anti-AnxA1 treatments**

WT and TNFR1<sup>-/-</sup> mice received four i.p. injections of BOC2 (N-t-butyloxycarbonyl-Phe-Leu-Phe-Leu-Phe) (Bachem Americas, California, USA), a pan-antagonist of FPRs [31], once a day starting on day 7 after DSS treatment (10 µg/Kg, 0.1 ml). BOC2 was dissolved in DMSO, and further diluted in PBS on the day of administration. Equivalent concentrations of DMSO were used as vehicle controls. In other experiments mice received i.p. 1µg of rabbit anti-murine AnxA1 antibody (Invitrogen, Grand Island, USA) in 100 µl PBS during 3 days starting on day 1 of DSS treatment.

## 2.12 Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 software (GraphPad Software, San Diego, CA). Differences between groups were determined by two-way analysis of variance followed by a Student-Newman-Keuls test or Mann-Whitney U-test. Data were expressed as mean  $\pm$  SEM;  $p < 0.05$  was considered statistically significant.

## 3. Results

### 3.1 AnxA1 expression in colon from UC patients in remission under anti TNF- $\alpha$ therapy

Previously, IFX treated Crohn's patients presented increase of AnxA1 plasma levels and up-regulation of AnxA1 mRNA transcription in peripheral mononuclear cells [23]. We wondered if biological agents such as IFX could also modify levels of AnxA1 in colonic mucosa in IBD patients. To address this question we explored the expression and localization of AnxA1 in biopsy sections of UC patients either untreated or in remission upon anti-TNF- $\alpha$  therapy. For comparative purposes we also included UC patients receiving GC as well as healthy volunteers (Table 1). The representative section in Fig. 1A shows lower expression of AnxA1 in epithelial cells from an untreated active UC patient (asterisks); in contrast, higher AnxA1 immunostaining was observed in crypts and surface epithelium in biopsies from patients with GC- or IFX-induced remission (arrows) (Fig. 1B, C), although without reaching the AnxA1 immunostaining observed in biopsies from healthy volunteers (Fig. 1D). The intensity of epithelial AnxA1 expression is displayed in the Figure 1E in densitometric units. Our previous findings [24] together with the higher levels of AnxA1 found in patients under anti-TNF- $\alpha$  therapy prompted us to evaluate the interplay between TNF- $\alpha$  and AnxA1 levels.

### 3.2 TNFR1<sup>-/-</sup> mice exhibit early resolution of acute colitis

To evaluate the putative TNF- $\alpha$ /AnxA1 axis in colitis we used the DSS acute model in C57BL/6 and TNFR1<sup>-/-</sup> mice. Signs were evaluated until day 18, as we previously found that the acute colitis reaches a complete clinical resolution in the knockout group (data not shown). Oral administration of DSS for 5 days provoked colitis in both strains; in the acute period, around days 5-7, WT and TNFR1<sup>-/-</sup> animals showed similar clinical signs with increased DAI and weight loss compared with control groups (Fig. 2A, B). By day 8, TNFR1<sup>-/-</sup> mice exhibited a significant reduction of DAI as well as weight recovery compared with WT animals (Fig. 2A, B). Macroscopically, the colon length shortening found in WT animals on days 10 and 18 ( $p < 0.05$ ) was not present in DSS-treated TNFR1<sup>-/-</sup> mice (Fig. 2C).

We wondered whether the resolving profile of mice with attenuated TNF- $\alpha$  signaling could be associated with differences in the inflammatory response in the intestinal mucosa. Microscopic observation of colon sections showed no histopathological changes in control groups of both strains (Fig. 3A, B). In contrast, DSS-treated WT and TNFR1<sup>-/-</sup> mice exhibited marked cellular infiltration into colonic mucosa by day 5 (Fig. 3C, D). The localization of polymorphonuclear cells (PMN) in TNFR1<sup>-/-</sup> mice was mainly intravascular while in WT animals, cells infiltrated massively the LP (Fig. 3C and D, insets). Yet, the histopathological score in knockout group was significantly lower ( $p < 0.05$ ) as other tissue alterations taken into account in this parameter such as ulcers, crypt alterations and edema were smaller (Fig. 3I). On days 10 and 18, the histopathological analysis revealed transmural inflammation with lymphoid follicles, polypoid processes and atypical dysplastic crypts with abscesses, particularly in WT mice (Fig. 3E, G). TNFR1<sup>-/-</sup> mice instead showed more preserved histoarchitecture with signs of regeneration (Fig.

3F, H) and re-epithelialization by squamous epithelium (Fig. 3H, inset). The scoring sum of histopathological alterations displayed significant differences between both mouse strains (Fig. 3I).

Additionally, PMN infiltration into the colon mucosa was measured indirectly by MPO activity. As shown in Fig. 3J, DSS-treated WT and TNFR1<sup>-/-</sup> mice presented mild increase in colonic levels of MPO on day 5, compared with control animals. Subsequently, on days 10 and 18, MPO was barely detectable in knockout mice whereas WT group showed significantly higher levels of the enzyme. Moreover, in colon explant culture supernatants, TNFR1<sup>-/-</sup> mice exhibited lower levels of the pro-inflammatory cytokine IL-6 both in acute (day 5) and in resolution (days 10-18) steps of DSS-induced colitis compared with WT animals (data not shown).

### **3.3 AnxA1 expression increases in the intestinal mucosa of TNFR1<sup>-/-</sup> during colitis resolution**

To address if early recovery of colitis in TNFR1<sup>-/-</sup> mice could be related to different amount of AnxA1 in intestinal mucosa we evaluated AnxA1 immunoreactivity in colon sections of TNFR1<sup>-/-</sup> and WT mice. Even before the inflammatory challenge, control TNFR1<sup>-/-</sup> mice exhibited more intense expression of AnxA1 than control WT animals (Fig. 4A and D). Furthermore, during the recovery period (days 10-18), mice with reduced TNF- $\alpha$  signaling showed higher expression of AnxA1 (Fig. 4E and F), with specific localization in the crypt epithelium (arrows) (Fig. 4F). None reactivity was detected in immunohistochemistry controls lacking the anti-AnxA1 antibody (Fig. 4G).

Also, AnxA1 was detected in supernatants of colon explant cultures before and at the end of the recovery period (Fig. 4H), as previously found by others in bronchoalveolar lavage fluid [28]. Consistent with the immunofluorescence results, control and DSS-treated TNFR1<sup>-/-</sup> mice exhibited significantly higher AnxA1 levels released by colonic mucosa (Fig. 4I).

We also assessed if immune cells could be contributing to the AnxA1 supply in the tissue. For this purpose, we isolated LP cells from WT and TNFR1<sup>-/-</sup> colons and we evaluated surface/intracellular AnxA1 expression by flow cytometry. Again, before the inflammatory challenge, the frequency of AnxA1<sup>+</sup> CD11b<sup>+</sup> cells was higher in control knockout animals (Fig. 4I), although on day 18, the contribution of this subset was similar in both strains. Interestingly, the frequency AnxA1<sup>+</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells augmented significantly by day 18 in TNFR1<sup>-/-</sup> mice ( $p < 0.05$ ), which is consistent with a recent study demonstrating the ability of AnxA1<sup>+</sup> T cells to attenuate T cell-driven inflammatory responses [32].

### **3.4 Attenuation of the early recovery of colitis in TNFR1<sup>-/-</sup> mice upon blockade of AnxA1 receptors**

To confirm that the milder colitis in absence of TNF- $\alpha$  signaling was due to AnxA1 expression/activity we treated the TNFR1<sup>-/-</sup> C57BL/6 strains with BOC2, the pan-antagonist of FPR [32]. This receptor mediates the biological effects of AnxA1 and AnxA1 derived peptide Ac2-26 [5]. On day 7 after DSS treatment we injected i.p. BOC2 during 4 days and we evaluated colitis outcome and resolution until day 11, both in WT and TNFR<sup>-/-</sup> C57BL/6 strains. BOC2 had no effect when given alone to control WT and TNFR<sup>-/-</sup> mice (Fig. 5). The colitis outcome in

WT mice remained unmodified upon BOC2 treatment, in agreement with a less prevailing activity of AnxA1 when the TNF- $\alpha$  signaling was intact (Fig. 5A and B). Remarkably in the DSS+BOC2 TNFR1<sup>-/-</sup> group the antagonist was able to prevent the early DAI attenuation and the observed weight recovery (Fig. 5C and D). Moreover, colon sections of DSS+BOC2 TNFR1<sup>-/-</sup> group showed strong tissue damage and profuse inflammation, as can be seen in representative microphotographs (Fig. 6A) associated to higher MPO levels (Fig. 6B), demonstrating that FPR blockade overrides the anti-inflammatory/pro-resolving function of increased AnxA1. In additional experiments, TNFR1<sup>-/-</sup> mice were injected i.p with anti-AnxA1 antibody during 3 days after starting DSS dispense. Although the neutralizing anti-AnxA1 dose administered was not enough to change the clinical signs in the evaluated mice, the treatment provoked the increase of IL-6 production by colon explants (data not shown).

#### 4. Discussion

Many reports support the intimate relationship of TNFR1 expression and signaling with the pathogenesis of inflammatory diseases. Some studies have explored the presence of protein biomarkers in serum/plasma of patients with rheumatoid arthritis treated with IFX, one of the major anti-TNF- $\alpha$  drugs [33, 34]. Mainly, up- or down- differentially regulated proteins were apolipoproteins, components of the complement system and acute phase reactants [33, 34].

Herein we found that patients who were experiencing an UC flare, with diarrhea, bloody feces, abdominal pain and others clinical and biochemical signs did not express AnxA1 in epithelium. In contrast, UC patients in remission treated with anti-TNF (low number of clinical signs,



colonoscopy and histopathology with moderate grade) presented increased AnxA1 protein in intestinal mucosa, particularly in the crypts and surface epithelium. This result was similar to those two patients receiving GC, which presented more clinical signs, probably because the AnxA1 is a GC-inducible protein. Even though the number of patients was limited, the data were in agreement with our recent work which revealed the increased expression of systemic AnxA1 in mononuclear cells from patients with Crohn's Disease after IFX treatment [23] and prompted us to evaluate the interplay between TNF- $\alpha$  and AnxA1 levels in colitis.

To validate clinical observations, we used the well known model of DSS-induced colitis in TNFR1<sup>-/-</sup> and WT C57BL/6 mice. In our hands, both strains developed a similar colitis until days 7-8. However, clinical signs, histopathology and inflammatory biomarkers, such as MPO, returned to baseline levels in knockout earlier than in WT mice, suggesting a detrimental role for TNFR1 in the resolution of acute inflammation. In agreement, other studies demonstrated that TNFR1 ablation attenuated tissue damage after trinitrobenzene sulphonic acid (TNBS) induced colitis [35], which was related to control of NF- $\kappa$ B activity [36]. Yet, previous reports demonstrated that TNFR1 or TNFR2 deficiency led to exacerbation of colitis signs compared with WT counterparts [36, 37] and that TNFR1<sup>-/-</sup> strain had more weight loss and increased mortality after TNBS instillation or DSS intake, respectively [37-39]. Mice lacking NEMO in intestinal epithelial cells (NEMO<sup>IEC-KO</sup> mice) at young age develop severe pancolitis affecting all parts of the colon distal from the caecum; double NEMO<sup>IEC-KO</sup>/TNFR1-deficient mice did not show macroscopic or histological signs of colitis demonstrating that TNFR1 signalling is crucial for disease pathogenesis in this model [40]. Overall, these studies highlight the complexity of the TNF signaling via TNFR1 or TNFR2 in colitis during the onset and perpetuation of intestinal

inflammation, which may be affected by different TNFR expression patterns and distinct colitis models used. In fact, the background of the strains, the mechanism of inflammation induction, the acute or chronic colitis model, the innate vs. Th1 immune response shape different experimental conditions that are useful to study the TNF-TNFR axis but do not necessarily reproduce the disease mechanism.

The blockade of TNF-TNFR signaling seems to induce the amelioration of inflammatory diseases through apoptosis induction, cytokine suppression and activation of regulatory cells [35-37]. Consistently, we found higher AnxA1 expression in the colonic epithelium of TNFR1<sup>-/-</sup> mice before the inflammatory challenge and along the resolution of DSS-colitis, in agreement with previous reports [41]. On the other hand, epithelial AnxA1 expression was abundant in crypts and surface epithelium only in TNFR1<sup>-/-</sup> at later stages, which was concomitant with the weight gain, lower DAI and histological recovery in these animals. Together the abrogation of TNF- $\alpha$  signaling facilitated AnxA1-mediated activity in gut function in agreement with the recent study from Leoni [14] that demonstrates the influence of AnxA1 in the “final act” during resolution of inflammation, which requires efficient tissue repair and restoration of mucosal homeostasis. As suggested previously, the constant release of AnxA1 by innate immune cells, would have a pro-resolving effect on the inflammatory response [42]. Still, as many other endogenous anti-inflammatory and pro-resolving mediators, we believe that AnxA1 may be functioning to counteract the properties of proinflammatory factors and to ensure a prompt resolution of inflammation in the TNFR1<sup>-/-</sup> colitis model.

Furthermore, LP CD11b<sup>+</sup> and T cells also seemed to be an important source of AnxA1 in the later stage of the study that ended with fully clinical resolution of DSS-colitis in TNFR1<sup>-/-</sup> mice. Interestingly, the effects of AnxA1 evaluated in three distinct models of T cell-mediated inflammation indicate that T cell-expressed AnxA1 functions to attenuate T cell-driven inflammatory responses via T cell-intrinsic effects on intracellular signaling, proliferation, and Th1/Th17 cytokine release [32]. Whether AnxA1 has effects on the regulatory T cells that may contribute to the anti-inflammatory actions of this protein on colitis resolution is a possibility that remains to be addressed.

In terms of AnxA1 availability, the two mouse strains secreted this protein in colon explant supernatants. Yet, at the resolution step, TNFR1<sup>-/-</sup> mice presented more AnxA1 than WT animals. Possibly, the bioactive N-terminal peptide could have been playing the protective role in the mucosa in the late stage of colitis. This result suggests that cleaved N-terminal peptide could be binding to FPR1 in the TNFR1<sup>-/-</sup> mouse mucosa promoting the pro-resolving profile.

The in vivo blockade of the AnxA1 signaling by BOC2, a pan-antagonist of the FPR, reverted partially the early recovery of colitis in TNFR1<sup>-/-</sup> mice. The finding suggests that the biological actions of AnxA1, enhanced by the lack of TNF- $\alpha$  signaling, involve FPR pathways. A study using a chronic DSS-colitis model revealed that in FPR1<sup>-/-</sup> mice, there is no re-epithelialization of the ulcers, suggesting that this AnxA1 receptor has a protective role in the gut inflammation [43]. Also, FPR2 seems to play a more important role in maintaining colonic homeostasis and inflammatory responses than FPR1, as shown by decreased epithelial cell proliferation and shortened crypts in acute DSS-colitis animals [44]. Furthermore, BOC2 and

others FPR antagonists abrogated the effect of AnxA1 and its Ac2-26 peptide in several models, such as uveitis [9], mesenteric ischemia/reperfusion [45], sepsis [46], peritonitis [47, 48], pleurisy [48] and recently in colitis [14].

Despite its wide tissue distribution and the role of AnxA1 in crucial cellular events, very little is known about the regulation of its expression. The novelty of our work is to identify the lack of TNF- $\alpha$  signaling as a condition that modulates the expression, secretion and function of AnxA1 on the pro-resolving process of colitis. Altogether, the human and animal studies suggest a role of AnxA1 in mediating the anti-inflammatory and protective effects of anti-TNF- $\alpha$  therapy. Besides, this molecule could represent a helpful biomarker for monitoring therapeutic efficiency in IBD patients.

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## **Conflict of Interest Disclosure**

The authors declare no conflict of interest.

## 6. References

- [1] Perretti M, Ahluwalia A, Harris JG, Harris HJ, Wheller SK, Flower RJ. Acute inflammatory response in the mouse: exacerbation by immunoneutralization of lipocortin 1. *Br J Pharmacol* 1996;117: 1145-54.
- [2] D'Amico M, Di Filippo C, La M, Solito E, McLean PG, Flower RJ, et al. Lipocortin 1 reduces myocardial ischemia-reperfusion injury by affecting local leukocyte recruitment. *FASEB J* 2000; 14: 1867-69.
- [3] de Coupade C, Ajuebor MN, Russo-Marie F, Perretti M, Solito E. Cytokine modulation of liver annexin 1 expression during experimental endotoxemia. *Am J Pathol* 2001; 159: 1435-43.
- [4] Oliani SM, Paul-Clark MJ, Christian HC, Flower RJ, Perretti M. Neutrophil interaction with inflamed postcapillary venule endothelium alters annexin 1 expression. *Am J Pathol* 2001; 158: 603-15.
- [5] Bandeira-Melo C, Bonavita AG, Diaz BL, Silva PM, Carvalho VF, Jose PJ, et al. A novel effect for annexin 1-derived peptide ac2-26: reduction of allergic inflammation in the rat. *J Pharmacol Exp Ther* 2005; 313: 1416-22.
- [6] Damazo AS, Sampaio AL, Nakata CM, Flower RJ, Perretti M, Oliani SM. Endogenous annexin A1 counter-regulates bleomycin-induced lung fibrosis. *BMC Immunol* 2011;12: 59.
- [7] Facio FN Jr, Sena AA, Araújo LP, Mendes GE, Castro I, Luz MA, et al. Annexin 1 mimetic peptide protects against renal ischemia/reperfusion injury in rats. *J Mol Med (Berl)* 2011; 89: 51-63.

- [8] Patel HB, Kornerup KN, Sampaio AL, D'Acquisto F, Seed MP, Girol AP, et al. The impact of endogenous annexin A1 on glucocorticoid control of inflammatory arthritis. *Ann Rheum Dis* 2012; 71: 1872-80.
- [9] Girol AP, Mimura KK, Drewes CC, Bolonheis SM, Solito E, Farsky SH, et al. Anti-inflammatory mechanisms of the annexin A1 protein and its mimetic peptide Ac2-26 in models of ocular inflammation in vivo and in vitro. *J Immunol* 2013;190: 5689-701.
- [10] Ernst S, Lange C, Wilbers A, Goebeler V, Gerke V, Rescher U. An annexin 1 N-terminal peptide activates leukocytes by triggering different members of the formyl peptide receptor family. *J Immunol* 2004; 172: 7669-76.
- [11] Babbin BA, Laukoetter MG, Nava P, Koch S, Lee WY, Capaldo CT, et al. Annexin A1 regulates intestinal mucosal injury, inflammation, and repair. *J Immunol* 2008;181: 5035-44.
- [12] Ouyang N, Zhu C, Zhou D, Nie T, Go MF, Richards RJ, et al. MC-12, an annexin A1-based peptide, is effective in the treatment of experimental colitis. *PLoS One* 2012; 7: e41585.
- [13] Vong L, Ferraz JG, Dufton N, Panaccione R, Beck PL, Sherman PM, et al. Up-regulation of Annexin-A1 and lipoxin A(4) in individuals with ulcerative colitis may promote mucosal homeostasis. *PLoS One* 2012; 7: e39244.
- [14] Leoni G, Alam A, Neumann PA, Lambeth JD, Cheng G, McCoy J, et al. Annexin A1, formyl peptide receptor, and NOX1 orchestrate epithelial repair. *J Clin Invest* 2013;123: 443-54.
- [15] Cosnes J, Gower-Rousseau C, Seksik P, Cortot A. Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology* 2011;140: 1785-94.
- [16] Maloy KJ, Powrie F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature* 2011; 474: 298-306.

- [17] Khor B, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. *Nature* 2011; 474: 307-17.
- [18] Ordas I, Mould DR, Feagan BG, Sandborn WJ. Anti-TNF monoclonal antibodies in inflammatory bowel disease: pharmacokinetics-based dosing paradigms. *Clin Pharmacol Ther* 2012; 91: 635-46.
- [19] Bosani M, Ardizzone S, Porro GB. Biologic targeting in the treatment of inflammatory bowel diseases. *Biologics* 2009; 3: 77-97.
- [20] Rutgeerts P, Sandborn WJ, Feagan BG, Reinisch W, Olson A, Johanns J, et al. Infliximab for induction and maintenance therapy for ulcerative colitis. *N Engl J Med* 2005; 353: 2462-76.
- [21] Roda G, Caponi A, Benevento M, Nanni P, Mezzanotte L, Belluzzi A, et al. New proteomic approaches for biomarker discovery in inflammatory bowel disease. *Inflamm Bowel Dis*. 2010;16: 1239-46.
- [22] Arijs I, Quintens R, Van Lommel L, Van Steen K, De Hertogh G, Lemaire K, et al. Predictive value of epithelial gene expression profiles for response to infliximab in Crohn's disease. *Inflamm Bowel Dis* 2010;16: 2090-98.
- [23] Sena A, Grishina I, Thai A, Goulart L, Macal M, Fenton A, et al. Dysregulation of anti-inflammatory annexin A1 expression in progressive Crohns Disease. *PLoS One* 2013; 8: e76969.
- [24] Wirtz S, Neufert C, Weigmann B, Neurath MF. Chemically induced mouse models of intestinal inflammation. *Nat Protoc* 2007; 2: 541-6.
- [25] Bento AF, Leite DF, Claudino RF, Hara DB, Leal PC, Calixto JB. The selective nonpeptide CXCR2 antagonist SB225002 ameliorates acute experimental colitis in mice. *J Leukoc Biol* 2008; 84: 1213-21.

- [26] Geboes K, Riddell R, Ost A, Jensfelt B, Persson T, Löfberg R. A reproducible grading scale for histological assessment of inflammation in ulcerative colitis. *Gut* 2000; 47: 404-9.
- [27] Porporatto C, Bianco ID, Riera CM, Correa SG. Chitosan induces different L-arginine metabolic pathways in resting and inflammatory macrophages. *Biochem Biophys Res Commun* 2003; 304: 266-72.
- [28] Tsao FH, Meyer KC, Chen X, Rosenthal NS, Hu J. Degradation of annexin I in bronchoalveolar lavage fluid from patients with cystic fibrosis. *Am J Respir Cell Mol Biol* 1998; 18: 120-8.
- [29] Debret R, El Btaouri H, Duca L, Rahman I, Radke S, Haye B, et al. Annexin A1 processing is associated with caspase-dependent apoptosis in BZR cells. *FEBS Lett* 2003; 546: 195-202.
- [30] Davies MD, Parrott DM. Preparation and purification of lymphocytes from the epithelium and lamina propria of murine small intestine. *Gut* 1981; 22: 481-8.
- [31] La M, D'Amico M, Bandiera S, Di Filippo C, Oliani SM, Gavins FN, et al. Annexin 1 peptides protect against experimental myocardial ischemia-reperfusion: analysis of their mechanism of action. *FASEB J* 2001; 15: 2247-56.
- [32] Yang YH, Song W, Deane JA, Kao W, Ooi JD, Ngo D, et al. Deficiency of annexin A1 in CD4<sup>+</sup> T cells exacerbates T cell-dependent inflammation. *J Immunol* 2013;190: 997-1007.
- [33] Sekigawa I, Yanagida M, Iwabuchi K, Kaneda K, Kaneko H, Takasaki Y, et al. Protein biomarker analysis by mass spectrometry in patients with rheumatoid arthritis receiving anti-tumor necrosis factor-alpha antibody therapy. *Clin Exp Rheumatol* 2008; 26: 261-7.
- [34] Dwivedi RC, Dhindsa N, Krokhin OV, Cortens J, Wilkins JA, El-Gabalawy HS. The effects of infliximab therapy on the serum proteome of rheumatoid arthritis patients. *Arthritis Res Ther* 2009;11: R32.



- [35] Yang Y, Wang H, Dou Y, Wang Y, Han G, Wang R, et al. Colitogenic role of tumour necrosis factor (TNF) receptors in trinitrobenzene sulphonic acid colitis: TNF-R1 ablation does not affect systemic inflammatory response. *Clin Exp Immunol* 2011;165: 372-82.
- [36] Nakai M, Sudo K, Yamada Y, Kojima Y, Kato T, Saito K, et al. The role of the tumor necrosis factor receptor in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice. *Dig Dis Sci* 2005; 50: 1669-76.
- [37] Wang Y, Han G, Chen Y, Wang K, Liu G, Wang R, et al. Protective role of tumor necrosis factor (TNF) receptors in chronic intestinal inflammation: TNFR1 ablation boosts systemic inflammatory response. *Lab Invest* 2013; 93: 1024-35.
- [38] Ebach DR, Newberry R, Stenson WF. Differential role of tumor necrosis factor receptors in TNBS colitis. *Inflamm Bowel Dis* 2005;11: 533-40.
- [39] Stillie R, Stadnyk AW. Role of TNF receptors, TNFR1 and TNFR2, in dextran sodium sulfate-induced colitis. *Inflamm Bowel Dis* 2009; 15: 1515-25.
- [40] Nenci A, Becker C, Wullaert A, Gareus R, van Loo G, Danese S, et al. Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature* 2007; 446: 557-61.
- [41] Comera C, Brousset P, Moré J, Vergnolle N, Buéno L. Inflammatory neutrophils secrete annexin 1 during experimentally induced colitis in rats. *Dig Dis Sci* 1999; 44: 1448-57.
- [42] Perretti M, D'Acquisto F- Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. *Nat Rev Immunol* 2009; 9: 62-70.
- [43] Farooq SM, Stadnyk AW. Neutrophil infiltration of the colon is independent of the FPR1 yet FPR1 deficient mice show differential susceptibilities to acute versus chronic induced colitis. *Dig Dis Sci* 2012; 57: 1802-12.

- [44] Chen K, Liu M, Liu Y, Yoshimura T, Shen W, Le Y, et al. Formylpeptide receptor-2 contributes to colonic epithelial homeostasis, inflammation, and tumorigenesis. *J Clin Invest*. 2013;123: 1694-1704.
- [45] Gavins FN, Yona S, Kamal AM, Flower RJ, Perretti M. Leukocyte antiadhesive actions of annexin 1: ALXR- and FPR-related anti-inflammatory mechanisms. *Blood* 2003;101: 4140-47.
- [46] Gavins FN, Hughes EL, Buss NA, Holloway PM, Getting SJ, Buckingham JC. Leukocyte recruitment in the brain in sepsis: involvement of the annexin 1-FPR2/ALX anti-inflammatory system. *FASEB J* 2012; 26: 4977-89.
- [47] Gastardelo TS, Damazo AS, Dalli J, Flower RJ, Perretti M, Oliani SM. Functional and ultrastructural analysis of annexin A1 and its receptor in extravasating neutrophils during acute inflammation. *Am J Pathol* 2009; 174: 177-83.
- [48] Vago JP, Nogueira CR, Tavares LP, Soriani FM, Lopes F, Russo RC et al. Annexin A1 modulates natural and glucocorticoid-induced resolution of inflammation by enhancing neutrophil apoptosis. *J Leukoc Biol* 2012; 92: 249-58.

### Figure captions

**Figure 1. Human colonic mucosa from healthy volunteers and remittent UC patients under anti-TNF- $\alpha$  therapy express more AnxA1.** AnxA1 expression was evaluated in sections from human biopsies by immunohistochemistry. Representative photomicrographs from UC patients without therapy (A), treated with GC (B), anti-TNF- $\alpha$  (C) and healthy volunteers (D). AnxA1 immunostaining in crypts and surface epithelium (arrows). Densitometry analysis of epithelial AnxA1 expression in all samples evaluated (E). Comparisons were performed between groups with  $n \geq 3$ . Data are mean  $\pm$  SEM. Magnification: 200x. \* $p < 0.05$ .

**Figure 2. Earlier clinical resolution of DSS-colitis in TNFR1 $^{-/-}$  mice.** Mice received 3% DSS for 5 days and drinking water for the next 2 days; animals were evaluated daily during 18 days for clinical signs to assess (A) the Disease Activity Index (DAI), (B) the weight loss percentage and (C) the colon shortening on day 18. (A) and (B): ( $\square$ ) control WT, ( $\circ$ ) control TNFR1 $^{-/-}$ , ( $\blacksquare$ ) DSS-treated WT, ( $\bullet$ ) DSS-treated TNFR1 $^{-/-}$ . Gray box: acute stage. Data are mean  $\pm$  SEM.  $n = 6-8$  animals/group. Experiments were performed twice. (A) and (B) \*  $p < 0.05$  vs. DSS-treated TNFR1 $^{-/-}$ ; (C) \*  $p < 0.05$  vs. control.

**Figure 3. Tissue injury and inflammation in DSS-treated TNFR1<sup>-/-</sup> mice.** Histopathological analysis of representative sections from WT (C, E, G) and TNFR1<sup>-/-</sup> (D, F, H) mice on days 5, 10 and 18 after colitis induction. Neutrophil infiltrate localized outside (C) or mainly inside blood vessels (D) (white arrows, insets). Progression of DSS-colitis in WT with presence of ulcers (asterisk) and tissue infiltrate of PMN (C and inset), dysplasia (asterisk) and crypt abscesses (E and inset) and even pseudopolyps (PP) and luminal abscesses at later stages (G and inset). WT and TNFR1<sup>-/-</sup> control groups (A, B); histopathological score on days 5, 10 and 18 after colitis induction (I); MPO (myeloperoxidase) activity (J). Magnification: 200x. Data are mean  $\pm$  SEM. n = 6-8 animals/group. Experiments were performed twice. # p<0.05 vs. control; ## p<0.01 vs. control; ### p<0.001 vs. control; \*p<0.05 vs. WT; \*\*p<0.01 vs. WT.

**Figure 4. AnxA1 expression increases in the resolution stage of colitis in TNFR1<sup>-/-</sup> mice.** Frozen colon sections from control TNFR1<sup>-/-</sup> and WT mice (A, D) or after 10 and 18 days of colitis induction (B-F) were used for AnxA1 labeling by immunofluorescence. Immunoreactivity for AnxA1 in epithelial cells (white arrows) at later stage of colitis (F); Immunohistochemistry control (G). Supernatants of colon explants (1mg of tissue, 10 $\mu$ l of total protein) from control or DSS-treated WT and TNFR1<sup>-/-</sup> mice were tested for the presence of AnxA1 by Western blotting with a polyclonal rabbit anti-AnxA1 (H); flow cytometry analysis of isolated LP CD11b, CD4 and CD8 cells stained for AnxA1 (I). Data are mean  $\pm$  SEM. n = 4 animals/group at each time studied. Magnification: 200x. \* p<0.05 vs. WT colitis; # p<0.05 vs. TNFR<sup>-/-</sup> colitis.

**Figure 5. Blocking AnxA1 activity exacerbates clinical signs of colitis in TNFR1<sup>-/-</sup> mice.**

WT and TNFR1<sup>-/-</sup> mice received 3% DSS for 5 days and drinking water for the next 2 days; BOC2 was injected i.p. on days 7-10; animals were evaluated during 11 days for clinical signs (A-D) Disease Activity index (DAI) and percentage of weight loss in control (□), BOC2 (Δ), DSS (■) and DSS+BOC2 (▲) groups in WT (A,B) and TNFR1<sup>-/-</sup> (C, D) mice; data are mean ± SEM. n = 4 animals/group. \*p<0.05 vs. DSS group in TNFR1<sup>-/-</sup> mice.

**Figure 6. Blocking AnxA1 activity impairs histopathological signs in TNFR1<sup>-/-</sup> mice.** WT and TNFR1<sup>-/-</sup> mice received 3% DSS for 5 days and drinking water for the next 2 days; BOC2 was injected i.p. on days 7-10; animals were evaluated during 11 days for clinical signs. (A) Representative sections of colon mucosa obtained on day 11 from control, BOC2, DSS and DSS+BOC2 groups in WT and TNFR1<sup>-/-</sup>; (B) MPO (myeloperoxidase) activity in ng/mg tissue protein. Data are mean ± SEM. n = 4 animals/group. Magnification: 200x.

**Table 1. Clinical data of UC patients and healthy volunteers**

Characteristics		Treatment groups		
		GC	anti-TNF- $\alpha$	Healthy volunteers
<b>Age</b>		31 $\pm$ 0	28 $\pm$ 2.7	60 $\pm$ 8
<b>Sex</b>	<b>female</b>	-	-	66%
	<b>male</b>	100%	100%	34%
<b>Histopathology</b>	<b>moderate</b>	50%	100%	-
	<b>severe</b>	50%	-	-
<b>Colonoscopy</b>	<b>moderate</b>	100%	100%	-
	<b>severe</b>	-	-	-
<b>Treatment time (years)</b>		0.4 $\pm$ 0	0.8 $\pm$ 0.4	-
<b>Number of clinical signs</b>		4.5 $\pm$ 0.5	1 $\pm$ 1	-
<b>Epithelial AnxA1 expression</b>		++	++	+++

GC = glucocorticoids. Clinical signs include diarrhea, bloody feces, abdominal pain, fever, urgency and extraintestinal manifestations. Numbers are expressed as mean  $\pm$  SEM or percentages (%).

Fig. 1. Sena et al

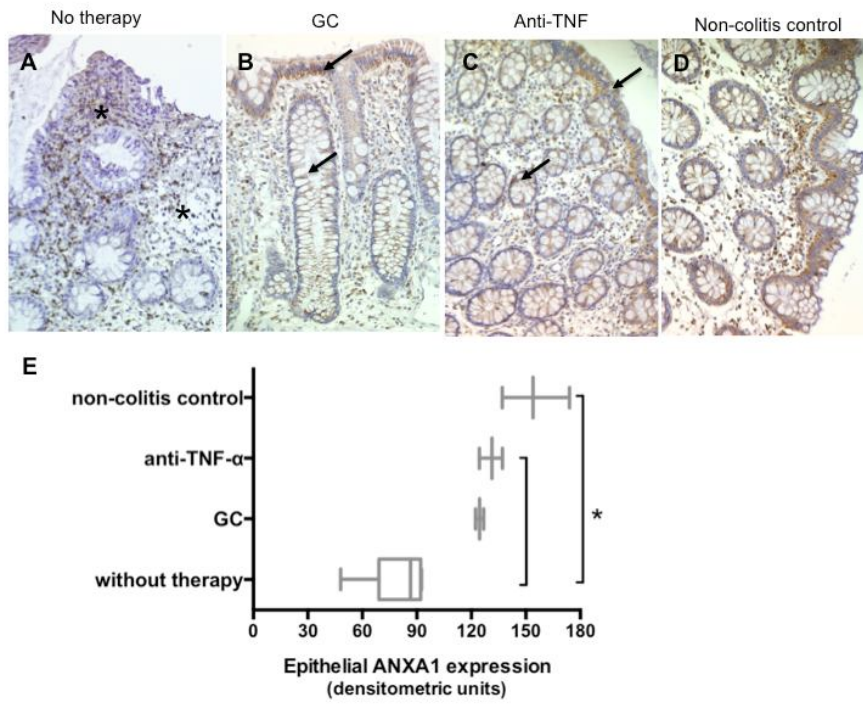


Fig. 2. Sena et al

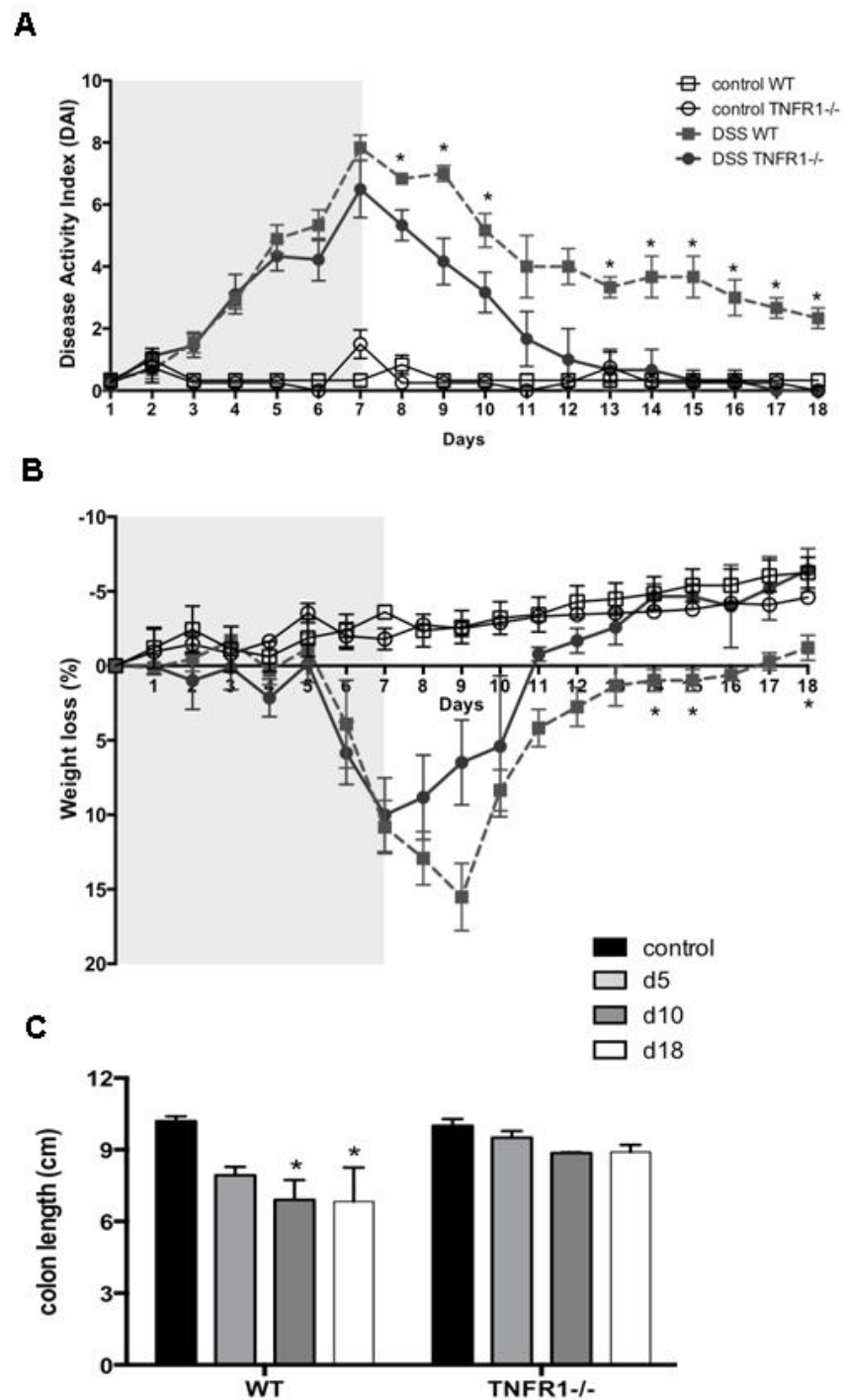




Fig. 3. Sena et al

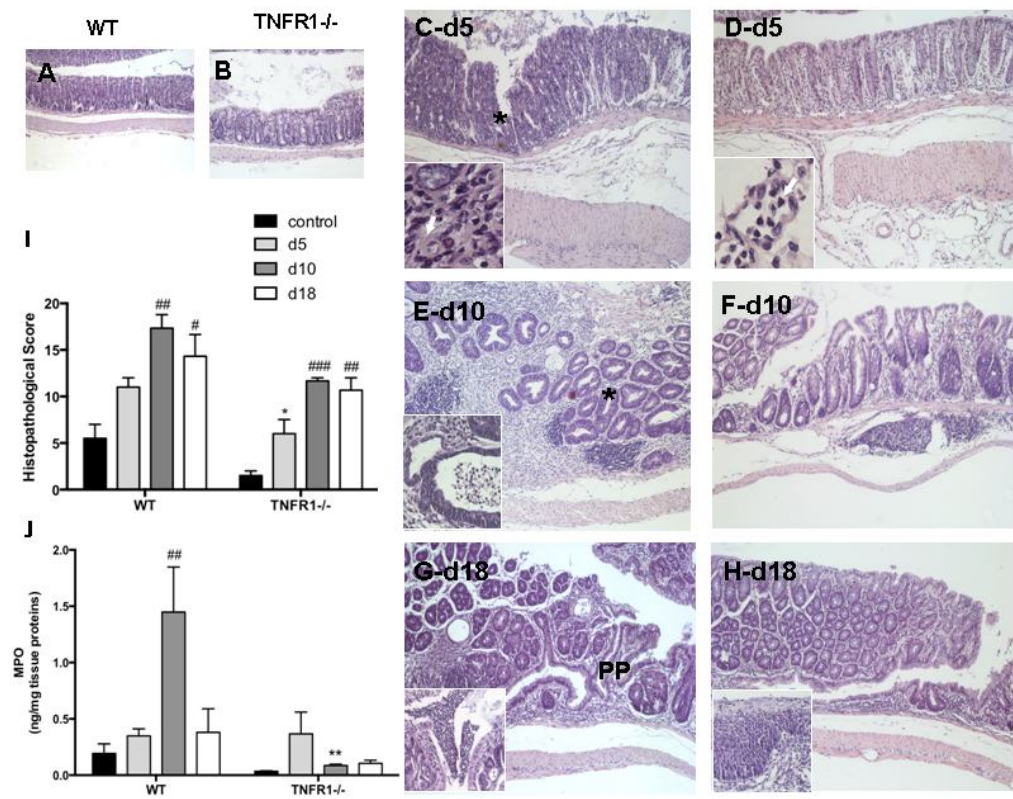


Fig. 4. Sena et al

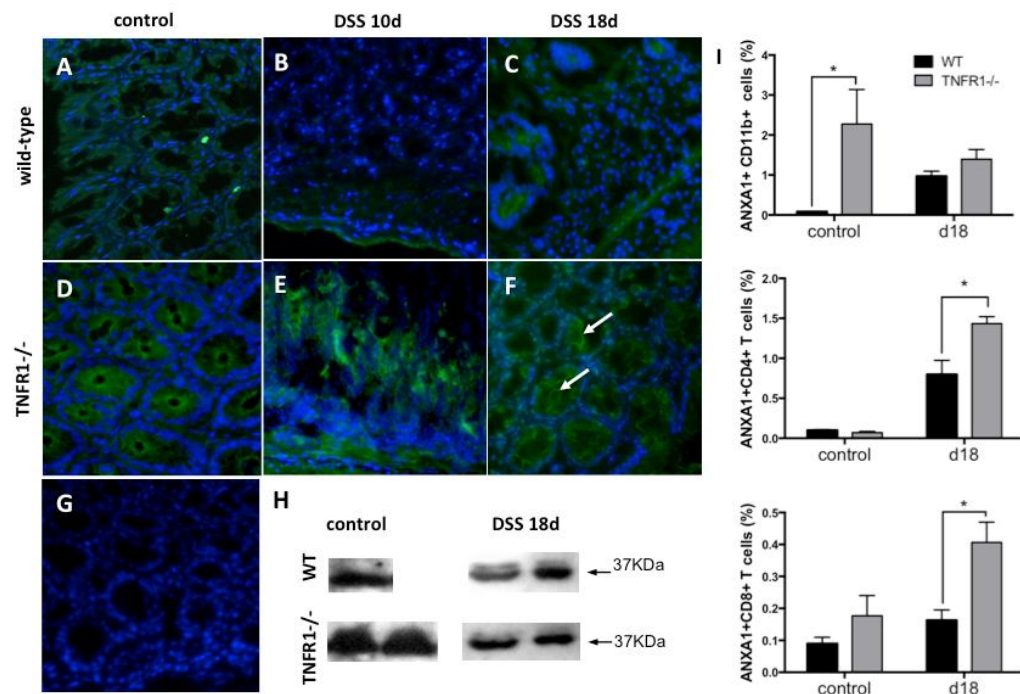


Figure 5 Sena et al

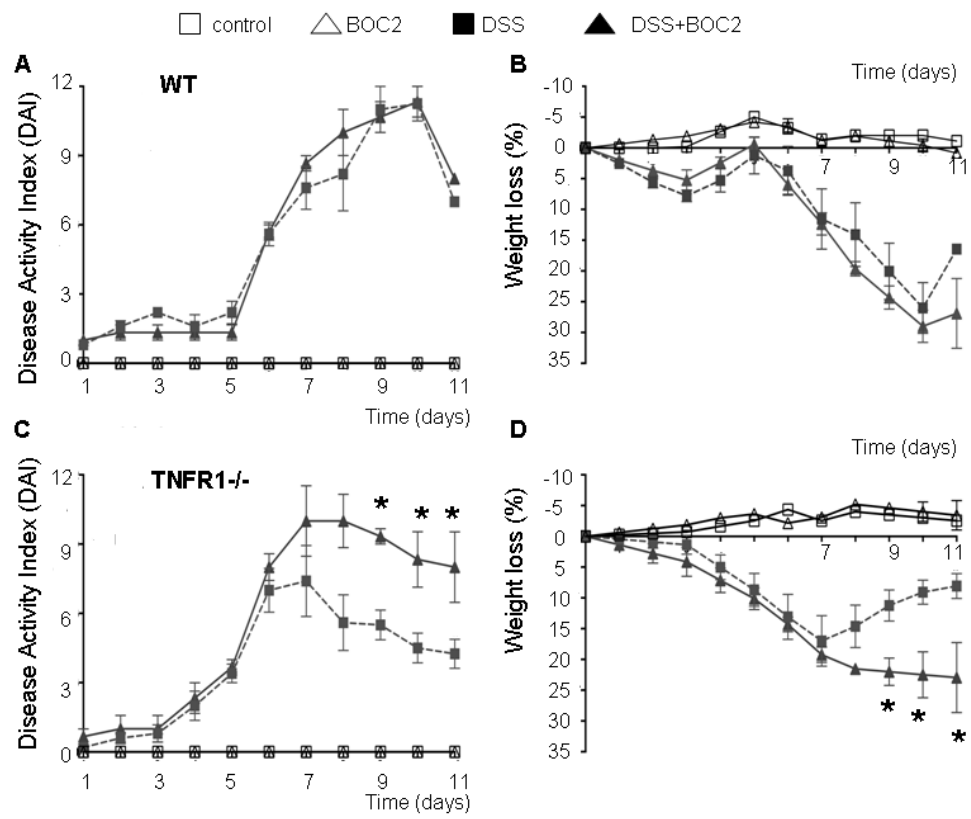


Figure 6 Sena et al

