



Role of the protein annexin A1 on the efficacy of anti-TNF treatment in a murine model of acute colitis



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ABSTRACT

TNF- α is involved in the mechanisms that initiate inflammatory bowel diseases (IBDs). Anti-TNF- α drugs, such as infliximab (IFX), cause non-responsiveness and side effects, indicating the need to investigate alternative therapies for these diseases. The anti-inflammatory protein, annexin A1 (AnxA1), has been associated with the protection of the gastrointestinal mucosa. To further address the role of endogenous AnxA1 on the TNF- α blockade efficacy in a murine model, we assessed colitis induced by Dextran Sulfate Sodium (DSS) in wild-type (WT) and *AnxA1*^{-/-} Balb/c mice treated with IFX. We consistently observed endogenous AnxA1 prevented clinical and physiological manifestations of experimental colitis treated with IFX, additionally the manifestation of the disease was observed earlier in *AnxA1*^{-/-} mice. Rectal bleeding, diarrhea, histological score, epithelial damages and collagen degradation caused by DSS were prevented following IFX treatment only in WT mice. IL-6 increased during colitis in WT and *AnxA1*^{-/-} mice, decreasing under IFX treatment in WT. The influx of neutrophils and TNF- α secretion were largely elevated in *AnxA1*^{-/-} mice when compared to WT mice. In the group WT/DSS + IFX, phagocytes were more susceptible to apoptosis following treatment with IFX. Endogenous expression of AnxA1 increased after DSS and decreased with IFX treatment, demonstrating an attenuated inflammatory response. The data indicate that AnxA1 contributes to the establishment of intestinal homeostasis after blocking of TNF- α was used as a treatment of IBD, constituting a key molecule in the mechanism of action and a potential biomarker of therapeutic efficacy.

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1. Introduction

Inflammatory bowel diseases (IBDs) (e.g. ulcerative colitis (UC) and Crohn's disease (CD)) are conditions characterized by inflammation of the lamina propria, which cause abdominal pain, diarrhea and rectal bleeding [1]. The pathogenicity of IBDs include genetic predisposition, aberrant immunological responses, epithelial dysfunction and altered microbial–host relationship [2]. Infiltrating inflammatory cells produce and are activated by a range of pro-inflammatory cytokines in the affected mucosa [3,4]. This cascade is followed by a loss of epithelial integrity, increased intestinal permeability and altered resident microbiota

that propagates the inflammatory process and attenuates the tissue regeneration [5].

In the acute stage of mucosa inflammation, tumor necrosis factor alpha (TNF- α) is a key cytokine. TNF- α is predominantly produced by macrophages in response to bacterial infections. Among others, TNF- α induces intestinal epithelial damage, increased adhesion molecule expression and enhanced vascular permeability, allowing the extravasation of cells and fluids [2,6]. This mechanism proposes TNF- α involvement in the development of inflammatory responses that activates IBDs. Since the secretion of TNF- α compromises the gastrointestinal physiology [7], TNF- α neutralizing therapy has been successfully utilized [8].

The efficacy of anti-TNF- α antibody, Infliximab (IFX), was demonstrated by inducing remission of the disease in patients with UC [9]. IFX is an IgG monoclonal antibody, that has constant regions that are from human and variable regions that are originally from mouse immunoglobulin [10]. This antibody strongly

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binds to the soluble and transmembrane forms of TNF- α , inhibiting this cytokine from exerting its inflammatory properties [10]. The inhibition of TNF- α signaling subsequently activates several anti-inflammatory effects, including mucosal healing, decreased pro-inflammatory cytokine release and T cells apoptosis [11]. However, some patients are tolerant to this therapy or still present with side effects, mainly related to opportunistic diseases, autoimmunity and hypersensitivity [12]. The non-responsiveness to IFX treatment highlights the need to investigate alternative mediators related to the therapeutic efficacy following the TNF- α blockade.

In this context, our group has recently demonstrated that treatment with IFX could induce the transcription, and subsequent expression, of the anti-inflammatory protein annexin A1 (AnxA1) [13]. This study reported that CD patients lose the ability to express systemic and mucosal AnxA1, which is recovered following IFX treatment. The systemic up-regulation of transcriptional expression and release of plasma AnxA1 was correlated with improved quality of life in these patients [13]. Further data demonstrated that biopsies from healthy volunteers and patients undergoing anti-TNF- α therapy with remittent UC, presented with higher AnxA1 expression than those with the active disease [14]. Together, our results demonstrate that AnxA1 is a potential key protein in anti-TNF- α therapy.

AnxA1 is a glucocorticoid induced protein [15,16], whose N-terminal domain exerts anti-inflammatory roles [17]. In the gastrointestinal tract, AnxA1 mediates the expression of cytokines such as, TNF- α , IL-6 and IL-10 [18]. Additionally, AnxA1 has been shown to mediate stomach wound healing after indomethacin treatment [19] and tissue damage recovery during IBDs [20]. Although the AnxA1 anti-inflammatory roles are well established, the function of this protein on IBDs and its interactions with TNF- α are not well understood.

We hypothesize that endogenous AnxA1 is involved in the mechanism of action of TNF- α neutralization. Herein, wild-type and AnxA1 deficient mice strains are used to investigate the relevance of this protein on the clinical and immunomorphological effects of IFX in a murine model of DSS induced colitis.

2. Materials and methods

2.1. Ethics statement and animals

Female wild-type (WT) and AnxA1 null (*AnxA1*^{−/−}) Balb/c mice aged 8- to 10-weeks-old or 20–25 g were obtained from the Animal House at the School of Pharmaceutical Sciences, University of São Paulo (Brazil). The animals were housed with a 12 h light-dark cycle and allowed food and water *ad libitum*. The experiments were performed in strict accordance with the Brazilian laws of protection and this study was approved by the Committee on the Ethics of Animal Experiments of the São Paulo Federal University (UNIFESP), São Paulo, Brazil (Permit Number: 6850200715) and University of São Paulo (Permit number: 01200.003570/1998-08).

2.2. Colitis induction and clinical analysis

Colitis was induced by Dextran Sulfate Sodium (DSS, MW 40,000, Dextran Products Limited, Canada), at a concentration of 5% in sterilized drinking water [14]. Fresh DSS solution was replenished every two days (days 0, 2 and 4). Over seven days, development of induced colitis was assessed by analysis of the following parameters: percentage of body weight loss, diarrhea and rectal bleeding (*Hemocult Sensa*). The Disease Activity Index (DAI) was calculated with consideration of the sum of the diary values of each score above. On day 6, mice were euthanized using nasal anesthesia (Isoflurane: 2-chloro-2-(difluoromethoxy)-1,1,1-t

trifluoro-ethane). The large intestine was removed and fragmented for cytokine (proximal colon), colorimetric (middle colon) and histopathological and immunohistochemical (distal colon) analysis.

2.3. Experimental design and anti-TNF- α treatment

WT and *AnxA1*^{−/−} mice were randomly divided into the following groups ($n = 6$ animals/group): oral DSS administration and intraperitoneal (i.p.) sterilized saline (group DSS); DSS administration and i.p. anti-TNF- α (Infliximab – IFX: REMICADE® Janssen-Cilag Pharmaceuticals Ltda, Argentina) at 1 mg/kg (group DSS + IFX). Mice from control groups received sterilized water only and were injected i.p. with either saline (Sham) or IFX, to allow a comparison between sick and healthy conditions. The i.p. injections were performed on day 1.

2.4. Myeloperoxidase measurement

Neutrophil infiltration was indirectly assessed by myeloperoxidase (MPO) measurement as previously described [14]. Briefly, fragments of the middle colon were weighted to a correct calculation for the concentration. These samples were homogenized in a 5% EDTA/NaCl buffer (pH 4.7) and centrifuged at 10,000 rpm, 4 °C for 15 min. The pellet was resuspended in a 0.2% NaCl, 1.6% NaCl-5% glucose and 0.5% hexadecyl trimethyl ammonium bromide (pH 5.4) solution. The samples were frozen in liquid nitrogen and thawed at 37 °C, three times. Finally, the supernatant was collected and 50 μ L was used for the colorimetric assay. The enzymatic reaction was performed in a 96 well plate, and MPO was detected using tetramethylbenzidine (TMB, Millipore, USA). The absorbance was measured using a spectrophotometer at 450 nm and the concentration of MPO in ng/ml was calculated in comparison to a standard curve generated from a solution of *horseradish peroxidase* (HRP, BD Biosciences, California, USA) diluted in NaPO₄. Results were expressed as mean \pm S.E.M. of the concentration (ng/mL/mg).

2.5. Histological analyses

Samples of the distal portion of the colon were fixed in 4% buffered formalin for 24 h, dehydrated in graded ethanol and embedded in paraffin for immunohistochemical and histopathological analysis.

Histopathological analysis and histological score: 2 μ m sections were stained with hematoxylin & eosin and analyzed using a high-power objective (40 \times) on an Axioskop 2-Mot Plus Zeiss microscope (Carl Zeiss, Jena, Germany). To quantify the morphological aspects following DSS and IFX, a scoring system was employed. Values of 0, 1, 2 and 3 correlated to normal, mild abnormality, moderate damage and severe injury, respectively. The following parameters were considered: crypts and histoarchitecture changes, edema, ulcers and inflammatory cells located at the epithelium, lamina propria and submucosa [21]. Results were expressed as mean \pm S.E.M.

Quantification of collagen: The submucosal collagen was detected by the picosirius-hematoxylin staining under polarized light. Five randomized fields per animal were photographed (40 \times). Subsequently, 1 mm² grid squares was designed for each field. The points were quantified that coincide either with intact collagen fibers or with the spots of conjunctive tissue in which these fibers were degraded. The proportion of intact collagen fibers/total fibers was calculated in each field and then to each animal. Results were expressed as mean \pm S.E.M. of the intact collagen fibers proportion.

2.6. Immunohistochemical detection of annexin A1 and caspase-3

Sections of the colon (2 μ m) were incubated with sodium citrate buffer at 96 °C for 30 min. The endogenous peroxide activity was blocked with 3% hydrogen peroxide for 30 min, followed by incubation with either polyclonal rabbit anti-AnxA1 (Zymed Laboratories, Cód. 713400, Cambridge, UK) at 1:500 or polyclonal rabbit anti-caspase-3 (Abcam, Cód. 552293, Cambridge, UK) at 1:50, in 10% TBS-BSA (Sigma-Aldrich, San Luis, USA). The slides were incubated overnight at 4 °C. Additionally, negative control sections were incubated with 10% TBS-BSA instead of the primary antibody. Following the wash step, sections were incubated with a secondary Ab conjugated with HRP (Abcam, Cambridge, U.K.). Positive staining was detected using 3,3'-diaminobenzidine (DAB substrate; Invitrogen, USA). Finally, sections were counterstained with hematoxylin (Inlab Confiança, São Paulo, Brazil) and mounted. Analysis was conducted on Axioskop 2-Mot Plus Microscope (Carl Zeiss, Jena, GR), using AxioVision software for densitometric and quantitative analysis. Densitometric analysis was used to determine AnxA1 intensity in colonic epithelial and immune cells (40 \times) on an arbitrary scale from 0 to 255, and the data were expressed as mean \pm S.E.M. Caspase-3 marked phagocytes (e.g. neutrophils, monocytes and macrophages) were quantified and the number of immunoreactive cells was divided by the total number of phagocytes counted in the same slides (objective 63 \times). Results were expressed as mean \pm S.E.M. of the percentage of apoptotic phagocytes in mm².

2.7. Explant culture and cytokine levels measurement in colonic tissue

Colonic medial fragments were weighted and washed in sterile PBS and incubated with Dulbecco's Modified Eagle Medium (DMEM, Cultilab, Campinas, São Paulo, Brasil) for 24 h, supplemented with 10% fetal bovine serum (FBS, Cultilab) and antibiotics (Cultilab), one punch biopsy per 1 mL medium. The supernatants were collected and used for the assessment of IL-6 and TNF- α secreted levels by ELISA (BD Biosciences, California, USA), according to manufacturers instructions. The data were expressed as mean \pm S.E.M. of the concentration (ng/mL/mg).

2.8. Statistical analysis

The statistics were performed using GraphPad software version 6.00. First, we performed the normality test Kolmogorov–Smirnov to determine if the data distribution was either parametric or non-parametric. Then, differences between groups were evaluated by either a one-way ANOVA (clinical analysis) or a two-way ANOVA (other analysis), followed by either Bonferroni (parametric) or Dunn's test (non-parametric). A probability value less than 0.05 was considered to be significant.

3. Results

3.1. IFX prevents clinical manifestations of DSS induced colitis in WT, but not in *AnxA1*^{-/-} mice

Initially, we assessed the clinical alterations resulting from DSS-induced colitis and the effects of treatment with IFX. The body weight loss was significant in WT/DSS and *AnxA1*^{-/-}/DSS mice at days 5 and 6, in comparison to the respective sham groups (Fig. 1A and B). The scores for diarrhea and rectal bleeding are shown in Fig. 1C–F and increased in both lineages over days 2–6. The Disease Activity Index (DAI), defined as the sum of the scores above, was higher in the WT/DSS (days 2–6) and *AnxA1*^{-/-}/DSS (days 1–6) mice when compared to the sham groups (Fig. 1G and H) confirming that DSS induced intense colitis in both strains. It

is noteworthy to mention that symptoms of disease started earlier in the *AnxA1*^{-/-} group than in WT mice.

The administration of 1 mg/kg of IFX prevented diarrhea and blood loss, as well as reduced the DAI in the WT mice. Interestingly, knockout mice did not present with improvement in any clinical parameter following treatment with IFX, indicating that TNF- α blockade is not sufficient enough to attenuate the colitis symptoms in the absence of AnxA1. Taken together, the clinical data reports that WT and *AnxA1*^{-/-} mice develop DSS-induced colitis with similar intensity, although earlier in *AnxA1*^{-/-}. However, the anti-TNF- α treatment was efficient in preventing the disease manifestations, but only in the presence of AnxA1.

3.2. DSS-induced colitis causes morphological alterations and collagen degradation in the colon

WT and *AnxA1*^{-/-} mice exposed to DSS and treated or non with IFX, presented with shortening in the length of the large intestine (Fig. 2B and C). Only *AnxA1*^{-/-}/DSS animals had decreased intestinal weight (Fig. 2B). The anti-TNF- α treatment did not prevent these parameters in any studied strain (Fig. 2B and C).

After observing anatomic alterations, we questioned if this colitis model could induce microscopic alterations, specifically in collagen integrity. Therefore, we examined these fibers in the submucosal layers (Fig. 2D–K). In this region, the collagen was degraded in the WT/DSS mice (Fig. 2F) and, following IFX treatment, there was a trend indicating an increase in the integrity of these fibers (Fig. 2G), however, it was not significant (Fig. 2L). In *AnxA1*^{-/-}/DSS and *AnxA1*^{-/-}/DSS + IFX, the picrosirius-hematoxylin polarization showed areas of degraded collagen, interspersed by areas of intact fibers (Fig. 2J and K). We did not detect quantitative significant alterations on the collagen integrity in either group of mice (Fig. 2L).

3.3. Histological damage induced by DSS is restrained by IFX only in the presence of *AnxA1*

Fig. 3A–H illustrates histological results following seven days of DSS administration. The DSS produced alterations on the colonic histoarchitecture, compromising homeostasis in WT and *AnxA1*^{-/-} mice. In both strains, the epithelial layer was interrupted in particular regions with occasional ulcerations over the distal portion of the colon. On the mucosal and submucosal layers of WT/DSS and *AnxA1*^{-/-}/DSS mice, we observed edema and evident inflammatory infiltrate, mainly constituted by neutrophils and macrophages, indicating the severity of the disease. On the lamina propria, there were discontinuous areas presenting with either dysplastic or absent crypts and vacuolar hydropic degeneration.

In WT mice, the treatment preserved the epithelial layer along the colon and decreased the vacuolar hydropic degeneration and the submucosal edema. Alternatively, the *AnxA1*^{-/-} animals were observed to have remaining glandular alterations and ulcers, indicating that the IFX administration was not efficient in recovering these histological damages in the absence of AnxA1. Furthermore, alterations caused by the IFX administration were not detected, thus, we are confident that the anti-TNF- α antibody is not involved in the induction of these specific tissue injuries (data not shown).

To quantify the intensity of the induced colitis and the anti-TNF- α treatment efficacy, we scored the histological changes observed in each condition (Fig. 3I) as described in M&M section. The sham and IFX groups presented with very low values of morphological alterations. Following exposure to DSS, both WT and *AnxA1*^{-/-} mice presented with increased histological scores, compared to the sham group of mice. However, the treatment decreased this parameter in the WT/DSS + IFX mice, while no improvement was observed in the *AnxA1*^{-/-}/DSS + IFX group.

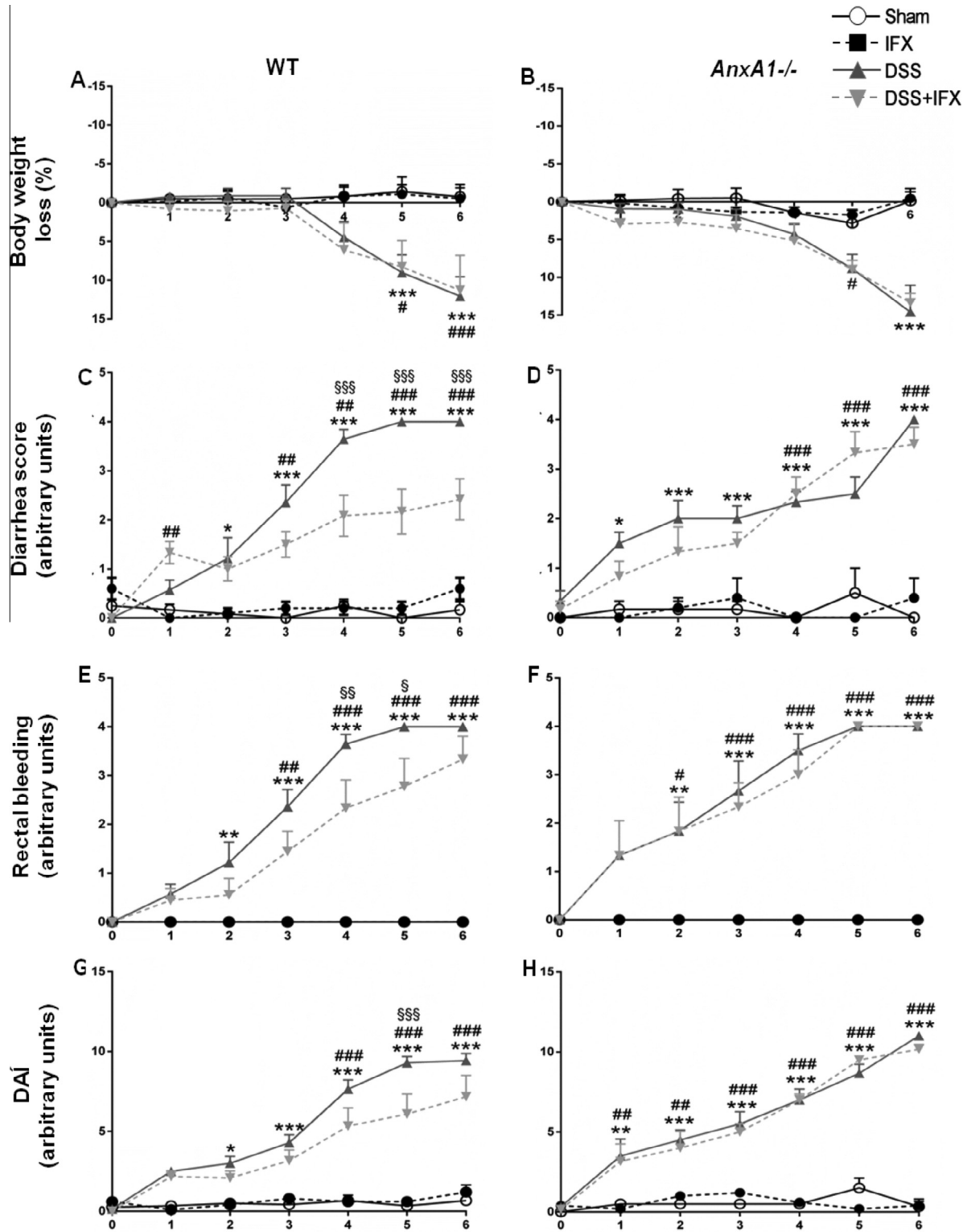


Fig. 1. Clinical parameters during experimental colitis and following anti-TNF- α treatment. (A and B) Loss of body weight. (C and D) Diarrhea score. (E and F) Rectal bleeding score. (G and H) Disease activity index (DAI). $n = 6$ animals/group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs Sham; # $p < 0.01$; ### $p < 0.001$ vs IFX; § $p < 0.01$; §§ $p < 0.001$ vs DSS.

3.4. *AnxA1* mediates the reduction of MPO and IL-6 secretion, and augments the proportion of phagocyte apoptosis following IFX treatment

MPO activity, a marker of neutrophil recruitment and infiltration, was quantified for each condition (Fig. 4A and B). Results

showed that WT mice had increased activity of this enzyme after DSS in comparison to the sham group (Fig. 4A). We hypothesize this to be the reason why we observed an augmented amount of neutrophils on the colonic tissue. The treatment with IFX did not reduce levels of MPO in either the WT or *AnxA1*^{-/-} groups. However, a 30-fold increase in MPO

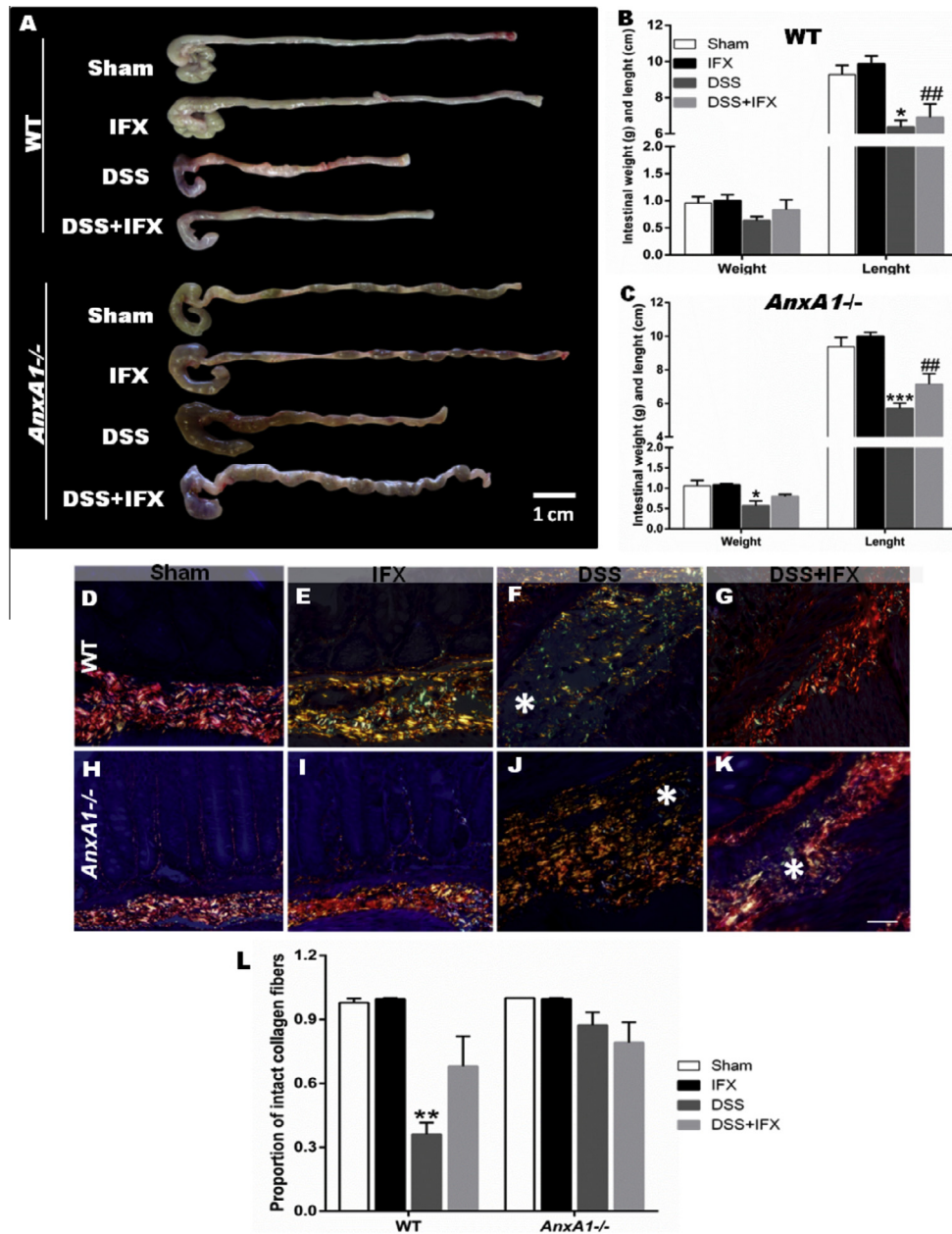


Fig. 2. Morphological analysis of the large intestine and submucosal collagen after colitis and anti-TNF- α treatment. (A) Anatomic evaluation of the large intestine, (B and C) Intestinal weight and length. (D–G) Collagen in WT. (H–K) Collagen in *AnxA1*^{-/-}. Sections: 2 μ m. Bar: 20 μ m. Staining: Picrosirius-Hematoxylin. (L) Collagen quantification. $n = 6$ animals/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs Sham; ## $p < 0.01$ vs IFX. Asterisk indicates degraded collagen fibers after DSS in WT (F) and in *AnxA1*^{-/-}/DSS and *AnxA1*^{-/-}/DSS + IFX (J and K).

level was detected in *AnxA1*^{-/-} mice in comparison to the WT group (Fig. 4A and B).

Furthermore, the concentrations of pro-inflammatory cytokines IL-6 and TNF- α were measured by ELISA assays (Fig. 4C–F). The exposure to DSS increased IL-6 expression in WT and *AnxA1*^{-/-} mice. Conversely, TNF- α was augmented only in the *AnxA1*^{-/-}/DSS, presenting with levels 150 times higher than the WT/DSS. The efficacy of the IFX treatment was evidenced by a reduction of IL-6 only in WT mice, highlighting the relevance of *AnxA1* on the efficacy of anti-inflammatory actions of IFX treatment.

Finally, we performed immunohistochemical analysis for caspase-3 to assess apoptosis of phagocytes in the colon (Fig. 4G). In the WT/DSS group, the percentage of neutrophils, monocytes and macrophages marked with caspase-3 was not altered. Nevertheless, in the WT/DSS + IFX mice, increased apoptosis was

observed in comparison to WT/sham and WT/DSS groups. *AnxA1*^{-/-} mice did not present differences on the total apoptotic phagocytes/total phagocytes when compared to WT mice. Nevertheless, the treatment with IFX in the *AnxA1*^{-/-}/DSS (*AnxA1*^{-/-}/DSS + IFX group) did not improve the apoptosis of phagocytes. The percentage of apoptotic phagocytes was significantly lower in the *AnxA1*^{-/-}/DSS + IFX group than that observed in the WT/DSS + IFX group (Fig. 4G).

3.5. Endogenous *AnxA1* is modulated in the intestinal epithelial and immune cells following DSS administration and TNF- α blockade

The immunoreactivity of the *AnxA1* protein was evaluated on the colonic tissue in each of the different studied conditions (Fig. 5). Sections were incubated with 10% TBS-BSA instead of the

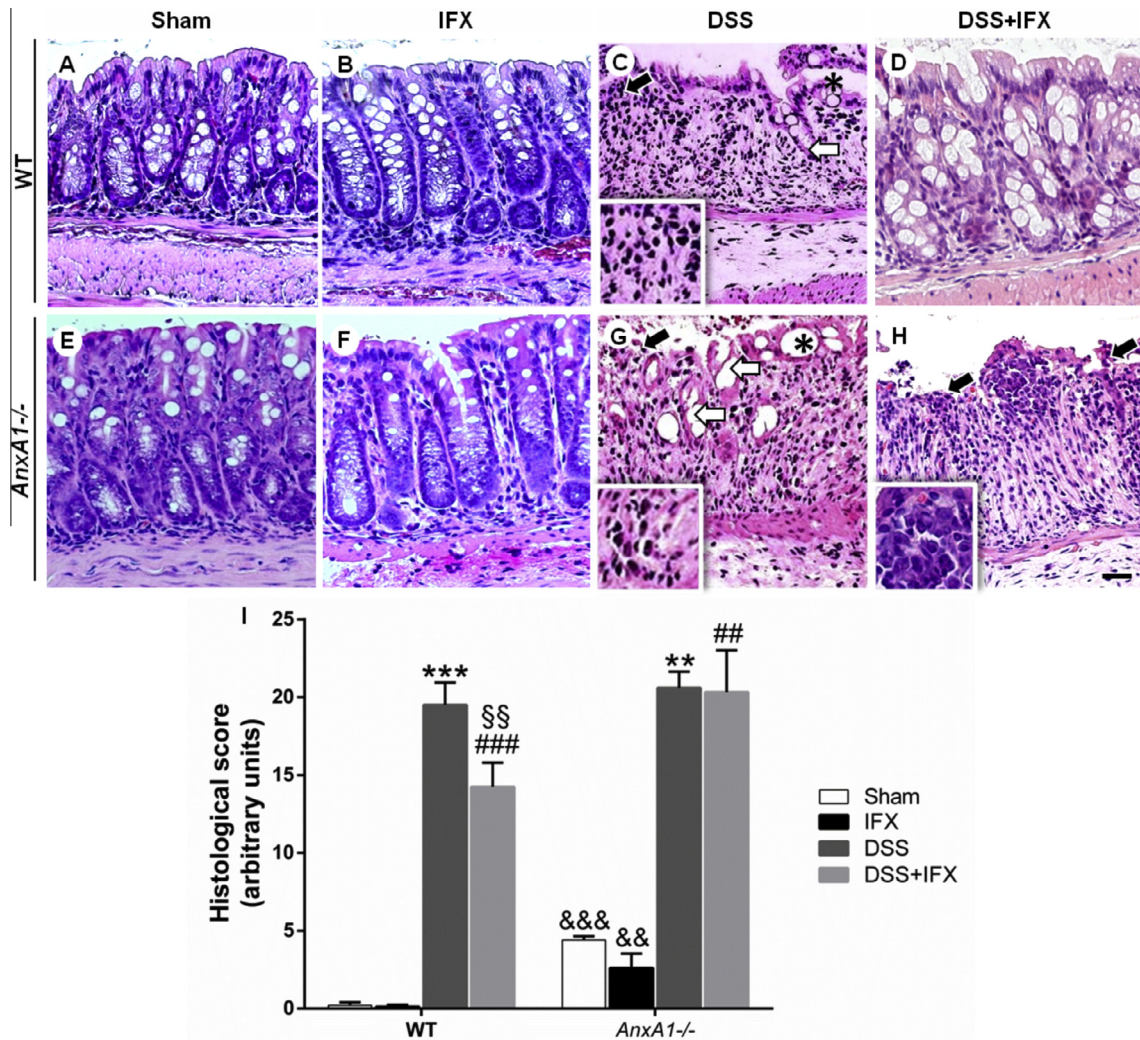


Fig. 3. Histopathological analysis of the distal colon after colitis and effects of the treatment with anti-TNF- α . (A and E) Normal intestinal morphology, (B and F) Preserved structure after IFX, (C and G) Altered histoarchitecture of the colon, (D and H) Tissue injuries attenuated by IFX in WT, but not in *AnxA1*^{-/-} mice. Ulcer (black arrows). Dysplastic crypts (white arrows). Vacuolar hydropic degeneration (asterisks). Mucosal inflammatory infiltrate (details: C, G and H). Inclusion: paraffin. Staining: Hematoxylin–Eosin. Sections: 2 μ m. Bar: 20 μ m. (I) Histological score. $n = 6$ animals/group. *** $p < 0.001$ vs Sham; ** $p < 0.01$; ### $p < 0.001$ vs IFX; §§ $p < 0.01$ vs DSS; && $p < 0.01$, &&& $p < 0.001$ vs respective WT.

primary antibody to provide a negative control for the reaction (Fig. 5F).

In the colonic normal condition (sham), AnxA1 was detected in the epithelial cells, which compose the excretory portion of tubular glands. In the epithelium of healthy mice treated with IFX, the immunostaining for this protein was less observed (Fig. 5B).

Conversely, in the WT/DSS group, AnxA1 expression was significantly increased in the epithelial cells and immune cells (mainly neutrophils and macrophages) (Fig. 5C). The previous blockade of TNF- α with IFX also promoted alterations to AnxA1 endogenous expression, showing lower levels in both epithelial cells and leukocytes (Fig. 5D) when compared to the non-treated mice. The densitometry analysis confirmed the described observations, showing higher AnxA1 expression during colitis and lower levels under IFX treatment (Fig. 5E).

4. Discussion

Current therapies used to suppress IBD activity still result in side effects and ineffectiveness, highlighting the need to research alternative messengers and biomarkers as potential treatment

options [22]. In the present study, we observed that the presence of the anti-inflammatory protein, AnxA1, is related to the efficiency of the IFX treatment in experimental colitis, constituting a potential biomarker of therapeutic efficacy.

Several studies demonstrated that the colitis induced by DSS in the murine model mimics the clinical manifestations and the consequent increase of the DAI in humans [19,23,24], and it is a suitable model to detect the efficiency of IFX treatment [24]. Indeed, in this study, DSS induced a typical set of alterations that are characteristic of acute colitis, such as body weight loss, diarrhea and rectal bleeding, which were significantly reduced in the WT mice. Interestingly, these symptoms were not significantly reduced in *AnxA1*^{-/-} mice treated with IFX, showing, for the first time, the involvement of endogenous AnxA1 in the clinical improvement following the TNF- α blockade in colitis. Moreover, clinical manifestation was detected earlier in *AnxA1*^{-/-} mice, corroborating the AnxA1 endogenous mediation on colitis [19].

Diarrhea and blood loss in colitis are associated with loss of epithelial cells, which are responsible for the absorption of water [25]. The impairment of the epithelial barrier during colitis is mediated by TNF- α , which also induces apoptosis of intestinal

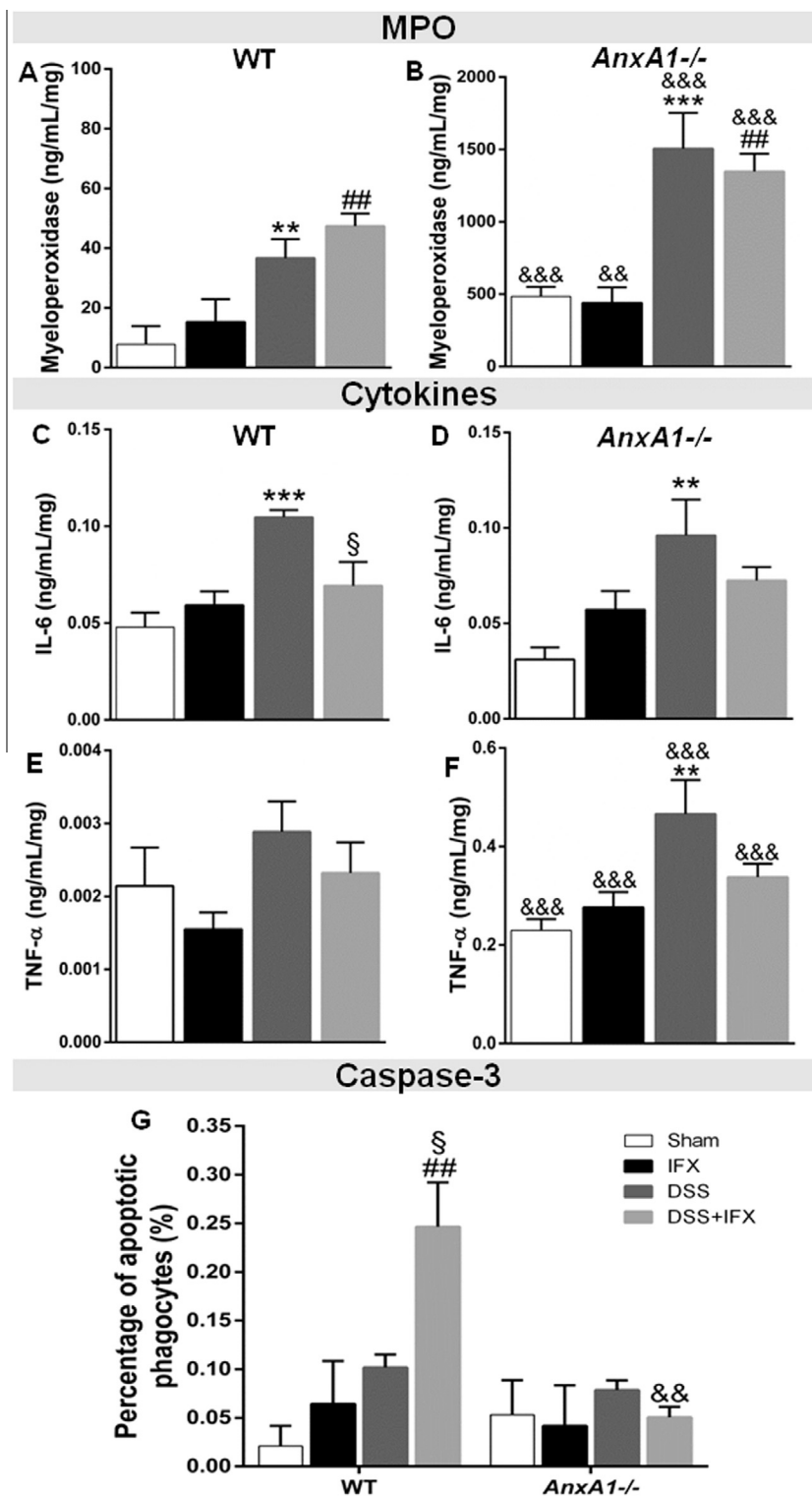


Fig. 4. Expression of MPO, IL-6, TNF- α and apoptosis of phagocytes on colonic tissue and effects of treatment with anti-TNF- α . (A and B) MPO activity, (C–F) IL-6 and TNF- α secretions, (G) Proportion of apoptotic phagocytes. $n = 6$ animals/group. Mean \pm S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs Sham; # $p < 0.05$, ## $p < 0.01$ vs IFX; && $p < 0.01$, &&& $p < 0.001$ vs respective WT.

epithelial cells and necroptosis of goblet cells [2,26,27]. We investigated the interrelationships of these effects during colitis, and results showed diarrhea, blood loss and omitted areas of ulceration in the distal region in the colon of the WT and AnxA1^{-/-} mice. Additionally, treatment with IFX largely prevented the loss of epithelial cells, ulceration and avoided rectal bleeding and diarrhea in WT

mice. The absence of therapeutic effects of IFX in AnxA1^{-/-} mice corroborated the key role of AnxA1 on IFX mechanisms. The exact mechanism of AnxA1 on IFX effects is not well understood, but a prominent role of AnxA1 on epithelial integrity is hypothesized, as AnxA1 promotes proliferation and migration of epithelial cells, playing an important role in tissue regeneration [19,20]. Moreover,

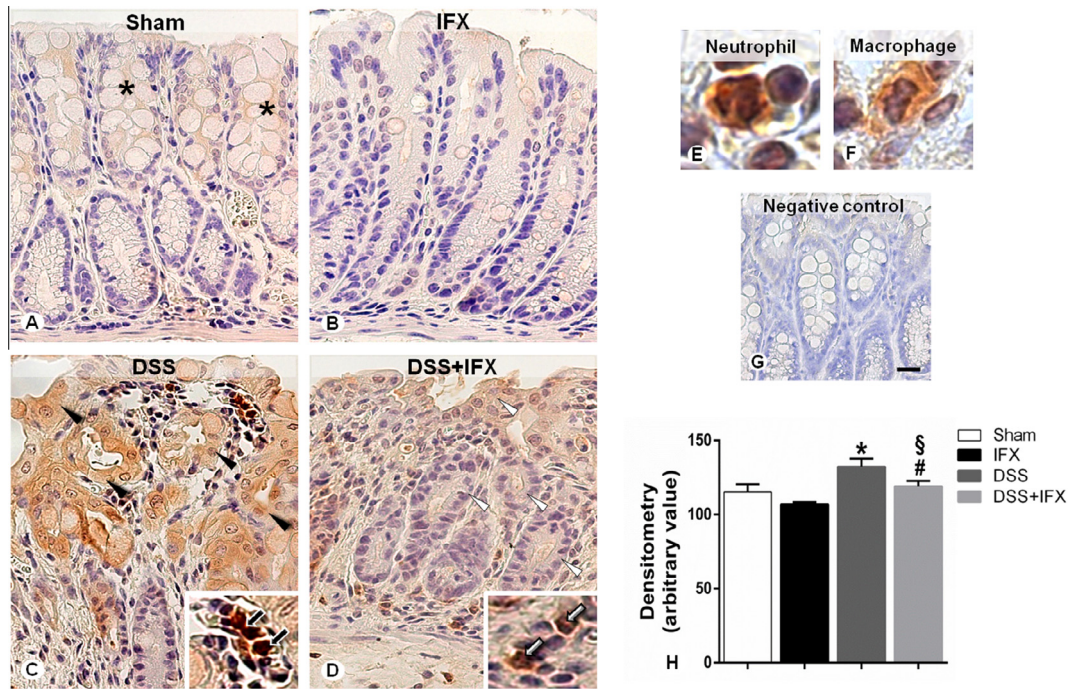


Fig. 5. Influence of colitis and IFX treatment on AnxA1 colonic expression. (A and B) AnxA1 expression on the secretory portion of mucosal glands (asterisks), (C) AnxA1 in the epithelial (black arrowheads) and inflammatory cells (black arrows) during colitis, (D) AnxA1 in the epithelial (white arrowheads) and inflammatory cells (white arrows) after colitis treatment, (E) AnxA1 immunoreactivity in neutrophil, (F) Macrophage positive to AnxA1, (G) Negative control, (H) Densitometry: AnxA1 after DSS and IFX treatment. Sections: 2 μ m. Bar: 20 μ m. Counterstain: Hematoxylin. $n = 6$ animals/group. * $p < 0.05$ vs Sham; # $p < 0.05$ vs IFX; § $p < 0.05$ vs DSS.

this supports tight junction integrity, such as in the blood–brain-barrier [28–30]. Furthermore, we speculate that the preservation of the epithelial layer by IFX is not related to the direct blockade of the pro-inflammatory actions of TNF- α on the epithelial cells, but instead is indirectly linked with the AnxA1 action in protecting the mucosal histoarchitecture [19,20].

DSS administration altered a large portion of intestine, which was observed by decreased length in samples collected from WT and *AnxA1*^{-/-} mice, and reduced weight of the intestine of the *AnxA1*^{-/-} mice. The IFX did not prevent the decrease of intestinal weight and length in both strains. Additionally, histological evaluation provided evidence of degradation of the collagen fibers in the WT/DSS group, but not in the *AnxA1*^{-/-}/DSS group. The degradation of the extracellular matrix components were demonstrated in intestinal explants from CD and UC patients, which presented augmented expression of MMPs-1, -2, -3 and -9 when compared to control patients [31], corroborating other *in vivo* studies [32,33]. The protein AnxA1 is also related to the upregulation of MMP-9 in breast cancer [34] and mediates the production of MMP-1 by TNF- α in rheumatoid arthritis [35]. In our study, the submucosal collagen was not damaged in the WT/DSS + IFX group when compared to the WT/DSS group. However, AnxA1 and IFX appear to influence tissue remodeling in inflammatory conditions [35,36] and in the culture of intestinal explants from patients with IBDs [31], further studies are needed to investigate this question.

The damage of the epithelial barrier in colitis allows the entrance of microbiota into the subjacent tissue, and the immune response occurs to control the action of potential pathogens [37]. Neutrophils and monocytes/macrophages are essential cells that support this process [38,39] by phagocytizing the pathogens, secreting pro-inflammatory cytokines, such as IL-6 and TNF- α , expressing metalloproteases and generating reactive oxygen species, which contributes to elimination of microorganisms in the tissue [26]. Therefore, phagocytes are also intrinsically related to the pathogenesis of IBDs, and recent data have shown that an

influx and actions of neutrophils and macrophages in the inflamed gut mucosal is required to provide an efficient host defense during colitis, nevertheless it may not be exacerbated [26,40–44]. Our data demonstrated enhanced neutrophil influx in DSS, corroborating previous data [45,46], and that it was approximately 30 times higher in the *AnxA1*^{-/-} mice than in WT mice. The higher neutrophil influx in *AnxA1*^{-/-} mice was expected, as AnxA1 is a potent inhibitor of neutrophil migration from blood into tissues during acute inflammation [47]. Based on recent data, showing that adequate neutrophil migration in colitis is required for the development and resolution of inflammation, we infer that AnxA1 also controls colitis by avoiding an exacerbated neutrophil accumulation in the gut mucosa. Moreover, the relevance of neutrophils in colitis is reinforced by the fact that IFX treatment reduced DSS effects in WT mice, but did not reduce neutrophil influx. This observation corroborates similar neutrophil influx in WT and tumor necrosis factor receptor 1 knockout mice (*TNFR1*^{-/-}) in DSS induced colitis [14].

Monocytes, neutrophils and macrophages secrete high amounts of IL-6 and TNF- α during inflammation [26,48]. Based on our data, the expected elevated levels of these cytokines in the explants of inflamed DSS-induced colitis samples were observed and comparable to those found in the literature [45,46,48–50]. Nevertheless, the amount of cytokines, specifically TNF- α , was highly secreted by explants collected from *AnxA1*^{-/-} mice (approximately 150-fold), which may indicate the higher amounts of infiltrating neutrophils and enhanced ability of both neutrophils and macrophages from *AnxA1*^{-/-} to secrete the cytokines [41]. TNF- α is related to the more accentuated occurrence of ulcers, expression of adhesion molecules and vascular permeability, leading to increased blood loss and leukocyte transmigration. Certainly, this set of alterations confirm the results obtained from *AnxA1*^{-/-} mice, in which the elevated secretion of TNF- α correlates with more extensive ulcers and significantly higher levels of MPO in comparison to WT mice.

The relevance of IL-6 in the activation of IBDs was previously described [51,52]. This cytokine is involved in protecting the host against microorganisms, by activating antigen presenting cells and T-cells and stimulating the production of other pro-inflammatory cytokines [26]. Further, we observed that the expression of IL-6 increased following DSS administration in WT and *AnxA1*^{-/-} mice. Additionally, the IL-6 levels were reduced following the TNF- α blockade in the WT mice, but not in the *AnxA1*^{-/-} mice. Alternative models of inflammation have already described and report that IL-6 is negatively regulated by the AnxA1 protein [53,54]. Moreover, this cytokine was reduced in patients with rheumatoid arthritis treated with inhibitors of TNF- α [55]. These arguments show that IL-6 is directly influenced by the blockade of TNF- α and modulated by the AnxA1 protein.

The healing of injured tissue is a complex process, and apoptosis of inflammatory cells is one pivotal mechanism to return the tissue integrity [56]. Our data show that inducing apoptosis of leukocytes is one mechanism of IFX treatment, as it markedly enhanced the percentage of caspase-3 positive leukocytes in WT inflamed tissue, which was much higher than that observed in *AnxA1*^{-/-} tissue. Indeed, the role of AnxA1 as a inducer of monocyte and neutrophil apoptosis, has been fully shown *in vitro* and *in vivo* as a mechanism of resolution of inflammation [57–59]. Therefore, endogenous AnxA1 mediates IFX induced apoptosis of inflammatory cells in DSS induced colitis.

Although the possible participation of AnxA1 on the protection of the intestinal mucosa in models of IBDs has been increasingly described [13,20,50], the modulation of this anti-inflammatory protein after pharmacological strategies, such as the IFX, remains unclear. Concerning the importance of understanding how the endogenous AnxA1 expression and roles are modulated by the intestinal inflammation and the anti-TNF- α intervention, we evaluated the expression of this protein on the colonic tissue.

Previous reports have shown that the levels of endogenous AnxA1 were significantly increased in the epithelial and glandular cells, as well as in immune cells transmigrated to the tissue, as showed in other inflammatory conditions [19,60]. Additionally, we observed that IFX reduced AnxA1 expression in animals exposed to DSS. These data support that the blockade of TNF- α was sufficient to control mucosal inflammation, indicated by the reduced expression of endogenous AnxA1 following anti-inflammatory intervention, and the association with the resolution in several models of inflammation [47,61].

In conclusion, the results indicate that the endogenous AnxA1 protein is involved in promoting morphological and physiological preservation on the colon during inflammatory conditions treated by anti-TNF- α biological therapy, constituting an important anti-inflammatory mediator, as well as a potential biomarker of therapeutic efficacy.

Competing interests

The authors declare no competing interests exist.

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