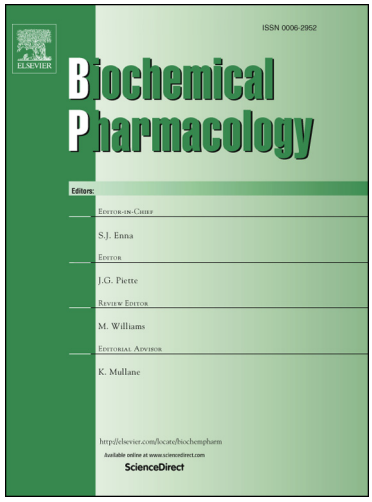


Role of the protein annexin A1 on the efficacy of anti-TNF treatment in a murine model of acute colitis

Marina de Paula-Silva, Bibiana Elisabeth Barrios, Lisa Macció Maretto, Angela Aparecida Sena, Sandra Helena Poliselli Farsky, Silvia Graciela Correa, Sonia Maria Oliani

PII: S0006-2952(16)30138-1
DOI: http://dx.doi.org/10.1016/j.bcp.2016.06.012
Reference: BCP 12572

To appear in: *Biochemical Pharmacology*

Received Date: 26 April 2016
Accepted Date: 17 June 2016

Please cite this article as: M.d. Paula-Silva, B.E. Barrios, L.M. Maretto, A.A. Sena, S.H.P. Farsky, S.G. Correa, S.M. Oliani, Role of the protein annexin A1 on the efficacy of anti-TNF treatment in a murine model of acute colitis, *Biochemical Pharmacology* (2016), doi: http://dx.doi.org/10.1016/j.bcp.2016.06.012

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

Marina de **Paula-Silva** [a¶]**;** Bibiana Elisabeth **Barrios** [b]**;** Lisa **Macció Maretto** [b]**;** Angela Aparecida **Sena** [c]**;** Sandra Helena Poliselli **Farsky** [d]; Silvia Graciela **Correa** [b]**;** Sonia Maria **Oliani** [a,c*]

[a] Post-graduation in Structural and Functional Biology, São Paulo Federal University (UNIFESP), São Paulo, São Paulo, Brazil

[b] Center of Investigation in Biochemistry and Clinical Immunology, Cordoba National University (UNC), Córdoba, Córdoba, Argentina

[c] Department of Biology, Laboratory of Immunomorphology, São Paulo State University (UNESP), São José do Rio Preto, São Paulo, Brazil.

[d] Department of Clinical and Toxicological Analyses, University of São Paulo (USP), São Paulo, São Paulo, Brazil

*Corresponding author at: Cristóvão Colombo Street, 2265, São José do Rio Preto, Brazil. Phone: +55 1732212381.

E-mail adress: smoliani@ibilce.unesp.br (S.M. Oliani).

Competing interests: the authors declare no competing interests existent.

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 sulphate 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.

Keywords: annexin A1, TNF-α, colitis, infliximab, therapy, biomarker.

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 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 hypersensibility [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-wks-old or 20-25g 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 Sulphate 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 (*Hemoccult 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-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 Pharmaceutics 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 10000 rpm, 4 ºC 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 ºC, three times. Finally, the supernatant was collected and 50 µL 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 $NaPO_4$. Results were expressed as the mean ± 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 hours, dehydrated in graded ethanol and embedded in paraffin for immunohistochemical and histopathological analysis.

Histopathological analysis and histological score: 2 µm sections were stained with hematoxylin & eosin and analyzed using a high-power objective (40X) 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 the mean ± S.E.M.

Quantification of collagen: The submucosal collagen was detected by the picrosirius-hematoxylin staining under polarized light. Five randomized fields per animal were photographed (40x).

Subsequently, 1 mm^2 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 the mean ± S.E.M. of the intact collagen fibers proportion.

2.6. Immunohistochemical detection of Annexin A1 and Caspase-3

Sections of the colon (2 µm) were incubated with sodium citrate buffer at 96 ºC 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 (40x) on an arbitrary scale from 0 to 255, and the data were expressed as the mean ± 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 63x). Results were expressed as the mean ± S.E.M. of the percentage of apoptotic phagocytes in mm^2.

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 the mean ± 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 one-way ANOVA (clinical analysis) or 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 Figures 1C-1F 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 1 G 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 (Figs 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 (Figs 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

Figure 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.

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

Furthermore, the concentrations of pro-inflammatory cytokines IL-6 and TNF-α were measured by ELISA assays (Figs 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 (Figs 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 (Figure 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 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 (Figure 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 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, 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 phagocyting 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 effecient 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 was 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]. Furhter, 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 mechanim 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, remain 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.

5. Acknowledgements

This work was supported by grants of Coordination for the Improvement of Higher Education Personnel (CAPES), National Council for Scientific and Technological Development (CNPq) and the São Paulo Research Foundation (FAPESP – Grant 2014/07328-4). Dextran Sulphate Sodium and the lyophilized REMICADE® were kind gifts of Dextran Products Limited (Canada) and Janssen-Cilag Pharmaceutics Ltda, respectively. We also thank PhD Ivanna Novotny Nuñes for all the technical support during the experiments.

6. References

[1] C. Abraham, R. Medzhitov, Interactions between the host innate immune system and microbes in inflammatory bowel disease, Gastroenterology. 140 (2011) 1729–1737. doi:10.1053/j.gastro.2011.02.012.

[2] V. Kumar, A.K. Abbas, N. Fausto, J.C. Aster, Fundamentos de Robbins & Cotran - Patologia: Bases Patológicas das Doenças, 8ª ed, Elsevier, Rio de Janeiro, 2010.

[3] B. Egger, M. Bajaj-Elliott, T.T. MacDonald, R. Inglin, V.E. Eysselein, M.W. Büchler, Characterisation of acute murine dextran sodium sulphate colitis: cytokine profile and dose dependency., Digestion. 62 (2000) 240–248. doi:10.1159/000007822.

[4] I.J. Fuss, W. Strober, The role of IL-13 and NK T cells in experimental and human ulcerative colitis., Mucosal Immunol. 1 Suppl 1 (2008) S31–S33. doi:10.1038/mi.2008.40.

[5] M. Dabek, L. Ferrier, R. Roka, K. Gecse, A. Annahazi, J. Moreau, et al., Luminal cathepsin g and protease-activated receptor 4: a duet involved in alterations of the colonic epithelial barrier in ulcerative colitis., Am. J. Pathol. 175 (2009) 207–214. doi:10.2353/ajpath.2009.080986.

[6] P. Parham, The Immune System, fourth edi, Garland Science, 2014.

[7] D.R. Clayburgh, M.W. Musch, M. Leitges, Y.X. Fu, J.R. Turner, Coordinated epithelial NHE3 inhibition and barrier dysfunction are required for TNF-mediated diarrhea in vivo, J. Clin. Invest. 116 (2006) 2682–2694. doi:10.1172/JCI29218.

[8] W.Y. Chey, Infliximab for patients with refractory ulcerative colitis., Inflamm. Bowel Dis. 7 (2001) 30–33. doi:10.1002/ibd.3780070507.

[9] N.. Khan, H.. Asim, G.. Lichtenstein, Safety of anti-TNF therapy in inflammatory bowel disease during pregnancy, Expert Opin. Drug Saf. 12 (2014) 1699–708.

[10] N. Thalayasingam, J.D. Isaacs, Anti-TNF therapy, Best Pract. Res. Clin. Rheumatol. 25 (2011) 549–567. doi:10.1016/j.berh.2011.10.004.

[11] M. Apostolaki, M. Armaka, P. Victoratos, G. Kollias, Cellular mechanisms of TNF function in models of inflammation and autoimmunity, Curr. Dir. Autoimmun. 11 (2010) 1–26. doi:10.1159/000289195.

[12] A. Uyanikoglu, F. Ermis, F. Akyuz, B. Pinarbasi, B. Baran, T. Aydogan, et al., Infliximab in inflammatory bowel disease: Attention to adverse events, Eur. Rev. Med. Pharmacol. Sci. 18 (2014) 2337–2342.

[13] A. Sena, I. Grishina, A. Thai, L. Goulart, M. Macal, A. Fenton, et al., Dysregulation of Anti-Inflammatory Annexin A1 Expression in Progressive Crohns Disease, PLoS One. 8 (2013) 1–13. doi:10.1371/journal.pone.0076969.

[14] A.A. Sena, L.P. Pedrotti, B.E. Barrios, H. Cejas, D. Balderramo, A. Diller, et al., Lack of TNFRI signaling enhances annexin A1 biological activity in intestinal inflammation, Biochem. Pharmacol. (2015) 1–10. doi:10.1016/j.bcp.2015.09.009.

[15] R.J. Flower, Lipocortin and the mechanism of action of the glucocorticoids, Br. J.

Pharmacol. 94 (1988) 987–1015.

[16] M. Perretti, F.N.E. Gavins, Annexin 1: an endogenous anti-inflammatory protein., News Physiol. Sci. 18 (2003) 60–64. doi:10.1152/nips.01424.2002.

[17] M. Perretti, R.J. Flower, Annexin 1 and the biology of the neutrophil., J. Leukoc. Biol. 76 (2004) 25–29. doi:10.1189/jlb.1103552.

[18] N. Ouyang, C. Zhu, D. Zhou, T. Nie, M.F. Go, R.J. Richards, et al., MC-12, an annexin A1-based peptide, is effective in the treatment of experimental colitis., PLoS One. 7 (2012) e41585. doi:10.1371/journal.pone.0041585.

[19] B.A. Babbin, M.G. Laukoetter, P. Nava, S. Koch, W.Y. Lee, C.T. Capaldo, et al., Annexin A1 regulates intestinal mucosal injury, inflammation, and repair., J. Immunol. 181 (2008) 5035–5044. doi:10.4049/jimmunol.181.7.5035.

[20] G. Leoni, A. Alam, P. Alexander Neumann, J.D. Lambeth, G. Cheng, J. McCoy, et al., Annexin A1, formyl peptide receptor, and NOX1 orchestrate epithelial repair, J. Clin. Invest. 123 (2013) 443–454. doi:10.1172/JCI65831.

[21] K. Geboes, R. Riddell, A. Ost, B. Jensfelt, T. Persson, R. Löfberg, A reproducible grading scale for histological assessment of inflammation in ulcerative colitis., Gut. 47 (2000) 404–9. doi:10.1136/gut.47.3.404.

[22] G. Roda, A. Caponi, M. Benevento, P. Nanni, L. Mezzanotte, A. Belluzzi, et al., New proteomic approaches for biomarker discovery in inflammatory bowel disease, Inflamm. Bowel Dis. 16 (2010) 1239–1246. doi:10.1002/ibd.21212.

[23] J.J. Kim, M.S. Shajib, M.M. Manocha, W.I. Khan, Investigating Intestinal Inflammation in DSS-induced Model of IBD, J. Vis. Exp. (2012) 1–6. doi:10.3791/3678.

[24] L.R. Lopetuso, V. Petito, V. Cufino, V. Arena, E. Stigliano, V. Gerardi, et al., Locally injected Infliximab ameliorates murine DSS colitis: Differences in serum and intestinal levels of drug between healthy and colitic mice, Dig. Liver Dis. 45 (2013) 1017–1021. doi:10.1016/j.dld.2013.06.007.

[25] L.W. Peterson, D. Artis, Intestinal epithelial cells: regulators of barrier function and immune homeostasis., Nat. Rev. Immunol. 14 (2014) 141–53. doi:10.1038/nri3608.

[26] M.F. Neurath, Cytokines in inflammatory bowel disease., Nat. Rev. Immunol. 14 (2014) 329–42. doi:10.1038/nri3661.

[27] K. Wang, G. Han, Y. Dou, Y. Wang, G. Liu, R. Wang, et al., Opposite Role of Tumor Necrosis Factor Receptors in Dextran Sulfate Sodium-Induced Colitis in Mice, 7 (2012) 1–

10. doi:10.1371/journal.pone.0052924.

[28] F.N.E. Gavins, E.L. Hughes, N.A.P.S. Buss, P.M. Holloway, S.J. Getting, J.C. Buckingham, Leukocyte recruitment in the brain in sepsis: Involvement of the annexin 1-FPR2/ALX anti-inflammatory system, FASEB J. 26 (2012) 4977–4989. doi:10.1096/fj.12-205971.

[29] E. Cristante, S. McArthur, C. Mauro, E. Maggioli, I.A. Romero, M. Wylezinska-Arridge, et al., Identification of an essential endogenous regulator of blood-brain barrier integrity, and its pathological and therapeutic implications., Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 832–41. doi:10.1073/pnas.1209362110.

[30] E. Maggioli, S. McArthur, C. Mauro, J. Kieswich, D.H.M. Kusters, C.P.M. Reutelingsperger, et al., Estrogen protects the blood–brain barrier from inflammation-induced disruption and increased lymphocyte trafficking, Brain. Behav. Immun. 51 (2016) 212–222. doi:10.1016/j.bbi.2015.08.020.

[31] M.J. Meijer, M.A.C. Mieremet-Ooms, W. Van Duijn, A.M. Van Der Zon, R. Hanemaaijer, J.H. Verheijen, et al., Effect of the anti-tumor necrosis factor-?? antibody infliximab on the ex vivo mucosal matrix metalloproteinase-proteolytic phenotype in inflammatory bowel disease, Inflamm. Bowel Dis. 13 (2007) 200–210. doi:10.1002/ibd.20051.

[32] B. von Lampe, Differential expression of matrix metalloproteinases and their tissue inhibitors in colon mucosa of patients with inflammatory bowel disease, Gut. 47 (2000) 63–73. doi:10.1136/gut.47.1.63.

[33] Q. Gao, M.J.W. Meijer, F.J.G.M. Kubben, C.F.M. Sier, L. Kruidenier, W. van Duijn, et al., Expression of matrix metalloproteinases-2 and -9 in intestinal tissue of patients with inflammatory bowel diseases, Dig. Liver Dis. 37 (2005) 584–592. doi:10.1016/j.dld.2005.02.011.

[34] H. Kang, J. Ko, S.W. Jang, The role of annexin A1 in expression of matrix metalloproteinase-9 and invasion of breast cancer cells, Biochem. Biophys. Res. Commun. 423 (2012) 188–194. doi:10.1016/j.bbrc.2012.05.114.

[35] C.E. Tagoe, N. Marjanovic, J.Y. Park, E.S. Chan, A.M. Abeles, M. Attur, et al., Annexin-1 mediates TNF-alpha-stimulated matrix metalloproteinase secretion from rheumatoid arthritis synovial fibroblasts., J. Immunol. 181 (2008) 2813–2820. doi:181/4/2813.

[36] I. Papaconstantinou, C. Zeglinas, M. Gazouli, K. Nastos, A. Yiallourou, P. Lykoudis, et al., Effect of infliximab on the healing of intestinal anastomosis: An experimental study in rats, Int. J. Surg. 12 (2014) 969–975. doi:10.1016/j.ijsu.2014.07.271.

[37] K.L. Wallace, L.-B. Zheng, Y. Kanazawa, D.Q. Shih, Immunopathology of inflammatory bowel disease., World J. Gastroenterol. 20 (2014) 6–21. doi:10.3748/wjg.v20.i1.6.

[38] M.C. Grimm, W.E. Pullman, G.M. Bennett, P.J. Sullivan, P. Pavli, W.F. Doe, Direct evidence of monocyte recruitment to inflammatory bowel disease mucosa., J. Gastroenterol. Hepatol. 10 (1995) 387–395.

[39] S.M. Farooq, A.W. Stadnyk, 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. 57 (2012) 1802–1812. doi:10.1007/s10620-012-2082-y.

[40] E.L. Campbell, S.P. Colgan, Neutrophils and inflammatory metabolism in antimicrobial functions of the mucosa, J. Leukoc. Biol. 98 (2015) 517–522. doi:10.1016/j.smim.2015.03.007.

[41] E.L. Campbell, W.J. Bruyninckx, C.J. Kelly, L.E. Glover, N. Eóin, B.E. Bowers, et al., Influence Resolution of Inflammation, 40 (2015) 66–77. doi:10.1016/j.immuni.2013.11.020.Transmigrating.

[42] C.L. Zindl, J.-F. Lai, Y.K. Lee, C.L. Maynard, S.N. Harbour, W. Ouyang, et al., IL-22-producing neutrophils contribute to antimicrobial defense and restitution of colonic epithelial integrity during colitis., Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 12768–73. doi:10.1073/pnas.1300318110.

[43] S.P. Colgan, Neutrophils and inflammatory resolution in the mucosa, Semin. Immunol. 27 (2015) 177–183. doi:10.1016/j.smim.2015.03.007.

[44] S. Meshkibaf, A.J. Martins, G.T. Henry, S.O. Kim, Protective role of G-CSF in dextran sulfate sodium-induced acute colitis through generating gut-homing macrophages, Cytokine. 78 (2016) 69–78. doi:10.1016/j.cyto.2015.11.025.

[45] M. Perse, A. Cerar, Dextran sodium sulphate colitis mouse model: Traps and tricks, J. Biomed. Biotechnol. 2012 (2012) 1–13. doi:10.1155/2012/718617.

[46] S. Melgar, A. Karlsson, E. Michaëlsson, Acute colitis induced by dextran sulfate sodium progresses to chronicity in C57BL/6 but not in BALB/c mice: correlation between symptoms and inflammation., Am. J. Physiol. Gastrointest. Liver Physiol. 288 (2005) G1328–G1338. doi:10.1152/ajpgi.00467.2004.

[47] B. Stuqui, M. de Paula-Silva, C.P. Carlos, A. Ullah, R.K. Arni, C.D. Gil, et al., Ac2-26 Mimetic Peptide of Annexin A1 Inhibits Local and Systemic Inflammatory Processes Induced by Bothrops moojeni Venom and the Lys-49 Phospholipase A2 in a Rat Model, PLoS One. 10 (2015) e0130803. doi:10.1371/journal.pone.0130803.

[48] Y. Naito, T. Takagi, O. Handa, T. Ishikawa, S. Nakagawa, T. Yamaguchi, et al., Enhanced intestinal inflammation induced by dextran sulfate sodium in tumor necrosis factor-alpha deficient mice., J. Gastroenterol. Hepatol. 18 (2003) 560–569. doi:10.1046/j.1440-1746.2003.03034.x.

[49] C.O. Elson, R.B. Sartor, G.S. Tennyson, R.H. Riddell, Experimental models of inflammatory bowel disease., Gastroenterology. 109 (1995) 1344–1367. doi:10.1016/0016-5085(95)90599-5.

[50] L. Vong, J.G.P. Ferraz, N. Dufton, R. Panaccione, P.L. Beck, P.M. Sherman, et al., Up-regulation of Annexin-A1 and lipoxin A4 in individuals with ulcerative colitis may promote mucosal homeostasis, PLoS One. 7 (2012) 1–9. doi:10.1371/journal.pone.0039244.

[51] B. Möller, P.M. Villiger, Inhibition of IL-1, IL-6, and TNF-alpha in immune-mediated inflammatory diseases., Springer Semin. Immunopathol. 27 (2006) 391–408. doi:10.1007/s00281-006-0012-9.

[52] M. Allocca, M. Jovani, G. Fiorino, S. Schreiber, S. Danese, Anti-IL-6 Treatment for Inflammatory Bowel Diseases: Next Cytokine, Next Target, Curr. Drug Targets. 14 (2013) 1508–1521.

[53] Y.H. Yang, M.-L. Toh, C.D. Clyne, M. Leech, D. Aeberli, J. Xue, et al., Annexin 1 negatively regulates IL-6 expression via effects on p38 MAPK and MAPK phosphatase-1., J. Immunol. 177 (2006) 8148–8153. doi:10.4049/jimmunol.177.11.8148.

[54] Y.H. Yang, D. Aeberli, A. Dacumos, J.R. Xue, E.F. Morand, Annexin-1 regulates macrophage IL-6 and TNF via glucocorticoid-induced leucine zipper., J. Immunol. 183 (2009) 1435–1445. doi:10.4049/jimmunol.0804000.

[55] S.-N.J. Song, M. Iwahashi, N. Tomosugi, K. Uno, J. Yamana, S. Yamana, et al., Comparative evaluation of the effects of treatment with tocilizumab and TNF-α inhibitors on serum hepcidin, anemia response and disease activity in rheumatoid arthritis patients., Arthritis Res. Ther. 15 (2013) R141. doi:10.1186/ar4323.

[56] C. Ward, I. Dransfield, E.R. Chilvers, C. Haslett, A.G. Rossi, Pharmacological manipulation of granulocyte apoptosis: Potential therapeutic targets, Trends Pharmacol. Sci. 20 (1999) 503–509. doi:10.1016/S0165-6147(99)01391-7.

[57] E. Solito, A. Kamal, F. Russo-Marie, J.C. Buckingham, S. Marullo, M. Perretti, A novel calcium-dependent proapoptotic effect of annexin 1 on human neutrophils., FASEB J. 17 (2003) 1544–1546. doi:10.1096/fj.02-0941fje.

[58] E. Solito, C. de Coupade, S. Canaider, N.J. Goulding, M. Perretti, Transfection of annexin 1 in monocytic cells produces a high degree of spontaneous and stimulated apoptosis associated with caspase-3 activation., Br. J. Pharmacol. 133 (2001) 217–28. doi:10.1038/sj.bjp.0704054.

[59] J.P. Vago, C.R.C. Nogueira, L.P. Tavares, F.M. Soriani, F. Lopes, R.C. Russo, et al., Annexin A1 modulates natural and glucocorticoid-induced resolution of inflammation by

enhancing neutrophil apoptosis, J. Leukoc. Biol. 92 (2012) 249–258. doi:10.1189/jlb.0112008.

[60] C. Coméra, P. Brousset, J. Moré, N. Vergnolle, L. Buéno, Inflammatory neutrophils secrete annexin 1 during experimentally induced colitis in rats, Dig. Dis. Sci. 44 (1999) 1448–1457. doi:10.1023/A:1026668206020.

[61] F.N. Facio, A.A. Sena, L.P. Araújo, G.E. Mendes, I. Castro, M. a M. Luz, et al., Annexin 1 mimetic peptide protects against renal ischemia/reperfusion injury in rats, J. Mol. Med. 89 (2010) 51–63. doi:10.1007/s00109-010-0684-4.

7. Figure legends

Figure 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.

Figure 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 µm. Bar: 20 µm. 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).

Figure 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

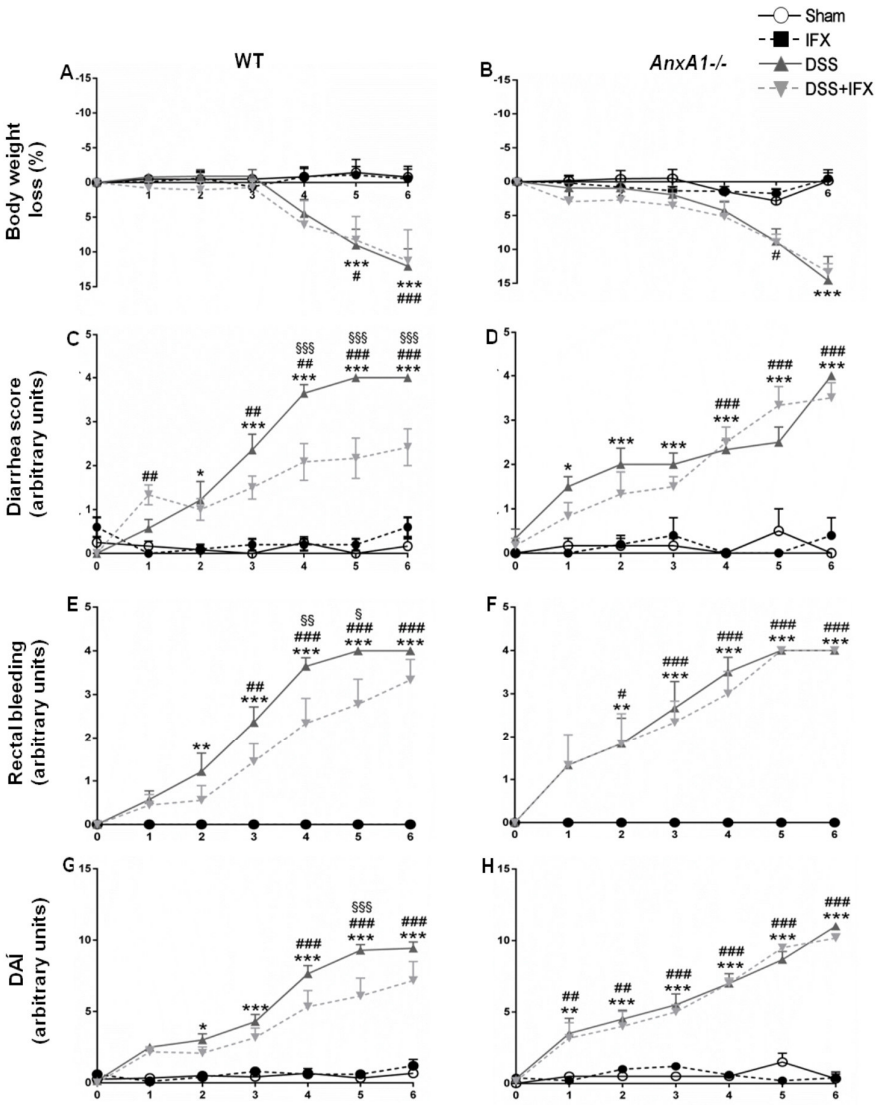
in *AnxA1*$^{-/-}$ mice. Ulcer (black arrows). Dysplasic crypts (white arrows). Vacuolar hydropic degeneration (asterisks). Mucosal inflammatory infiltrate (details: C, G and H). Inclusion: paraffin. Staining: Hematoxylin-Eosin. Sections: 2 µm. Bar: 20 µm. (I) Histological score. n = 6 animals/group. *** $p < 0.001$ vs Sham; ## $p < 0.01$, ### $p < 0.001$ vs IFX; §§ $p < 0.01$ vs DSS.

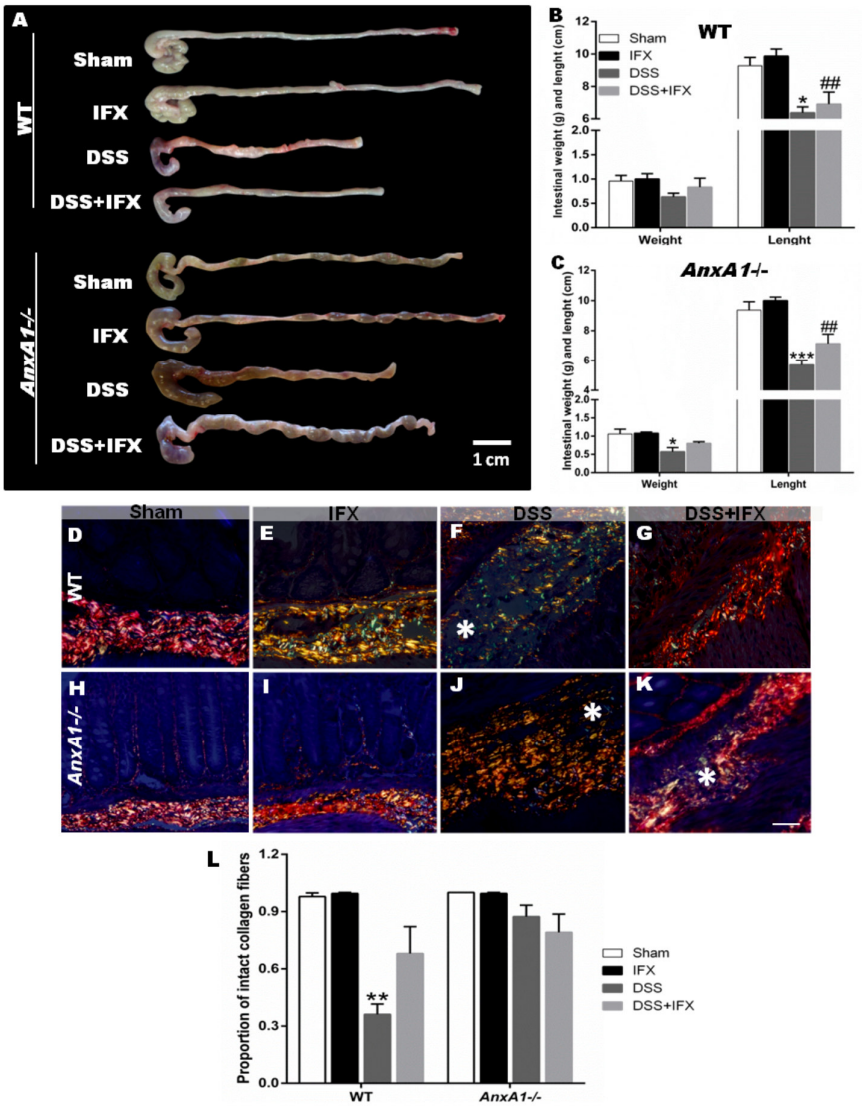
Figure 4. Expression of MPO, IL-6, TNF-α and apoptosis of phagocytes on colonic tissue and effects of treatment with anti-TNF-α.

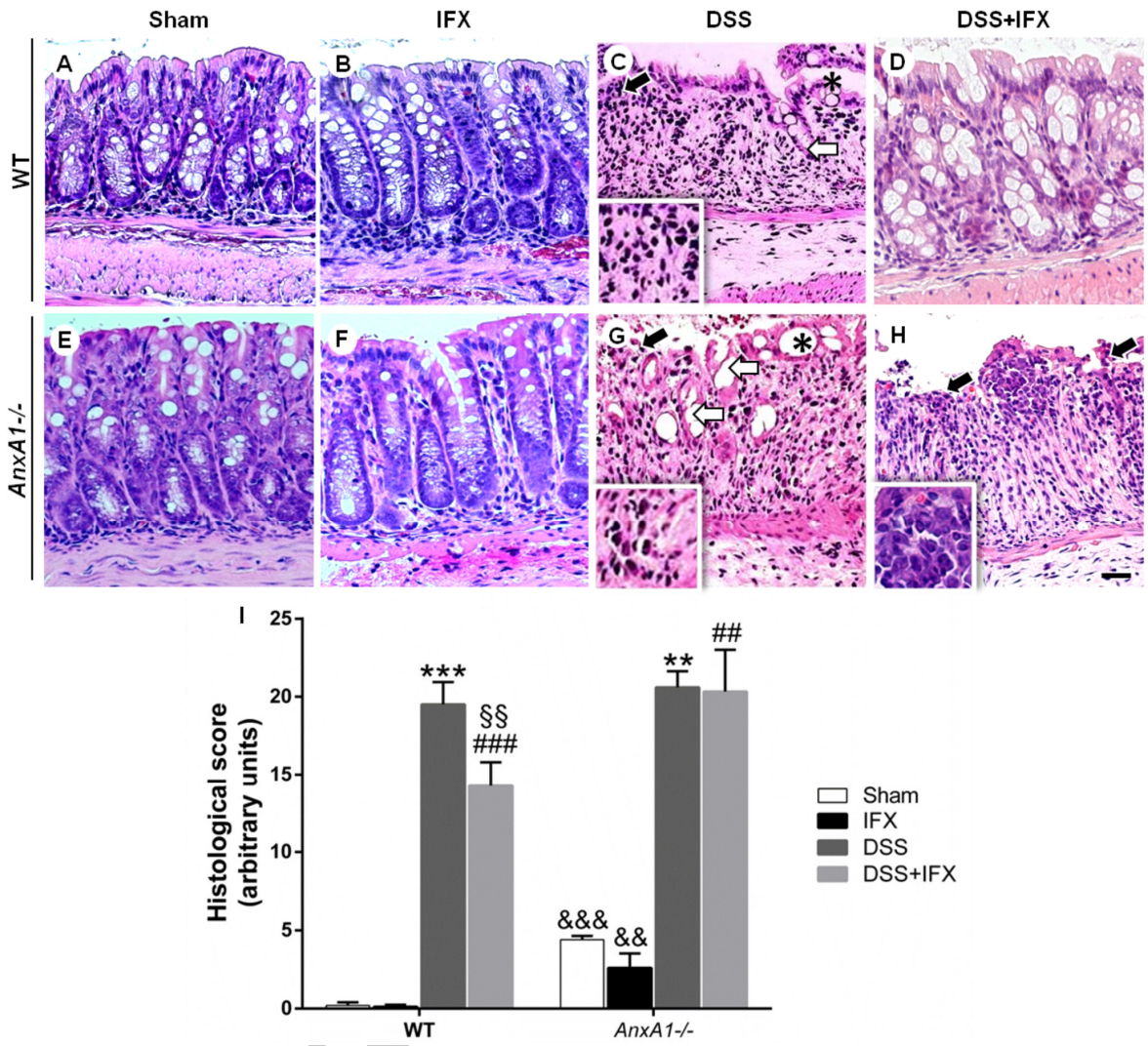
(A and B) MPO activity, (C-F) IL-6 and TNF-α secretions, (G) Proportion of apoptotic phagocytes. n=6 animals/group. Mean ± S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs Sham; # $p < 0.05$, ## $p < 0.01$ vs IFX; && $p < 0.01$, &&& $p < 0.001$ vs respective WT.

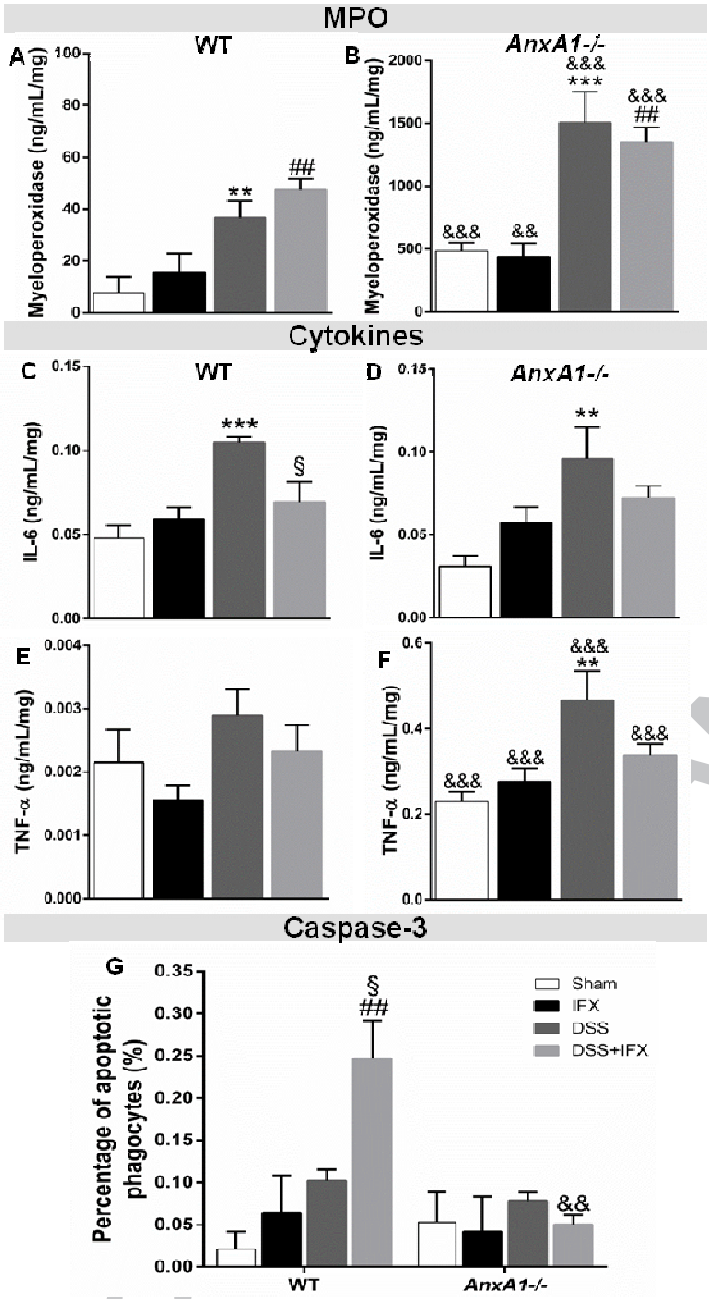
Figure 5. Influence of colitis and IFX treatment on AnxA1 colonic expression

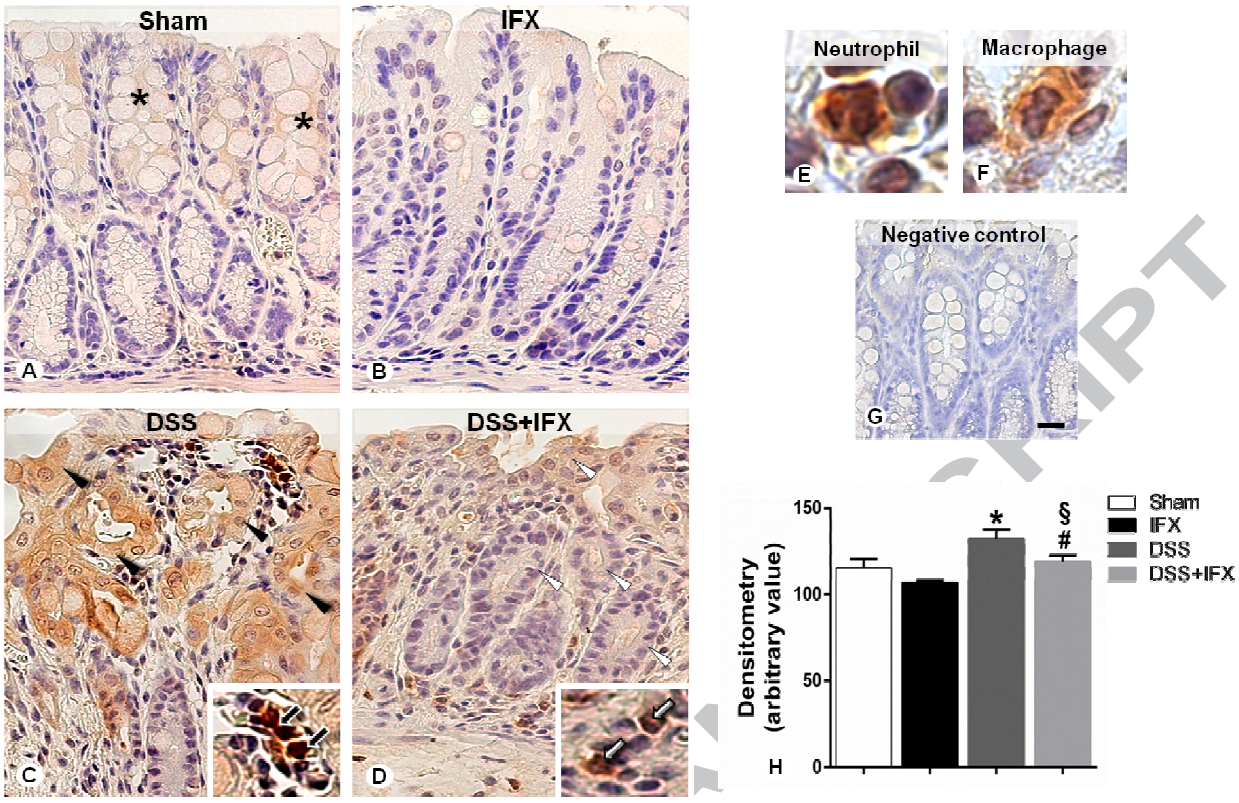
(A and B) AnxA1 expression on the secretory portion of mucosal glands (asterisks), (C) AnxA1 in the epithelial (black arrowheads) and inflammatory cells (black arrows) during colitis, (D) AnxA1 in the epithelial (white arrowheads) and inflammatory cells (white arrows) after colitis treatment, (E) AnxA1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

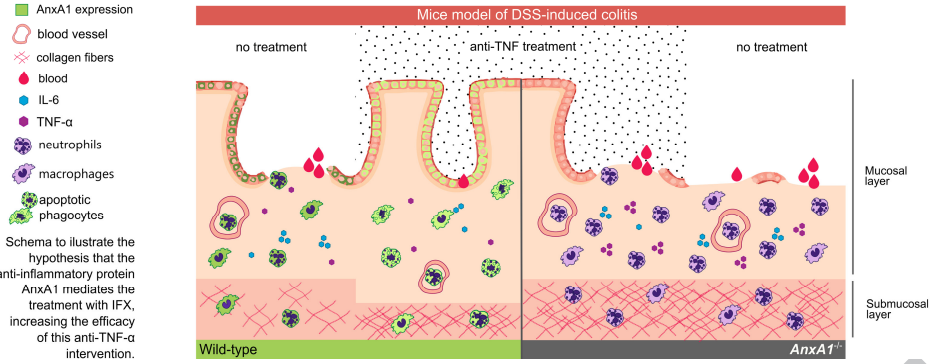












Schema to illustrate the hypothesis that the anti-inflammatory protein AnxA1 mediates the treatment with IFX, increasing the efficacy of this anti-TNF-α intervention.