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Preventive effect of Cardiotrophin-1 administration before DSS-induced ulcerative colitis in mice.

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ABSTRACT

Ulcerative colitis (UC) is a relatively frequent, chronic disease that impacts significantly the patient's quality of life. Although many therapeutic options are available, additional approaches are needed because many patients either do not respond to current therapies or show significant side effects. Cardiotrophin-1 (CT-1) is a cytokine with potent cytoprotective, anti-inflammatory, and antiapoptotic properties. The purpose of this study was to assess if the administration of CT-1 could reduce colon damage in mice with experimental UC.

UC was induced with 5% dextran sulfate sodium (DSS) in the drinking water. Some mice received i.v. dose of CT-1 (200 µg/kg) 2 hours before and 2 and 4 days after DSS administration. Animals were followed during 7 days after DSS. The severity of UC was measured by standard scores. Colon damage was assessed by histology and immunohistochemistry. Inflammatory mediators were measured by Western blot and PCR.

CT-1 administration to DSS-treated mice ameliorated both the clinical course (disease activity index), histological damage, inflammation (colon expression of TNF-α, IL-17, IL-10, INF-γ, and iNOS), and apoptosis.

Our results suggest that CT-1 administration before UC induction improves the clinical course, tissue damage and inflammation degree in DSS-induced UC in mice.

Keywords : apoptosis; cardiotrophin-1; colon; inflammation

Running head: Cardiotrophin-1 in ulcerative colitis.

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Introduction

Ulcerative colitis (UC) is a chronic, inflammatory bowel disease that disrupts colon structure and function. UC affects mainly young patients and is characterized by alternation of acute and remission phases. UC is associated to a higher incidence of dysplasia and colorectal cancer, and can impact significantly the patients' quality of life and working ability [1,2]. UC is most prevalent in northern Europe, especially in the United Kingdom and Scandinavia [3,4] and North America [5], whereas Asia is also expected to reach highest incidence over the next decade [6]. UC affects the colonic mucosa and submucosa with widespread superficial ulceration, destruction of epithelial architecture and integrity, crypt loss, submucosal edema, cellular infiltration and intense inflammation [7]. Although, the precise mechanisms are not yet fully understood, several mediators, including chemotactic peptides and pro-inflammatory cytokines are known to be involved [8].

Current therapies for UC include many different agents targeting different pathways involved in the disease induction. These agents comprise 5-aminosalicylic acid (5-ASA) precursors, such as Sulfasalazine, Mesalamine, and Budesonide, systemic (oral or intravenous) corticosteroids, thiopurines such Azathioprine and 6-mercaptopurine with steroid-sparing effects, anti-TNF-α antibodies such as Infliximab, Adalimumab, and Golimumab, calcineurin inhibitors such as cyclosporine, anti-integrin antibodies such as Vedolizumab, and Janus kinase inhibitors such as Tofacitinib and other similar molecules, as well as many different combinations of the above mentioned drugs. All these therapeutic approaches, their clinical use and results and their side effects have been evaluated in several recent reviews [9,10,11,] as well as briefly discussed in the Discussion section of this manuscript. All these reviews suggest that additional treatments focusing different targets are necessary due to the serious limitations of available options.

In the present study, we have assessed the utility of cardiotrophin-1 (CT-1), in an experimental model of dextran sodium sulphate (DSS)-induced UC in mice. CT-1 is a member of the interleukin 6 (IL-6) family of cytokines, also including many other cytokines [12]. CT-1 binds to and signals through a specific

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membrane receptor complex containing glycoprotein 130 (gp130) and leukemia inhibitory factor receptor (LIFR) [13-14]. CT-1 is expressed in a wide number of cell types and tissues and exerts potent anti-apoptotic effects on hepatocytes [15], cardiomyocytes [16] and neurons [17], and protective and anti-inflammatory effects in damaged heart [18,19], liver [20, 21], kidneys [22,23] and nervous system [17]. Recently we have reported that exogenous CT-1 attenuates experimental UC in mice, when given after the insult, thus suggesting a potential application of this substance in the treatment of ongoing relapses [24]. We now show that CT-1 is also able to partially prevent the development of UC when administered before the insult, thus suggesting a potential prophylactic application.

Materials and Methods

Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich (Madrid, Spain).

Animals

25 eight-week-old male mice purchased from Charles River Spain (Barcelona, Spain) were used. Only male littermates were used in order to obviate the possible interference of estrus. Animals were group-housed in a temperature and humidity controlled room, with a 12-hour dark/12-hour light cycle (Experimental Animal Facility, University of Salamanca), and allowed free access to standard chow and tap water.

Ethical considerations

All protocols were approved by the Animal Experimentation Ethics Committee of the University of Salamanca and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, National Academy of Science, USA, Declaration of Helsinki Principles on the Advice on Care and Use of Animals referred to in: law 14\/2 007 (3 July) on Biomedical Research, Conseil de l´Europe (published in Official Daily N. L358/1-358/6, 18-12-1986), Government Spanish (Royal Decree 223/1 988, (14 March) and Order of 13-10-1989, and Official Bulletin of the State b. 256, pp. 31349-31362, 28-10-1990).

Experimental model and study protocol

DSS administration is a most widely used animal model for the study of UC, for its simplicity, reproducibility and uniformity [25]. The DSS model exhibits many symptoms similar to those seen in human UC, such as diarrhea, bloody feces, body weight loss, mucosal ulceration, and shortening of the colorectum [26].

C57BL/6J mice were randomly distributed into three groups:

1. Colitis group (DSS; n = 10): Colitis was induced with 5% DSS (MW 36,000-50,000, MP Biomedical, Solon, OH, USA) in the drinking water through the experiment.41

2. Colitis + CT-1 treatment group (DSS + CT-1; n = 10): Mice received an i.v. dose of CT-1 (200 µg/kg) 2 hours before and 2 and 4 days after starting DSS.

3. Control group (Sham; n=5): Mice received neither DSS nor CT-1.

Animals were followed during 7 days after starting DSS.

To assess the effects of DSS, potential behavioral alterations and changes in body weight were monitored daily. Mice that showed extreme distress, became moribund, or lost more than 20% of initial body weight were euthanized. At the end of the study, mice were anesthetized with 60 mg/kg pentobarbital and blood was obtained by heart puncture. Then, animals were perfused through the cardiac puncture with heparin containing (5IU/mL) isotonic saline and the colon was dissected and cleaned. Colons were immediately trimmed in different pieces and some of them were fixed in buffered 4% formaldehyde for 24 hours for histological studies, whereas other samples were frozen in liquid nitrogen for other measurements.

Colitis severity quantification

Colitis severity was quantified using the disease activity index (DAI) that resulted from scoring body weight loss, stool consistency and presence or absence of fecal blood as described previously [27].

Histological studies

Fixed colon pieces were dehydrated in an ascending series of ethanol concentrations, cleared in xylene and embedded in paraffin. 3 µm colon sections were stained with hematoxylin and eosin for light microscopy analysis. Additional 3 µm sections were processed for immunohistochemistry as previously reported [24]. In brief, sections were deparaffined in xylene and rehydrated in graded ethanol concentrations. Endogenous peroxidase was blocked with 3% hydrogen peroxide, followed by primary antibody incubation. Primary antibodies used are described in Table 1.

Then, sections were washed three times in PBS and incubated with the Novolink Polymer Detection ® (RE7140-K, Novocastra, MA, USA), followed by reaction with 3,3´diaminobenzidine as chromogen. Ten images per slide were captured with an optical microscope. Then, images were digitalized and the number of cells stained by the antibodies was quantified by using ImageJ software (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, MD, USA).

Western Blot (WB) studies

WB studies with performed with tissue extracts from frozen colon samples, as previously described [28]. Tissue extracts were obtained by homogenizing colon samples with a tissue mixer (Ultra-Turrax T8, IKA®-Werke GmbH & Co. Staufen, Germany) at 4 ºC in homogenization buffer (140 mM NaCl, 20 mM Tris-HCl pH=7.5, 0.5 M ethylenediaminetetraacetic acid –EDTA-, 10% glycerol, 1% Igepal CA-630, 1 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL pepstatin A, 1 mM phenylmethylsulphonyl fluoride). Samples were separated by electrophoresis in 10-15% acrylamide gels (Mini Protean II system, BioRad, Madrid, Spain). Immediately, proteins were electrically transferred to an Immobilon-P membrane (Millipore, Billerica, MA, USA). Primary and secondary antibodies used are described in Table 1. WB quantification was performed with image quant software (GE-Healthcare, Madrid, Spain) after scanning the films on an Office jet 8500 scanner (Hewlett-Packard, Madrid, Spain).

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Table 1

Antibodies used for Western Blot and immunohistochemistry studies

Western Blot

Primary Antibody	Catalog number	Supplier
iNOS	#2977,	Cell Signaling Technology, Inc.,
CT-1	MAB438,	R&D Systems,
NFkB	#3034,	Cell Signaling Technology, Inc.,
pNFkB	#3031,	Cell Signaling Technology, Inc.,
STAT3	#9132,	Cell Signaling Technology, Inc.,
pSTAT3	#9145,	Cell Signaling Technology,
Cleaved caspase 3	#9661,	Cell Signaling Technology, Inc.,
Secondary Antibody	Catalog number	Supplier
Anti-rabbit IgG-HRP	4052-05	Southern Biotech.,
Anti-IgG rat IgG-HRP	sc-2006	Santa Cruz Biotechnology,
Anti-mouse IgG-HRP	1034-05	Southern Biotech.,

Immunohistochemistry

CD68	M0814	Dako Diagnósticos, Spain
iNOS	sc-651	Santa Cruz Biotechnology
Cleaved Caspase 3	#9661	Cell Signaling Technology

Suppliers

Cell Signaling Technology, Inc., Danvers, Massachusetts, USA

R&D Systems, Minneapolis, Minnesota, USA

Santa Cruz Biotechnology, CA, USA.

Southern Biotech., Birmingham, USA

Dako Diagnósticos, Barcelona, Spain

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Reverse transcription-Polymerase Chain Reaction (RT-PCR) studies

Total RNA was isolated from frozen colon samples using Nucleospin RNAII (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. Single-strand cDNA was generated from 2 µg of total RNA using poly-dT as primer with M-MLV reverse transcriptase (Promega Biotech Ibérica, Alcobendas, Spain). For RT-PCR, 1 µL of cDNA was used in a standard 50-µL PCR mixture with 400 nM of each primer and 2 U of FastStart Taq DNA polymerase Roche (Sigma Aldrich). PCR products were separated by electrophoresis on a 1% agarose gel and visualized by SybrSafe (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) staining.

Quantitative RT-PCR was performed in triplicate. Each 20 µL reaction contained 1 µL of cDNA, 400 nM of each primer, and 1x iQ SybrGreen Supermix (Bio-Rad, Madrid, Spain). Standard curves were run for each transcript to ensure exponential amplification and to rule out non-specific amplification. Gene expression was normalized to GAPDH expression. The reactions were studied with an iQ5 Real-time PCR detection system (Bio-Rad, Madrid, Spain). The specific primers used for PCR are shown in Table 2.

Table 2.

Sequences of the primers used and conditions of PCR studies

Gen	*Primer*	Secuencia (5'-3')	Amplicon (pb)	Tm (ºC)
TNF-α	*Fw*	AGCACAGAAAGCATGATCCG	212	60
	Rv	CTGATGAGAGGGAGGCCATT		
IFN-γ	*Fw*	TACACACTGCATCTTGGCTTTG	128	57,5
	Rv	CTTCCACATCTATGCCACTTGAG		
IL-10	*Fw*	ATGCTGCCTGCTCTTACTGACTG	216	58.8
	Rv	CCCAAGTAACCCTTAAAGTCCTGC		
IL-17	*Fw*	GCTCCAGAAGGCCCTCAGA	142	58.8
	Rv	AGCTTTCCCTCCGCATTGA		

GAPDH	*Fw*	GTCGGTGTGAACGGATTTG	153	55,9
	Rv	GAATTTGCCGTGAGTGGAGT		

Measurement of plasma levels of cytokines

Plasma levels of CT-1 (ELM-CT-1 RayBiotech, Norcross, GA, USA) , INF-γ, RYD-MIF00; R&D Systems, Minneapolis, MN, USA) and TNF-α (MTA00B, R&D Systems) were measured using commercial ELISAS, according to the manufacturers´ instructions.

Data statistical analysis

The area under the curve (AUC) was calculated in each animal by the addition of the areas of the contiguous trapezes. Statistical analysis was performed using the NCSS software. Normally distributed data were expressed as mean ± SEM. Differences between groups were assessed using one-way or two-way analysis of variance (ANOVA), as appropriate. Scheffe's post-hoc test was used for multiple comparisons. For data not conforming to a normal distribution, values were expressed as median and the Kruskal-Wallis Z value test was used for multiple comparisons. $P<0.05$ or $Z>1.96$ were considered statistically significant.

Results

Evolution of colitis

No animal died during the study. In the DSS group there was a manifest decrease in motility and loss of responses to stimuli, compared with sham animals. In the DSS+CT-1 group, these signs were much less pronounced. No significant differences in weight were observed (data not shown). Stool consistency score increased progressively in the DSS group during the observation period, and CT-1 partly prevented this increment (Figure 1A). The area under the curve (AUC) for stool consistency was 0 for sham animals, 4.51

± 0.60 arbitrary units (AU) in DSS-treated animals and 2.10 ± 0.33 AU in DSS+ CT-1 mice (Z>1.96). Fecal blood score also increased progressively in the DSS group; whereas in the animals that received DSS + CT-1, it was lower at all time points (data not shown). Disease activity index (DAI) was always lower in CT-1-treated than in untreated mice (Figure 1B). The area under the curve (AUC) for DAI was 1.51 ± 0.3 for sham animals, 5.61 ± 0.61 arbitrary units (AU) in DSS-treated animals and 3.94 ± 0.33 AU in DSS+ CT-1 mice (Z>1.96).

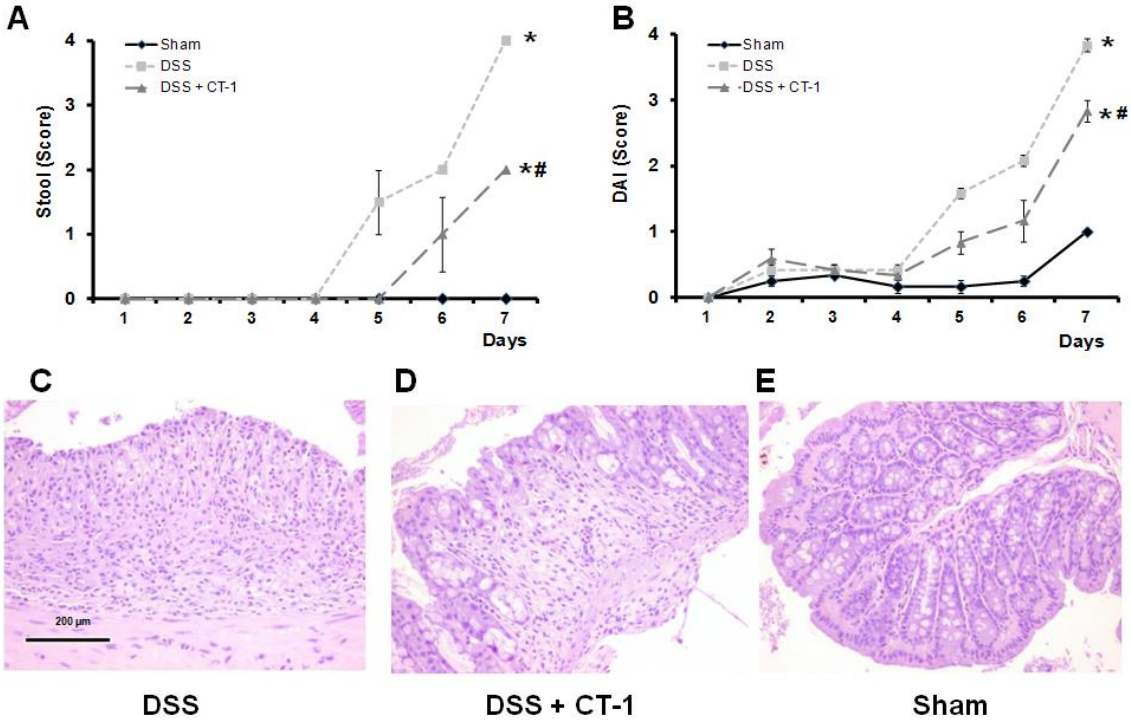

Figure 1

Figure 1. Effect of CT-1 on clinical and histological damage in DSS-induced colitis in mice. (A) Stool Consistency Score. (0: normal feces; 4: massive hemorrhage). (B) Disease activity index (DAI). (C, D, E) Representative histology images of colonic damage. Sections (4 µm) were stained with hematoxylin-eosin. Bar: 200 µm. Values are expressed as mean ± SEM (panels A and B: Sham, n=4; DSS, n=8; DSS+CT-1, n=8.). *: $p<0.05$ vs. Sham group; #: $p<0.05$ vs. DSS group.

Histological characterization

Hematoxylin-eosin staining of colon specimens revealed that mice treated with DSS showed typical alterations of ulcerative colitis. Ulcers were focally distributed through the inner surface of the colon and invaded the entire thickness of the colon wall, even penetrating the lamina propria. In ulcerated areas, total destruction of the crypts was seen with disappearance of the epithelium, including goblet cells. Granulation tissue and a large transmural inflammatory infiltrate occupied the ulcerated zones (Figure 1 C). Peripheral tissue surrounding colonic ulcers had normal structure, without alterations. In mice treated with DSS plus CT-1, even though ulcers were observed, mainly in the distal colon, these were fewer than those found in the control group and their size was also smaller. In many areas, although an infiltration of inflammatory cells was occasionally detected, the epithelium of the colon mucosa appeared fairly well preserved, including goblet cells and most of the colon had no structural alterations (Figure 1D). Hematoxylin-eosin-stained, proximal colon sections from the sham group showed intact epithelium, well defined crypt length, absence of edema or infiltrating cells in the mucosa and submucosa, and no ulcers or erosions (Figure 1E).

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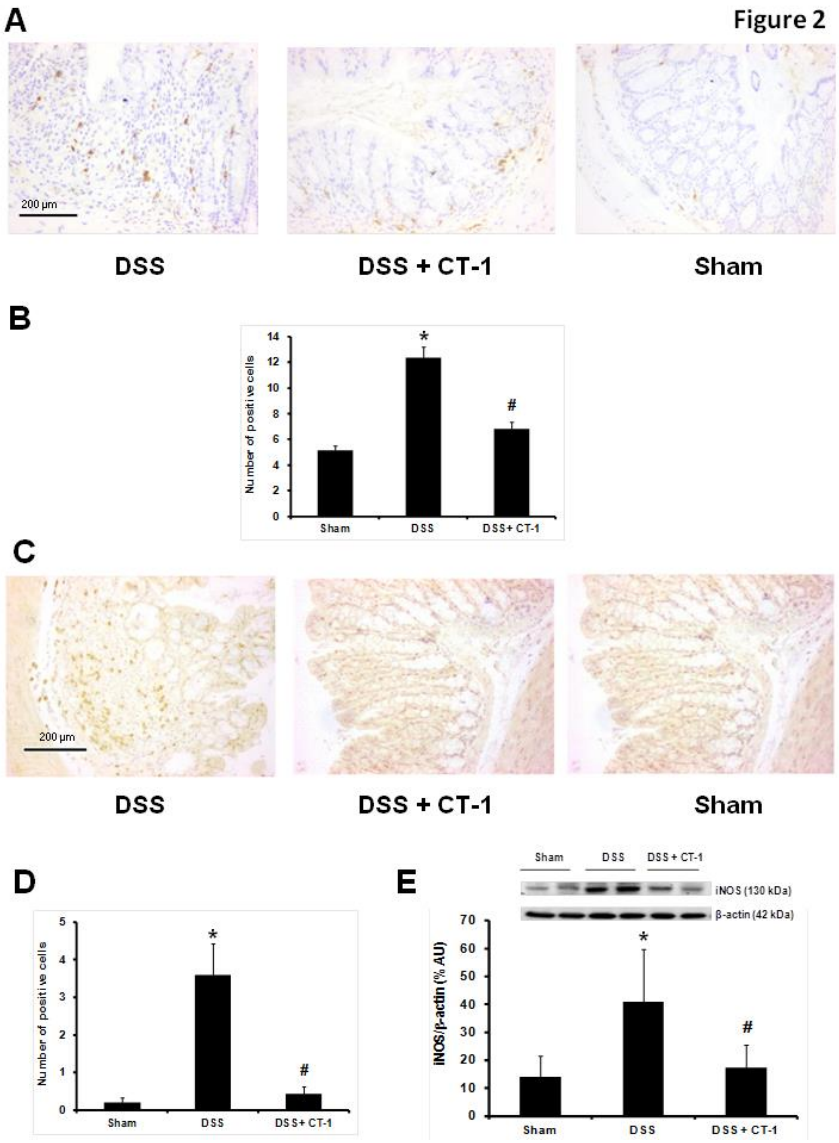

Figure 2. Effect of CT-1 on colonic inflammation in DSS-induced colitis in mice. (A) Representative images of CD-68 staining in colon. Bar: 200 µm. (B) Number of CD-68 positive cells. (C) Representative images of iNOS staining in colon. Bar: 200 µm. (D) Number of cells positive for iNOS staining. (E) Western blot analysis of iNOS and β-actin levels in colon tissue homogenates, expressed as % arbitrary units (% AU). Values are expressed as mean ± SEM. The number of slides quantified per group in panels A to D was as follows: Sham, n=40; DSS, n=60; DSS+CT-1, n=60. In panel E: Sham, n=4; DSS, n=5; DSS+CT-1, n=5. *: Z>1.96 vs. Sham group; #: Z>1.96 vs. DSS group.

Evaluation of colonic inflammation

Immunohistochemistry for CD-68 was performed to assess monocyte/ macrophage infiltration into the mucosa. A high number of CD-68 positive cells were observed in DSS-treated mice, whereas in the animals that received also CT-1, the number of CD-68 positive cells was markedly lower (Figure 2A, B).

Immunohistochemistry for inducible nitric oxide synthase (iNOS), an enzyme associated to inflammation, revealed that cells expressing iNOS were very scarce in the colonic tissue of animals from the sham group, but very abundant in the animals receiving DSS alone (Figure 2 C). The number of these cells was significantly lower in the DSS+CT-1 group than in DSS group (figure 2D). Western blot studies also demonstrated that iNOS levels in animals that received DSS alone were more than 5 times higher than those of sham animals. And that CT-1 reduced iNOS significantly to levels similar to those in sham animals (figure 2E).

Inflammation was also assessed by plasma levels and by colonic gene expression of several cytokines. Plasma TNF-α levels were much higher in the DSS group than in the sham group. Animals that received also CT-1 had plasma TNF-α levels significantly lower than those in the DSS group, and similar to those of the sham group (Figure 3A). Colonic TNF-α gene expression (PCR) was significantly higher in the DSS than in sham group, but without statistically significant differences with respect to animals that also received CT-1 (Figure 3B, C). Gene expression of IL-17 in colonic tissue was significantly higher in mice treated with DSS than in sham animals. In mice pre-treated with CT-1, colon IL-17 gene expression was significantly lower than in animals treated with DSS alone, and not significantly different from those in sham animals (Figure 3B, D). Gene expression of INF-γ in colonic tissue was significantly higher in mice treated with DSS than in sham animals. In mice that also received CT-1 in addition to DSS, gene expression of INF-γ in colon was not significantly different from that in sham animals (Figure 3B, E). Similar results were obtained for IL-10 expression (Figure 3B, F).

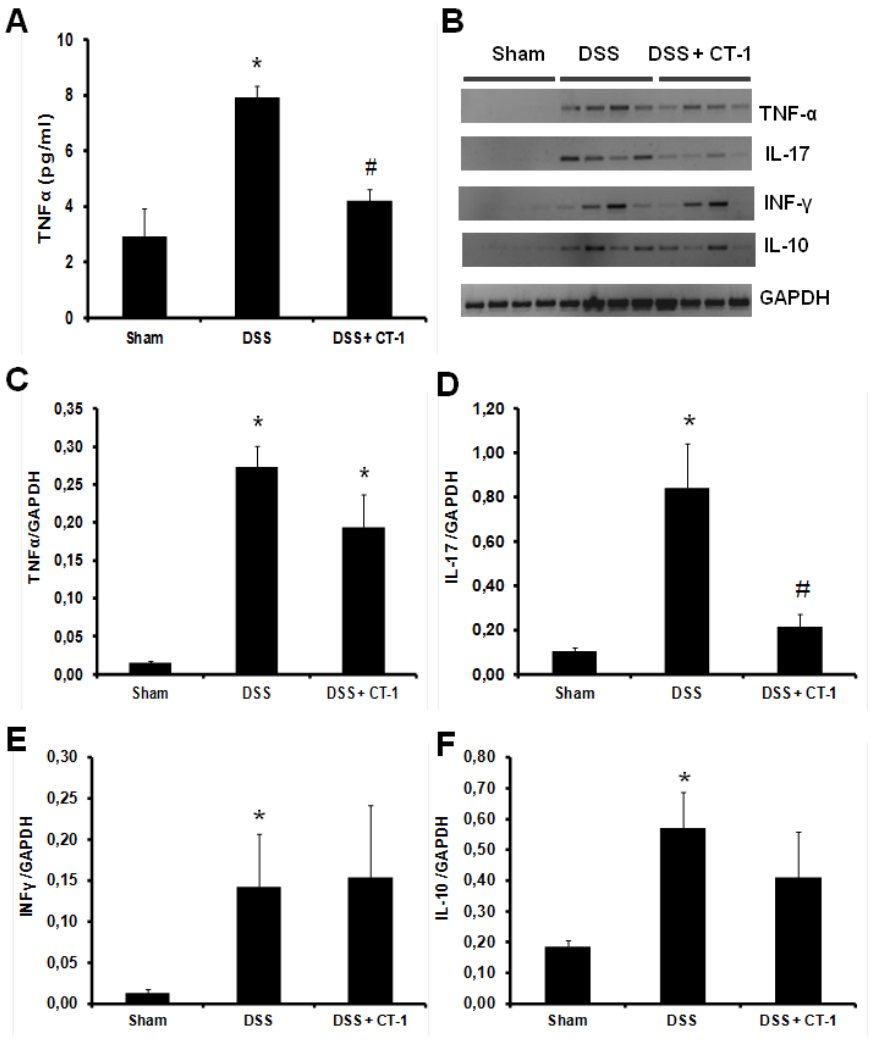

Figure 3

Figure 3. Effect of CT-1 on colon cytokine production in DSS-induced colitis in mice. (A) Plasma TNF-α levels expressed as pg/mL. (B) PCR analysis of TNF-α, IL-17, INF-γ, IL-10 and GAPDH levels in colon tissue homogenates. (C) TNF-α/GAPDH quantification. (D) IL-17/GAPDH quantification. (E) INF-γ/GAPDH quantification. (F) IL-10/GAPDH quantification. Values are expressed as mean ± SEM (Sham, n=4; DSS, n=8; DSS+CT-1, n=8). *: $p<0.05$ vs. Sham group; #: $p<0.05$ vs. DSS group.

NFκB activation was assessed by quantifying the levels of phospho-p65 (pp65) as a fraction of total p65 (RelA) in colonic tissue. Animals treated with DSS had a higher pp65/p65 ratio than controls, and co-treatment with CT-1 significantly

reduced the ratio to levels in controls (Figure 4 A). The ratio pStat-3/Stat-3 was significantly higher in animals receiving DSS in the drinking water than in sham animals. Co-treatment with CT-1 reduced the ratio pStat-3/Stat-3, but this reduction was not statistically significant when compared with the mice that received only DSS (Figure 4 B).

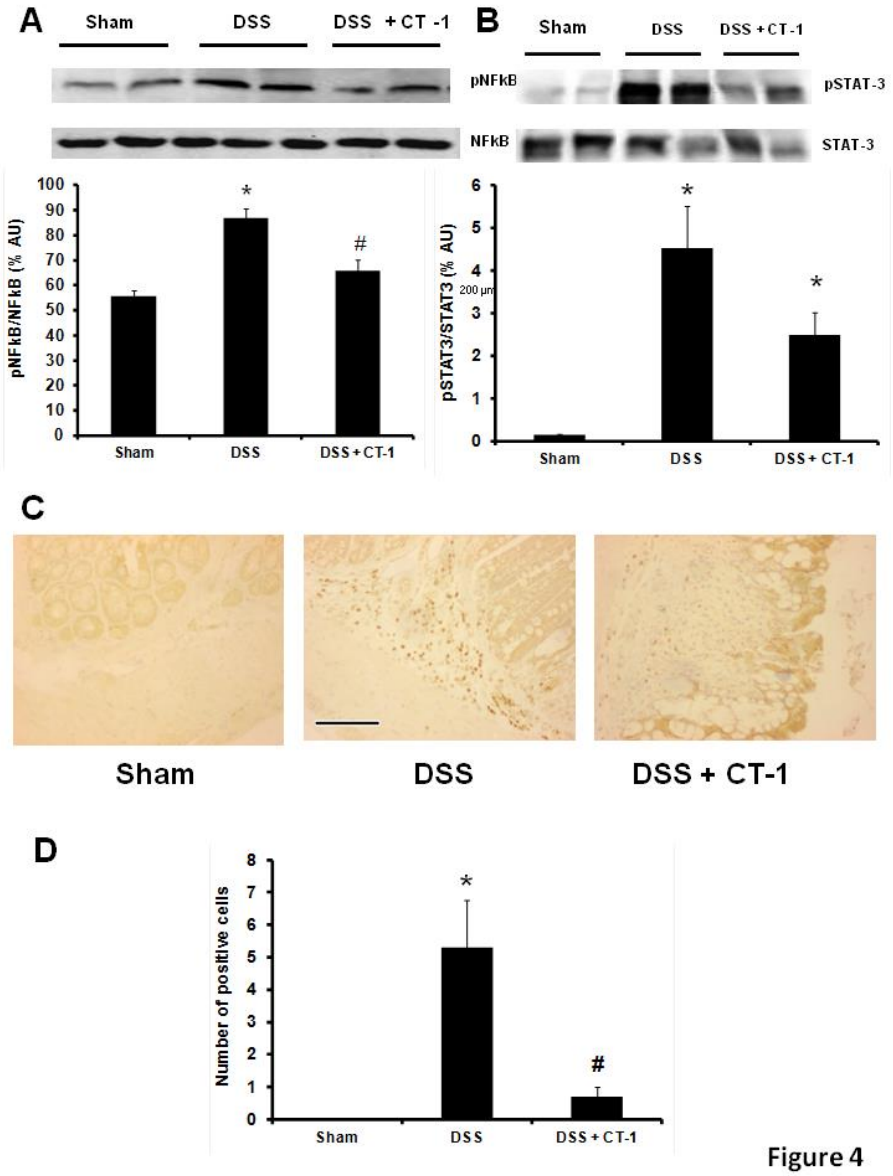

Figure 4

Figure 4. Effect of CT-1 on NFκB activation, STAT-3 activation and apoptosis in DSS-induced colitis in mice. (A) Western blot analysis of NFκB and NFκB activation (pNFκB) levels in colon tissue homogenates and expressed as % arbitrary units (% AU). (B) Western blot analysis of STAT-1 and STAT-3 activation (pSTAT-3) levels in colon tissue homogenates and expressed as % arbitrary units (% AU). (C) Representative images of cleaved

caspase 3 staining in colon; Bar: 200 µm. (D) Number of cleaved caspase 3 positive cells. Values are expressed as mean ± SEM (panels A and B: Sham, n=8; DSS, n=10; DSS+CT-1, n=10). Slides quantified per group in panels C and D: Sham, n=40; DSS, n=60; DSS+CT-1, n=60. *: $p<0.05$ vs. Sham group; #: $p<0.05$ vs. DSS group.

Apoptosis evaluation

Apoptosis was assessed by immunostaining through the number of cleaved caspase 3 positive cells. Representative images are shown in Figure 4C. The number of cleaved caspase 3 positive cells in colon was very low in control animals, but significantly higher in animals receiving DSS. In animals receiving CT1+ DSS this number was significantly lower than in the DSS group (Figure 4D).

CT-1 expression

Plasma levels of CT-1 were higher in animals receiving DSS than in control animals, but the difference was not statistically significant. Animals receiving CT-1+DSS showed higher plasma levels of CT-1 than mice receiving DSS alone, although the difference did not reach statistical significance either (Figure 5A). CT-1 content in colonic tissue was significantly higher in DSS-treated animals than in control mice. Mice receiving CT-1+DSS showed significantly higher level of CT-1 in the colon than animals receiving DSS alone (Figure 5B). CT-1 gene expression in colon was higher in DSS than in sham group, without statistically significant differences. In animals that also received CT-1, CT-1 gene expression in colon was significantly higher than in the DSS group (Figure 5C).

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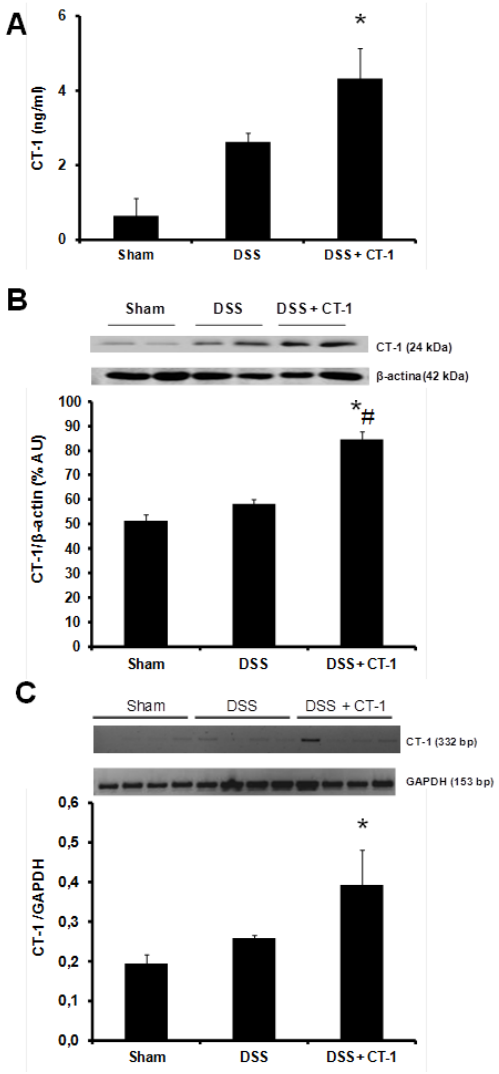

Figure 5

Figure 5. Effect of CT-1 on plasma levels and colonic production of CT-1 in DSS-induced colitis in mice. (A) Plasma CT-1 levels. Values are expressed as mean ± SEM (Sham, n=3; DSS, n=4; DSS+CT-1, n=4). (B) Western blot analysis of CT-1 and β-actin levels in colon tissue homogenates, expressed as % arbitrary units (% AU). Values are expressed as mean ± SEM (Sham, n=8; DSS, n=10; DSS+CT-1, n=10). (C) PCR analysis of CT-1 and GAPDH levels in colon tissue homogenates and CT-1/GAPDH quantification. Values are expressed as mean ± SEM (Sham, n=4; DSS, n=4; DSS+CT-1, n=4). *: $p<0.05$ vs. Sham group; #: $p<0.05$ vs. DSS group.

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Discussion

Ulcerative colitis (UC) is a chronic disease affecting the large intestine, with an increasing incidence worldwide. UC is a chronic immune-mediated inflammatory condition of the large intestine that is frequently associated with inflammation of the rectum but often extends proximally to involve additional areas of the colon. Management of UC must involve a prompt and accurate diagnosis, assessment of the patient's risk of poor outcomes, and initiation of effective, safe, and tolerable medical therapies. UC is a chronic condition for which therapy is required to induce and maintain remission; therapeutic decisions should be categorized into those for (i) induction and (ii) maintenance [9],

For patients with mild-to-moderate disease, 5-aminosalicylic acid (5-ASA) precursors, such as Sulfasalazine, Mesalamine, and Budesonide, have been used either by oral or topic rectal administration, or even a combination of both types of administration. Some of these products have notable side effects such as anemia,abnormal liver tests, nausea, headaches, fevers, and rash, more frequent after treatment with Sulfasalazine that with Mesalamine and Budesonide [10, 11]. In addition, in more severe cases of colitis, 5-ASA precursors show only a limited efficacy

Systemic corticosteroids are frequently used for induction of remission in cases of moderate-to-severe disease. Oral steroids are used in most cases, but in patients in which the disease present as acute severe ulcerative colitis, intravenous steroids should be used. Steroids are very effective in inducing remission but they are ineffective in maintaining remission and steroid treatment have to be withdrawn. In addition, after long term therapy, steroid toxicity is common, may involve virtually any organ system and damage is frequently irreversible [29] Furthermore, the risks of severe side effects increase when steroids are used together with other immunosuppressive therapies.

Thiopurines such as azathioprine (AZA) and 6-mercaptopurine (6-MP) have a steroid-sparing effect and are used for maintenance of remission of UC when steroids are withdrawn. Thiopurines have no role for induction of remission. AZA and 6-MP are slow-acting drugs, and it can take about 3 months before therapeutic concentrations are achieved, thus requiring a longer treatment with

steroids is often before the appearance of pharmacologic effect of thiopurines, thus increasing the risk of steroid toxicity. In addition, AZA and 6-MP have multiple side effects, being the most frequent elevation of plasma transaminases and leukopenia. Thiopurines are also associated with an increased risk of malignancy [30]

Anti-TNF-α antibodies (infliximab, adalimumab, and golimumab) have been used for both induction and maintenance of remission of either alone or in combination with corticosteroids [31]. Infliximab is a chimeric (combination of human and murine) IgG1 monoclonal antibody. Adalimumab and golimumab are 100% human antibodies. All of them binds with affinity to TNF-α and neutralizes its biologic activity. However, anti-TNF-α antibodies are not useful for one third of patients (i.e. “primary failures”), and in another third, they loses effect over time (“secondary failures”) [32]. Moreover, these treatments are often complicated by multiple side effects [33] and high price leading to differential access among European countries [34].

Calcineurin inhibitors such as cyclosporine has been reported to induce remission in severe-to-fulminant steroid-refractory colitis. Only limited data for the use of tacrolimus are now available, they are not recommended for typical use. Cyclosporine is not useful for long-term therapy. Although over 60% of patients with severe UC respond to intravenous cyclosporine, most will still ultimately require colectomy in 5–7 years [35]

Anti-integrins are other gamily of agents used for CU treatment. Integrins are proteins that regulate migration of leucocytes to the intestines. Vedolizumab, the first anti-integrin approved for use in UC, is a fully humanized recombinant monoclonal antibody that binds to alpha4–beta7 integrin and prevents migration of leucocytes to the gut. Vedolizumab has shown to be effective to induce and maintain remission in moderate-to-severe active UC, without important side effects. [36]

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway is a potent signaling cascade involved in many biological effects, including inflammation promotion. Janus kinase. JAK is a tyrosine kinase that is activated by many molecules, including interleukine-6 (IL-6), that plays a major

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role in CU-associated inflammation. IL-6,binds the IL-6r-Gp130 receptor complex and activates the JAK recruited to the intracellular domains of GP-130 receptors. Once activated, JAK proteins change their conformation, dimerize, phosphorylate, and activate their primary substrates, the STAT proteins. Tyrosine-phosphorylated STAT proteins homo- or hetero-dimerize and translocate to the nucleus, where they interact with coactivators and bind to specific regulatory elements in the promoter regions of thousands of different genes, including those codifying for proteins involved in the inflammatory process. [37]. Tofacitinib is a JAK inhibitor indicated for treatment of adult patients with moderate-to-severe UC, but it is not recommended for use in combination with other biologics or potent immunosuppressants such as a thiopurine or calcineurin inhibitor [38]. Other JAK inhibitors such as filgotinib, upadacitinib, TD-1473, peficitinib, and Pf-06651600/Pf-06700841, have been recently developed but for most of them there are only data from phase I and II trials [39]. Adverse effects of Tofacitinib are severe infections due to bacterial, mycobacterial, invasive fungal, viral, or other opportunistic pathogens. In addition opportunistic herpes zoster infections producing severe meningoencephalitis, as well as ophthalmologic, and disseminated cutaneous diseases have been observed. [38].

Other different therapeutic approach for UC treatment is the use of Mesenchymal stem cells, although the development of this treatment is in a very preliminary phase [40].

As above described, most drugs used for UC treatment become ineffective after some time of treatment and/or present severe side effects, thus being desirable the development of new therapeutic strategies targeting different mechanisms responsible for inducing the colon damage. Thus, we have assessed the possible effect of CT-1 on preventing colon damage and inflammation before UC induction.

In the whole, this study reveals that CT-1 administration before the induction of experimental UC with DSS, ameliorates both the clinical course of the disease and the histological damage in the colon. Protection appears to be provided by several mechanisms. Similarly as in several other organs including the lungs

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[41], heart [42] liver [43], and kidneys [23], CT-1 has a potent anti-inflammatory effect in this model, as revealed by lower plasma level of TNF-α, lower colonic expression of IL-1β, IL-17, less pronounced increased expression of iNOS, and lower colon infiltration of monocytes/macrophages. CT-1 also ameliorates colonic apoptosis in our model, as in other organs and cells such as isolated myocardiocytes [44,45], liver [43,46,47], pancreatic beta cells [48], neurons [49] and kidney epithelial cells [23].

When comparing the results of this study with those from previously published use of CT-1 in a curative strategy [24], both approaches ameliorate the course of disease and preserve colon tissue, although colon apoptosis and most indicators of inflammation are further minimized by the preventive strategy. With a therapeutic aim, both studies provide a potential tool for the management of ongoing UC as a complement to prophylaxis. Because prevention of relapses poses a more complicated intervention involving the monitoring of early signs and symptoms, and early biomarkers such as fecal calprotectin [50,51], patients handling and outcome might benefit from both approaches, preventive and curative.

Despite a similar outcome, the preventive and curative mechanisms behind CT-1 action might be, at least partly, different. In the case of the preventive effect, CT-1 might precondition the colon to oppose damage; whereas in the curative action, CT-1 might reverse damage mechanisms. This is hypothetically inferred from the different behavior of specific mediators. Whereas in the preventive model CT-1 significantly moderates the increase in colonic activation of STAT-3 and NFκB by DSS, in the curative model CT-1 has no effect on or, if anything, increases colonic STAT-3 and NFκB activation [24]. This is consistent with the dual role known to be played by NFκB in inflammation [52, 53]. During the initiation phase, NFκB contributes to the inflammatory process by increasing the expression of proinflammatory, chemokines and adhesion molecules, and and promotes epithelial cell proliferation and survival whereas during the resolution phase, NFκB favors leukocyte apoptosis [52,53]. This suggests that CT-1 may also act upstream of inflammation, likely by preventing tissue damage and cell death. Preventive and curative data on the role of NFκB would be in agreement with inflammation being the consequence of colon injury, as previously

hypothesized [52]. In the preventive scenario, CT-1 would prevent tissue damage and, consequently, the activation of NFκB and inflammation. In the curative scenario, some degree of damage would occur before CT-1 administration, which would give rise to an inflammatory response, and some colon apoptosis. Apoptosis progression would be prevented by CT-1 from the moment of treatment inception, in the presence of established inflammatory response, characterized by activation of NFκB, for the resolution phase. Similarly, a general reference of inflammation, namely the plasma level of TNF-α is also congruent with this hypothesis. In fact, plasma TNF-α in CT-1-treated mice with ulcerative colitis is almost normal (i.e. undistinguishable from the controls), whereas it was markedly lower to that found in untreated mice with colitis.

An attractive aspect of this study is that the clinical use of CT-1 as a therapeutic agent in IBD can progress in a very fast way because CT-1 has already been approved for human use in several conditions. CT-1 holds status of orphan drug by the US Food and Drug Administration (FDA) for the treatment of acute liver failure (designation request 11-3507) and for protecting the liver from ischemia/reperfusion injury associated to the transplantation procedures (designation request 07-2449). The European Medicines Agency (EMA) has also approved this status for the prevention of ischemic/reperfusion injury associated with solid organ transplantation (EU/3/06/396). A clinical trial to assess the safety, tolerability and pharmacokinetics of CT-1 in healthy volunteers has been registered in the US at ClinicalTrials.gov (https://clinicaltrials.gov/; identifier NCT01334697, checked on July 16, 2018). Because chronic, long-term, high dosage CT-1 administration is associated to organ fibrosis and functional impairment of the heart and kidney [54] and none of these effects have been observed when CT-1 is administered in low doses or for short periods [55], our suggested application of CT-1 therapy is an acute treatment (4-6 days) for attenuating the severity of UC-predicted relapses.

Conclusions

Our data demonstrates that CT-1 administration before the induction of UC with DSS, ameliorates both the clinical course and the histological damage in the

colon. This effect seems to be mediated by direct anti-inflammatory and anti-apoptotic effects, which course with activation of the Stat-3 and/or NFκB pathways. Thus, acute therapy with CT-1 could be envisaged as a possible new therapeutic tool against UC relapse.

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Lopez-Hernandez and Antonio Rodriguez-Perez; Writing – original draft, José M Lopez-Novoa; Writing–review & editing, Francisco J. Lopez-Hernandez, Antonio Rodriguez-Perez and José M Lopez-Novoa. All the authors approved the final version of the manuscript.

Conflicts of Interest:

The authors declare no conflict of interest. Dr. Juan Ruiz (Digna Biotech, Pamplona, Spain) collaborated in the design of the study. The sponsors had no role in the execution, interpretation, or writing of the study.

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